

Effect of Oral Antigen and Antibody Exposure at Birth on Subsequent Immune Status

A Study in Neonatal Pigs

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Key Words

CD4 T cells • CD8 T cells • Neonates • Oral tolerance • Sensitisation

Abstract

Background: The purpose of this work was to investigate the effects of early low-level exposure to either antigen or antibody alone on subsequent immune responses in entirely immunologically naïve animals. This is impossible in species with a permeable placenta such as rodents or humans, where both antigen and antibody can be transferred in utero. It is, however, possible in pigs, due to the impermeable placenta of the sow. Thus, neonatal piglets were used for this study. **Methods:** Newborn piglets were exposed to ovalbumin (OVA) at dosages similar to those used in rodents to sensitise, as well as to serum containing anti-OVA antibodies. **Results:** Both single low doses of OVA (10 and 1,000 mg per animal) induced classical oral tolerance following a systemic challenge: both doses reduced specific systemic IgG responses and tertiary in vitro recall proliferative responses by splenocytes and especially by mesenteric lymph node (MLN) cells. Additionally, dietary challenge had phenotypic effects on helper T cells in MLN, which could be reversed by OVA at birth. In contrast, giving antibody as serum collected from hyperimmune or orally tolerant pigs had no functional ef-

fects. **Conclusions:** Overall, our data support the hypothesis that contrary to previous work in rodents, very early exposure of neonatal pigs to a single small dose of antigen can reduce subsequent immune responses. This may have implications for human health. However, although these data point to a reducing/regulatory effect of low doses of antigen in very young animals, they cannot be extrapolated directly to allergy.

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Introduction

There is evidence that the number of individuals suffering allergic reactions to food is increasing [1, 2]. Much effort has been put into identifying the factors responsible for induction of, and susceptibility to, food allergy. Whilst it is established that there is a strong genetic component to food hypersensitivity [3], it is not yet sufficiently clear what additional contribution environmental factors in early life may make on the subsequent development of an inappropriate immune response, although the beneficial effects of maternal antibodies to pathogens are well established. The type of antigen, antigen dose and age at first exposure are all thought to play a part [4–8] in the subsequent immune response. Based mostly on ex-

perimental work in rodents, it was generally thought that premature exposure to novel dietary antigens may predispose to allergic reactions and advice had been issued that weaning infants onto solids should be delayed. The recommendations in 1998 by the Department of Health, Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment, that atopic mothers may wish to avoid dietary antigens such as egg white and nuts during pregnancy and lactation, follows a similar line of reasoning. However, recent results indicate that avoidance of such dietary components has not had the desired result [9, 10]; instead, initial exposure via inflamed skin has been suggested as a trigger for hypersensitivity [11, 12]. The opposite hypothesis has been put forward recently. Guidelines issued by many national and international allergological and paediatric associations do not recommend such dietary restrictions in the weaning period any more. Our studies support the view that early introduction of antigen into the diet of humans may be the key to inducing oral tolerance and may also prevent atopic reactions to food antigens [13, 14] in later life.

There is a clear need to dissect the immunological effects of both antigen and antibody very early in life/in utero, not just in rodents, but also in other species. Are any effects due to the antigen, antibody or a combination of both? It is not possible to expose animals with a permeable placenta, such as rodents, to one without the other. Our study in colostrum-deprived, neonatal pigs provides a challenging model in this context, allowing us to investigate in isolation various environmental effects acting at the same time in humans in utero. We have tried to answer the questions of the long-term effects of a single dose of antigen as well as that of allergen-specific antibody.

Additionally, this study added to our understanding of some of the immunological processes involved by demonstrating effects on cellular composition, in particular effects on memory T cells in mesenteric lymph nodes (MLN).

However, unfortunately there is as yet neither an antibody to porcine IgE nor a reliable experimental model of allergy in the pig, reducing its power to investigate allergic effects. Instead, the system of oral tolerance induction accompanied by the study of IgG responses, specific T-cell proliferation and phenotypic changes in total T-cell populations was used. Thus, our model system has both inherent strengths as well as weaknesses in comparison to rodent models investigating factors implicated in the immune responses to dietary antigen and susceptibility to food allergies in the human infant.

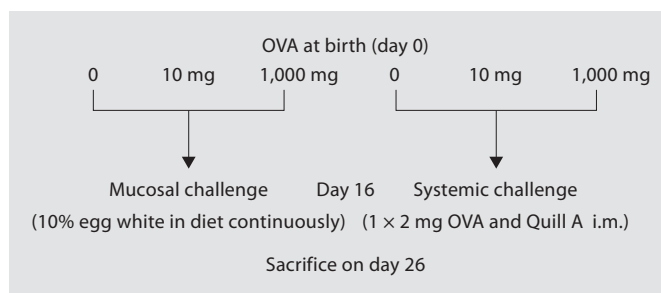


Fig. 1. Diagram showing the design for experiment 1.

Materials and Methods

Animals

Piglets were caught as born and transferred to a warmed, sterile box with a HEPA-filtered air supply on a commercial pig farm 20 miles from the University. On reception at the University facility, piglets were washed in a detergent bath (Virkon) and transferred to a specified pathogen-free isolation unit. By stomach tube, piglets were given 50 ml of Haemacel, a colloid solution designed for intravenous infusion to replace blood volume (Intervet UK, Milton Keynes, UK).

Husbandry

Six piglets were recovered from each of two separate sows; one piglet from each sow was allocated to one of six experimental treatment groups. This was repeated four times. For both experiments, numbers ranged between 6 and 8 pigs/treatment group, due to some losses. Piglets were given daily prophylactic broad-spectrum antibiotic therapy [enrofloxacin, (Baytril), at 5 mg/kg i.m. daily, from Bayer, Animal Health Division, Newbury, UK], to minimise the risk of opportunistic bacterial infection. An automatic feeding system delivered calibrated hourly amounts of a pig milk substitute at 200 g/l in water, free of ovalbumin (OVA) and porcine immunoglobulins (Piggi-Milk; Parnutt Foods, Sleaford, UK). A fluid replacement solution (Lectade) was provided for the first week to assist the animals in maintaining their hydration status. Pigs were maintained at 28°C, housed individually and fed the milk replacement solution hourly.

Experimental Design

Antigen/Antibody Exposure at Birth

In experiment 1, piglets were orally dosed by stomach tube with either: 1,000, 10 or 0 mg of OVA, pre-mixed with 50 ml of Haemacel (Intervet UK) and given immediately upon transfer to the pig unit. As the mean body weight at birth was approximately 1.5 kg, this represents an approximate dosage of 0.667 and 0.007 mg/g body weight, respectively. In experiment 2, 5 ml of serum from donor pigs were given by stomach tube, derived from hyper-immune, tolerised or control pigs of the same ancestry. These sera were raised as described below.

Antigen Challenge on Day 16

On day 16, the piglets were challenged either systemically (1 ml of 1 mg/ml OVA plus saponin delivered intraperitoneally)

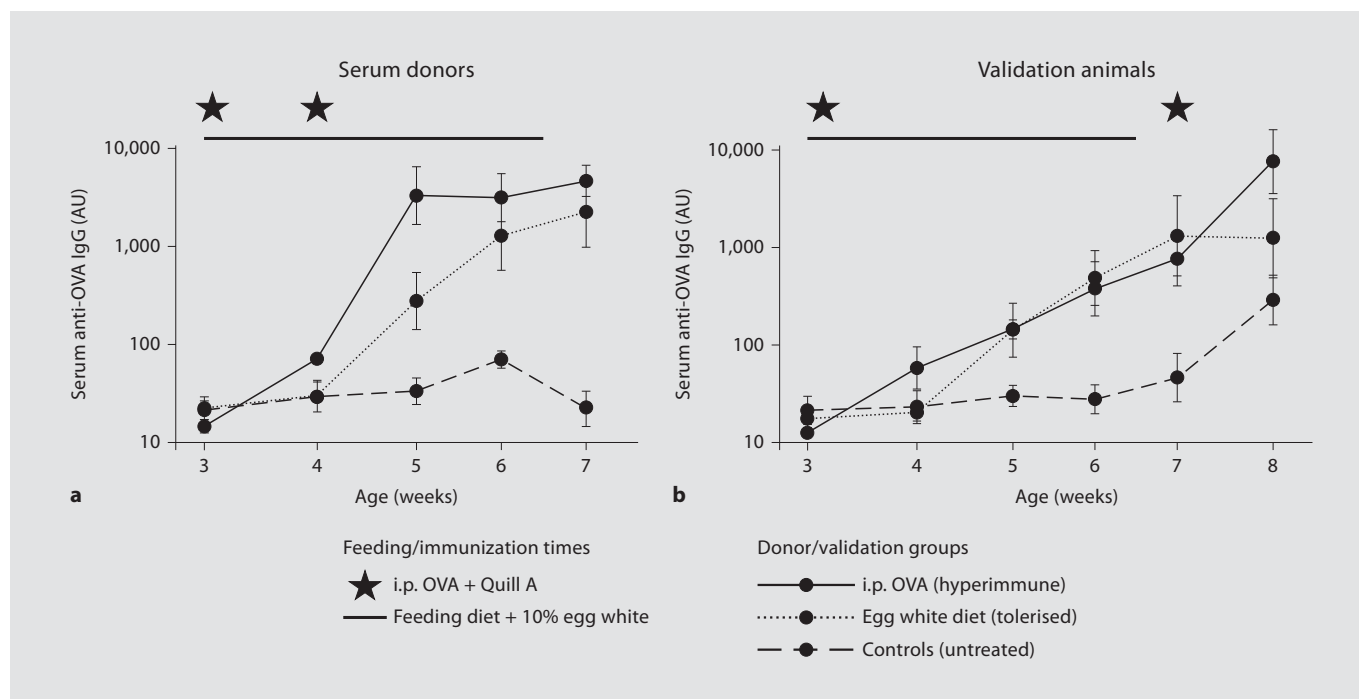


Fig. 2. Antibody titres on the y-axis are given in arbitrary units (AU) of anti-OVA IgG relative to a previously prepared high-titre hyperimmune reference serum with anti-OVA IgG titre of AU = 10,000. **a** Anti-OVA IgG responses in three groups of serum donors. Time course of antigen exposure and corresponding anti-OVA IgG responses in peripheral blood. Hyperimmune animals were injected with OVA and Quill A at the age of 3 and 4 weeks;

tolerised animals were fed an egg-white-containing diet from weeks 3–6. Control animals were age-matched but untreated. **b** Anti-OVA IgG responses in three further age- and treatment-matched validation groups of pigs; the hyperimmune group was injected OVA and Quill A at the age of 3 and 7 weeks, tolerised and control groups received injections at the age of 7 weeks. All animals were sacrificed aged 8 weeks.

or mucosally (10% egg white was introduced into the diet and fed continuously from that point in time). The single largest protein constituent of egg white is OVA, it constitutes about 90%.

On day 26, piglets were sacrificed (10 days after challenge) and tissues were taken for subsequent analysis as described. The design for experiment 1 is shown in figure 1, the experimental design for experiment 2 followed a similar layout, administering antibody-containing sera instead of OVA at birth (not shown).

Preparation of Hyperimmune or Tolerised Serum for Experiment 2

In order to obtain sera containing antibodies to OVA, we had to raise antiserum in two pig groups, a hyperimmune group injected intraperitoneally with OVA and saponin and a tolerised group fed a diet supplemented with egg white.

Thirty piglets from a local, commercial pig farm were selected at random subsequent to weaning at the age of 21 days from a group of eight litters that had been allowed to mix freely on the sows. The piglets were housed in groups of 10 in three pens and fed a standard commercial weaner diet. Hyperimmune serum was raised by two injections of 1,000 mg OVA i.p. in Quill A when piglets were 3 and 4 weeks old. Tolerised serum was raised by supplementing their standard diet with 10% egg white for a pe-

riod of 3.5 weeks, withdrawal of egg white for the final half week was in order to allow OVA to clear from the circulation. Half of each group of piglets were sacrificed aged 7 weeks, the other half used as 'validation animals', by challenging them with an injection of 1,000 mg OVA i.p. in saponin and sacrificing them after a further 7 days. However, the second intraperitoneal injection in the validation animals was given at the age of 7 instead of 4 weeks. The rationale for this different time course was the need to keep the frequency of injections the same for donors and validation animals, to allow the titre to maximise in the donor animals and to test the immune status of the validation animals at the point of donor sacrifice. The protocols for OVA exposure as well as the corresponding IgG responses are shown in figure 2. This protocol for demonstrating the hyperimmune or tolerised status has been used by this group on several previous occasions [15–17]. Sera were pooled and stored at -20°C until use.

ELISA

The humoral immune response was determined by anti-OVA IgG-specific ELISA. Briefly, ELISA plates (LabSystems) were coated with pre-optimised concentrations of OVA (5 $\mu\text{g}/\text{ml}$, Sigma, grade V) overnight at 4°C . Plates were blocked with PBS-Tween supplemented with 5% skimmed milk powder (Marvel) for 1 h

Table 1. Monoclonal antibodies and their source

Specificity	Antibody	Isotype	Donor
CD45	K252.1E4	IgG ₁	authors
CD3	STH 164	IgG _{2a}	Dr. M. Shimizu, Kyoto Biken Laboratories Inc., Kyoto, Japan
CD4	MIL17	IgG _{2b}	authors
CD8	MIL12	IgG _{2a}	authors
CD25	K231.3B2	IgG ₁	authors
CD45RC	MIL15	IgM	authors

and trebling dilution series of test sera and reference sera were applied. Binding of antibody was detected with a polyclonal goat anti-pig IgG antiserum, conjugated to alkaline phosphatase (Sigma, A1418) at optimal dilution in PBS-Tween. Finally, pNPD (Sigma 104 phosphatase substrate) in carbonate buffer was added and optical density read at 405 nm. A hyperimmune reference porcine serum, which had been raised previously by three injections of 1,000 mg OVA in Quill A in another animal, was assigned an arbitrary value of 10,000 antibody units and used to construct a standard curve. The quantity of antibody in test sera was determined by interpolation of optical density values from all dilutions falling within the straight section of the reference curve. All antibody values were expressed relative to the standard.

Other Ig responses such as IgA or IgM were not investigated, as we have shown previously that the dominant Ig response to dietary protein in pigs is IgG [15–17]. However, we cannot rule out that other Ig classes may have played a role in this system.

Cell Isolation

Leucocytes for flow-cytometric characterisation and in vitro culture were isolated from four tissues. MLN and spleen cells were isolated by tissue disruption in Hank's balanced salt solution (HBSS) with or without Ca and Mg and filtered through a cell strainer to obtain single cell suspensions. Splenocytes were obtained similarly and then isolated over a Ficoll/Hypaque density gradient; most remaining erythrocytes were lysed by hypotonic shock with Trizma-buffered ammonium chloride (4.5 g ammonium chloride, 1 g Trizma base Sigma 6066 in 1 litre H₂O) for 6 min. Peyer's patch cells were isolated by excising Peyer's patches from the remaining gut tissue, gently scraping off the mucosal cells and mechanically disrupting them by vigorous pipetting. This suspension was centrifuged and re-suspended in collagenase medium (RPMI Dutch Modification, Life Technologies, catalogue No. 22409-015), containing 100 units/ml of collagenase (collagenase grade V, Sigma, catalogue No. C9263), and shaken at 37°C for 1 h. The suspension was then passed through a cell strainer to obtain a single cell suspension, washed in HBSS twice and re-suspended. Jejunal lamina propria cells were isolated by a method similar to the one described previously [18]. Briefly, three inverted inflated gut sacs from proximal, mid and distal jejunum were incubated with 1 mM EDTA in calcium- and magnesium-free HBSS at 37°C on a shaking platform. Epithelial cells were removed by repeated incubations, for a total period of approximately 3 h. The tissue was then digested in collagenase medium as used for Peyer's patches for 2 × 45 min. Cells were washed,

pelleted and suspended in a 40% Percoll in HBSS solution, and underlain with a 75% Percoll solution. Cells were isolated from the interface and washed in HBSS.

In vitro Antigen-Specific Cell Proliferation

The development of OVA-reactive memory cells in spleen, MLN, Peyer's patches and jejunal lamina propria was determined by the degree of antigen-specific cell proliferation in isolated cell populations. 2×10^6 leucocytes/ml, quantified by counting in a haemocytometer in white cell counting fluid (1 ml H₂O + 99 ml glacial acetic acid) to exclude remaining erythrocytes, were cultured in DMEM (Life Technologies) supplemented with 100 mM glutamine, sodium pyruvate, penicillin/streptomycin, 2-mercaptoethanol, 2% normal pig serum and 0.1 mg/ml OVA for 4 days. In order to check cellular viability, cells were also activated with 5 µg/ml concanavalin A and cultured for 3 days. All cultures were incubated with a final pulse of tritiated thymidine for 8 h and counted in an LKB Wallac beta counter. The stimulation index was calculated as the ratio of counts in the presence of OVA/counts in the absence of OVA.

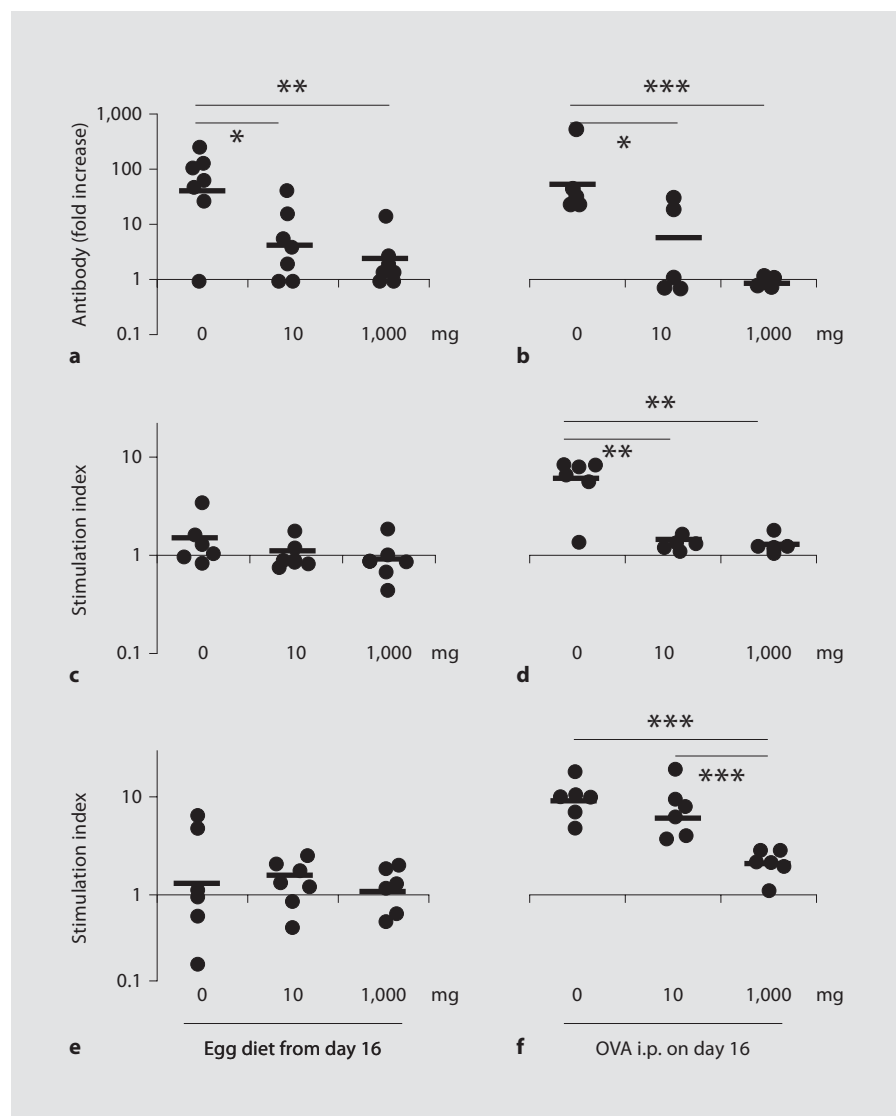
Flow-Cytometric Analysis

T cells from all four tissues were analysed by multi-colour flow cytometry as follows. CD3+ T cells were assessed as a proportion of CD45+ lymphocytes. Additionally, the proportions of CD4+ and CD8α^{high} cells as well as their memory and activation status were also assessed, using antibodies characteristic for this status. Although the expression of the same cell surface molecule may not necessarily imply the same cellular function in different species, it is generally accepted that the surface molecules CD3, CD45, CD4, CD8α, CD25 and CD45RC are functionally analogous in pigs to those in other species [19–24]: CD4 is indicative of Th cells, high levels of CD8α without CD4 indicate Tc cells, and CD25 is the IL2R and is found on activated T cells. CD45RC is present on naïve T cells and lost following activation. Antibody cocktail 1 contained antibodies to CD45 and CD3, and cocktail 2 antibodies to CD4, CD8α, CD25 and CD45RC.

In one respect, however, T-cell subsets in pigs are somewhat unusual [25–31]. In addition to the classical CD4+CD8– cells and the CD4–CD8α+β+ cells of other species, pigs have CD4+CD8α^{low}β–, CD4–CD8α^{low}β– and CD4–CD8– T cell subsets. Although Th and Tc are no longer considered accurate ways of referring to CD4+ and CD8+ T cells, the complex nature of CD4/CD8 co-expression in the pig made us continue to use these terms as 'shorthand'. When CD8α is expressed on cells at high intensity on lymphocytes which lack CD4, these cells have been referred to as Tc cells. Both CD4+CD8α– and CD4+CD8α+ cells have been referred to as Th cells. As mentioned, CD4+ Th cells in pigs can express low levels of CD8α (the so-called double-positive or CD4+CD8α+ subset) and this expression is indicative of antigenic experience [19], justifying the inclusion of CD8α expression on CD4+ Th cells as a memory marker. CD25 was never found on CD4–CD8α^{high} Tc cells and is therefore not shown for Tc. Table 1 lists all monoclonal antibodies and their sources used in this study.

1×10^6 leucocytes counted as above were suspended in 50 µl of phosphate-buffered saline containing 0.2% sodium azide (PBS-A) and incubated for 1 h with optimised concentrations of the antibody cocktails and isotype-matched controls in a total volume of 50 µl PBS-A. Cells were washed in PBS-A and incubated

Fig. 3. Immune responses after OVA exposure at birth (experiment 1). OVA dose is shown on x-axis. **a, c, e** Challenge by egg-white-containing diet from 16 days of age. **b, d, f** Challenged systemically by 1 mg OVA + Quill A i.p. at 16 days of age. **a, b** Fold increase in OVA-specific IgG in peripheral blood between days 16 and 26. **c–f** OVA-specific in vitro proliferation of MLN (**c, d**) and splenocytes (**e, f**) isolated on day 26. Statistical comparisons by ANOVA are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



with 50 μ l of corresponding conjugate-fluorochrome cocktails of pre-titrated isotype-specific affinity-purified goat anti-mouse antisera. Fluorochromes were FITC, phycoerythrin, biotin (Southern Biotechnology, Birmingham, Ala., USA) and Tricolor (Caltag-MedSystems, Claydon, UK) for 1 h. After a wash in PBS-A, the fourth fluorochrome was added in the form of SA-PE-TR (Caltag). Cells were washed in PBS-A. All steps were carried out at 4°C. Fluorescence was quantified using an EPICS XL flow cytometer (Coulter Electronics, Luton, UK). Isotype-matched pure mouse immunoglobulins were used to determine non-specific fluorescence of controls.

Statistical Analysis

Statistical analysis was carried out on log-transformed data, after applying the Shapiro-Wilk W test for normal data, for the serum IgG and cellular proliferation. Flow-cytometric data were analyzed untransformed. Data were then analyzed by ANOVA

(Bonferroni) with post hoc analysis by two-sample t test with equal variance. One-sample t tests for the serum IgG and cellular proliferation data were also done to test if individual groups were significantly different from 1.

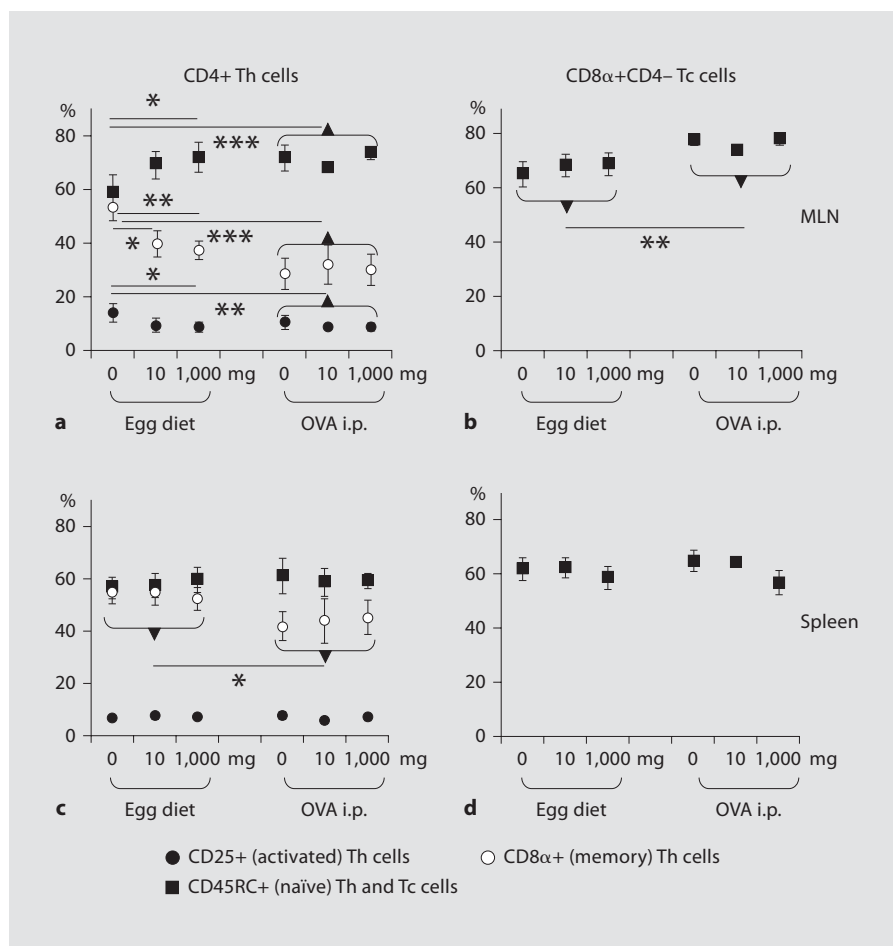
Results

Experiment 1

Serum IgG Responses

OVA exposure at birth in experiment 1 induced classical functional oral tolerance, as evidenced by a reduced systemic IgG antibody response on day 26 to the challenge with intraperitoneal OVA on day 16 (fig. 3b). Inter-

Fig. 4. T lymphocytes expressing cell surface molecules indicative of activation/memory status after OVA exposure at birth (experiment 1). OVA dose is shown on x-axis. Lymphocytes isolated on day 26 from MLN (**a, b**) and spleen (**c, d**). Proportion of CD4+ Th cells expressing CD25, CD8 α and CD45RC (**a, c**), and proportion of CD8 α +CD4- Tc cells expressing CD45RC (**b, d**). Values are means $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed homoscedastic t test.



estingly, this reduced antibody response was also observed and of a similar magnitude when piglets were challenged with an egg-white-containing diet (fig. 3a). Both the 10-mg as well as the 1,000-mg dose had a significant effect, although the effect of the high dose was more extensive.

In vitro T-Cell Proliferation

There were also clear effects of OVA exposure at birth on cellular function. However, these differed between challenge groups, even though they were accompanying a similar pattern of specific antibody production.

MLN cells and splenocytes from OVA-naïve animals challenged on day 16 via the intraperitoneal route responded with high in vitro antigen-specific proliferation 10 days later. The 1,000-mg dose of OVA exposure at birth effectively abrogated this in both spleen (fig. 3f) and MLN (fig. 3d). The smaller 10-mg dose also abrogated the proliferative responses of MLN (fig. 3d) but not of splen-

ic cells (fig. 3f). Piglets challenged with an egg-white-containing diet showed no in vitro OVA-specific cell proliferation 10 days later in MLN (fig. 3c) and spleen (fig. 3e). Therefore, antigen exposure at birth could not lead to any further reduction; it did not lead to any increase either.

No antigen-specific proliferation was seen for cells from the lamina propria or Peyer's patches in any experimental group (not shown), although cells were viable, as shown by proliferation to concanavalin A.

Cellular Composition and T-Cell Status in vivo

There were no effects of any of the experimental treatments on the proportion of CD3+ T cells within the CD45+ lymphocyte gate nor on the relative proportions of Th and Tc subsets in any of the tissues studied (not shown).

MLN - CD4+ Th Cells

However, we observed the effects of various experimental treatments on the activation and memory status of

T cells in MLN (fig. 4a, b). Th cells (fig. 4a) of OVA-naïve animals (0 mg OVA at birth, 1st data points) exposed to the egg-white-containing diet on day 16 had relatively high numbers of activated CD25+ and CD8α+ Th cells and, conversely, few naïve CD45RC+ Th cells. A comparison of these OVA-naïve piglets with those exposed to either 10 (2nd data points) or 1,000 mg (3rd data points) OVA at birth and then challenged with the egg diet showed that Th-cell activation and progression towards the CD45RC- phenotype was significantly reduced by OVA at birth. This effect was dose dependent. *p* values for the comparison between OVA-naïve animals and those given 1,000 mg OVA at birth were *p* < 0.05 for CD25+ Th cells, *p* < 0.01 for CD8α+ Th cells and *p* < 0.05 for CD45RC+ Th cells. Animals given 10 mg OVA at birth still had significantly fewer CD8α+ Th cells (*p* < 0.05) than the OVA-naïve controls. The differences for CD25 and CD45RC expression just failed to reach statistical significance. Thus oral antigen experience at birth prevented the increase in memory Th cells observed when OVA-naïve animals were challenged later by an OVA-containing diet.

All three groups of piglets without dietary exposure to egg white, but challenged with a single intraperitoneal OVA injection on day 16, had Th in MLN (fig. 4a; 4th, 5th and 6th data points) with a similar memory/activation status. Thus oral antigen exposure at birth had no effect on the number of memory Th cells when animals were challenged later by intraperitoneal injection. Oral OVA exposure at birth had left no obvious phenotypic effects on Th cells in these 26-day-old piglets, in spite of the functional differences.

Additionally, there was a significant effect of the challenge type on Th cells in MLN. The proportions of CD25+ and CD8α+ Th cells were significantly higher (*p* < 0.01 and *p* < 0.001, respectively), and those of CD45RC+ Th and Tc cells were significantly lower (*p* < 0.001 and *p* < 0.05, respectively) in the 0-mg OVA group subsequently challenged by diet (fig. 4a; 1st data points) in comparison to all of the piglets challenged with intraperitoneal OVA (fig. 4a; 4th, 5th and 6th data points). Piglets exposed to 10 and 1,000 mg of OVA and challenged by diet had proportions of Th cells intermediate between those with 0 mg OVA at birth and challenged by diet and all three groups which had been challenged intraperitoneally. Thus, the Th-cell status of piglets with OVA experience at birth and exposed to OVA in the diet was more similar to the intraperitoneally challenged animals, i.e. they did not show the same increase in activated/memory T cells as their counterparts which were OVA naïve at the point of dietary exposure.

MLN – CD8α+CD4- Tc Cells

The experimental effects on Tc in MLN (fig. 4b) were different from those observed on Th cells. There was no significant effect of OVA given at birth on CD45RC expression, i.e. on the memory status of Tc cells in MLN. There was only a significant effect of the challenge type (dietary versus intraperitoneal injection): the proportion of naïve Tc in MLN in the three dietary groups was significantly lower than in the intraperitoneally challenged piglets. In other words, dietary exposure led to increased numbers of memory Tc cells.

Spleen – Th and Tc Cells

The effect on splenocytes (fig. 4c, d) was less pronounced. Unlike in MLN, there was no effect of OVA exposure at birth in the spleen. Only the type of challenge led to significant effects; as in MLN, dietary challenge appeared to lead to more CD8α+ memory Th cells than intraperitoneal challenge (fig. 4c). Although a comparison of each of the dietary groups with all three intraperitoneally challenged groups just failed to reach statistical significance, a comparison of all three dietary groups together with all three intraperitoneally challenged groups was significant (*p* < 0.05). We observed no significant effects on splenic Tc (fig. 4d).

Jejunum and Peyer's Patches

There were no significant phenotypic effects on T cells isolated from the jejunum or Peyer's patches (not shown).

Experiment 2

In experiment 2, we investigated the effect of giving antibody to OVA at birth, taken from donor pigs, both as anti-OVA from donor pigs in the form of hyperimmune as well as in the form of tolerised serum, as described in the Materials and Methods section.

Immunological Status of Serum Donors

In order to obtain anti-OVA antibody, it was necessary to rear donor animals under 'hyperimmunising' and 'tolerising' conditions. Thus, hyperimmune animals were repeatedly injected with OVA and Quill A. Tolerised animals were fed an egg-white-containing diet. A third age-matched control group was also reared. Figure 2a shows IgG titres for the three groups of serum donor animals. Antibody titres were also followed in three more parallel 'validation' groups, used not as donors but in order to verify the immune status of their donor counterparts at the point of donor sacrifice (fig. 2b). Their second OVA

challenge was at the time point when the donor animals were sacrificed, i.e. week 7. The validation animals were sacrificed 1 week later and antibody titres were determined. Thus these validation groups were shown as classically 'orally tolerant' (no rise in antibody titre after intraperitoneal OVA at 7 weeks), hyperimmune (rise in antibody titre after second intraperitoneal OVA at 7 weeks) or antigen-naïve (rise in antibody titre after first intraperitoneal OVA at 7 weeks). It is apparent that the hyperimmunised and tolerised donor groups had IgG responses which were largely comparable in magnitude. However, antibody increases in the hyperimmunised donor animals were more rapid after the second boost and about twice as high at the point of sacrifice in comparison to the dietary group.

We have reported previously that in spite of a similar magnitude, the affinity of antibody responses by oral versus systemic exposure differs and may thus have different functional effects [15]. In fact, this previous observation was responsible for the use of antibody donors which had acquired IgG titres to OVA via the oral as well as via the systemic route. Figure 2b shows that hyperimmune animals responded to a secondary intraperitoneal challenge with an approximately tenfold rise in antibody titre. Antigen-naïve animals also responded to intraperitoneal challenge with increased titres. Tolerised animals did not show any increase in IgG titre at all, but maintained the IgG levels observed at the point of intraperitoneal challenge. We could thus show that in these 'validation' animals, which were challenged with intraperitoneal OVA at the same time point when donor animals were sacrificed, the diet group showed no rise in antibody titre (i.e. tolerance) after 1 week, in contrast to a responsive status of control and intraperitoneally primed donor animals. As the titre of the hyperimmune serum pool was somewhat higher (approximately 220% of that of the tolerised serum), tolerised serum was diluted with control serum until the same antibody titre was achieved for both pools.

Serum IgG Responses

The administration of these three sera to neonatal piglets had no effect on subsequent immune responses. After the *in vivo* challenges on day 16, most piglets in the six recipient experimental groups showed variable serum IgG responses to dietary (fig. 5a) and intraperitoneal challenge (fig. 5b). Such variability is often characteristic of a primary response [17]. Although mean responses were higher in the systemically challenged animals in comparison to those on the egg diet, there were no sig-

nificant differences within challenge groups due to the neonatal administration of antibody. Unlike antigen, administering these antibody-containing sera at birth had no effects on IgG responses to a later systemic or dietary challenge.

In vitro T-Cell Proliferation

Tertiary *in vitro* proliferative responses of MLN cells were also similar in all groups (fig. 5c, d). Finally, although the intraperitoneally challenged groups had higher mean *in vitro* proliferative responses of splenocytes than the dietary ones (fig. 5e, f), the administration of antibody to OVA at birth had no effect on these *in vitro* proliferative responses of splenocytes: all three groups were similar after the intraperitoneal challenge (fig. 5f). Thus, none of the functional tests showed any effects which were due to the antibody experience at birth.

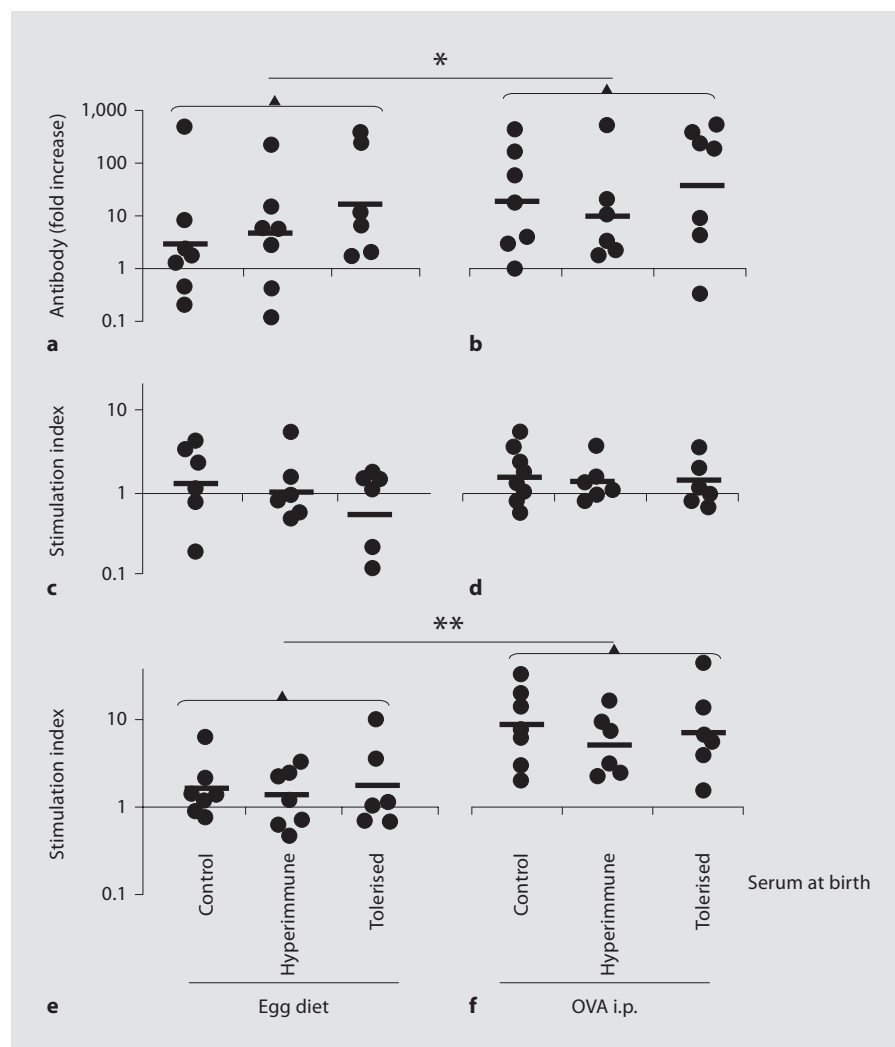
Cellular Composition and T-Cell Status *in vivo*

MLN – *Th* Cells. Interestingly, in spite of a lack of any functional effects, there was one phenotypic difference in MLN after administering the different sera. Again, the three groups undergoing a systemic intraperitoneal challenge had similar *Th* (fig. 6a) cell populations. However, although comparisons within the three dietary challenge groups showed no significant differences between the control group and those given hyperimmune or tolerised sera (fig. 6a), a direct comparison of CD4+CD8 α + memory *Th* cells of piglets given tolerised with those given hyperimmune serum showed significantly fewer such memory cells in those animals given the tolerised serum. Additionally, the status of *Th* cells of piglets given tolerised serum and challenged by diet was comparable to *Th* cells of the three intraperitoneally challenged groups. However, piglets given hyperimmune serum and challenged by diet had significantly higher numbers of CD4+CD8 α + memory *Th* cells compared to the intraperitoneally challenged groups.

MLN – *Tc* Cells. Again, as in experiment 1, the presence of antibody at birth appeared to have little effect on *Tc* cells (fig. 6b). As in experiment 1, an effect of the diet on *Tc* cells was also obvious in the form of significantly lower numbers of CD45RC+ naïve (i.e. more memory) *Tc* cells for all intraperitoneally challenged animals compared to those challenged by diet.

Spleen – *Th* and *Tc* Cells. There were no effects of the anti-OVA sera or of the type of challenge on the phenotype of splenocytes (fig. 6c, d).

Fig. 5. Immune responses after anti-OVA serum exposure at birth (experiment 2). **a, b** Fold increase in OVA-specific IgG in peripheral blood between days 16 and 26. **c–f** OVA-specific in vitro proliferation of MLN cells (**c, d**) and splenocytes (**e, f**) isolated on day 26. **a, c, e** Challenge with egg-white-containing diet from 16 days of age. **b, d, f** Challenged systemically with 1 mg OVA + saponin i.p. at 16 days of age. * $p < 0.05$, ** $p < 0.01$, ANOVA and/or two-tailed homoscedastic t test.



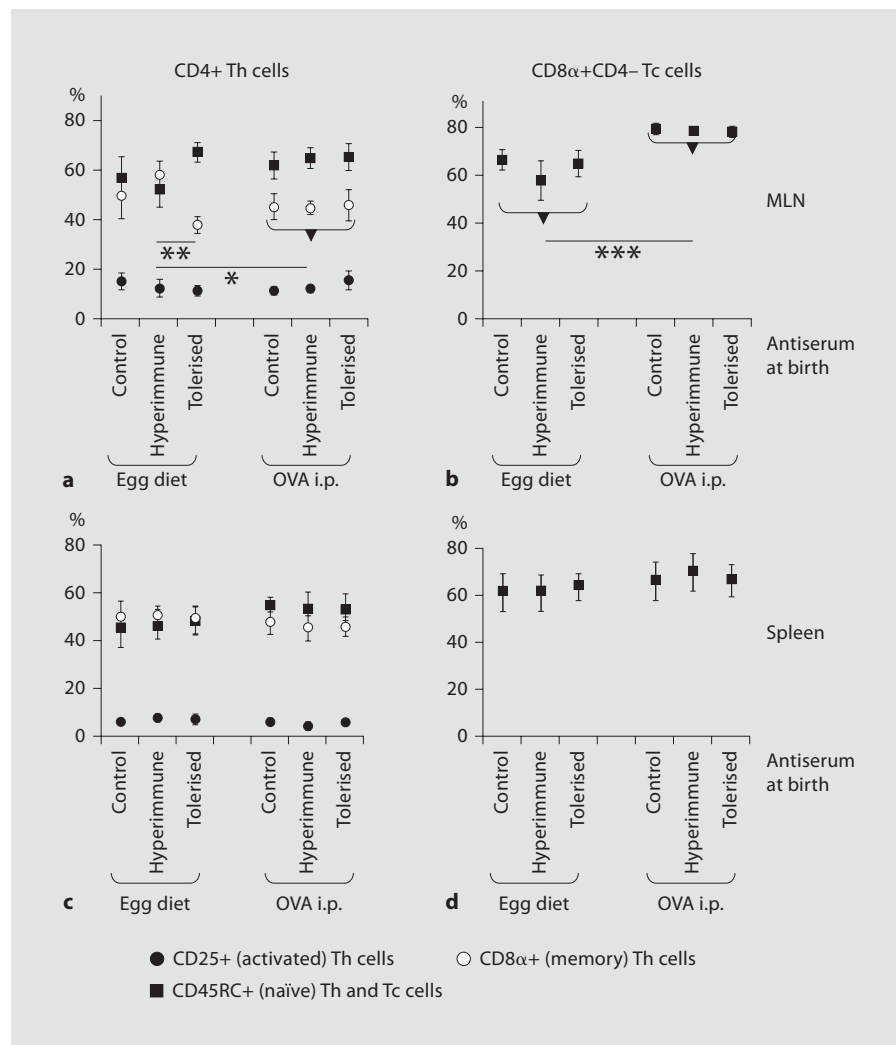
Discussion

The phenomenon where oral antigenic exposure leads to reduced systemic antibody and memory T-cell responses to a later systemic challenge has been used frequently to explore the mechanisms involved in the immunological responses to food and other harmless antigens in the healthy normal rodent and has been termed 'oral tolerance'. However, in humans, oral tolerance may also refer to tolerance of the oral exposure to an antigen irrespective of the immunological answers. Thus, using this term may be confusing. When referring to classical 'oral tolerance', this work is referring to the reduced antibody and T-cell responses caused by an earlier oral exposure following a secondary systemic challenge in the rodent and pig model. This reduction is only in compar-

ison to antigen-naïve or systemically primed animals and does not imply a total absence of such responses. The fact that antibody responses to dietary exposure continue to occur over a prolonged period is clearly demonstrated by the presence of such antibodies in our serum donor animals.

The link between classical oral tolerance and lack of atopy, with a corresponding link between sensitisation and atopy, is by no means fully explored. Nevertheless, experiments in rodents have been partially responsible for the dietary advice to atopic mothers to avoid premature exposure of human infants to potential allergens. Our results, demonstrating that the exposure of neonatal, immunologically naïve pigs to a protocol likely to induce sensitisation in neonatal rodents induced classical oral tolerance, as defined by reduced IgG and memory T-cell

Fig. 6. T lymphocytes expressing cell surface molecules indicative of activation/memory status after anti-OVA serum exposure at birth (experiment 2). Lymphocytes isolated on day 26 from MLN (**a, b**) and spleen (**c, d**). Proportion of CD4+ Th cells expressing CD25, CD8 α and CD45RC (**a, c**) and proportion of CD8 α +CD4- Tc cells expressing CD45RC (**b, d**). Means $\pm$ SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed homoscedastic t test.



responses in this species, may throw further doubt on the validity of this advice.

We also believe that it was important to demonstrate an immunologically unresponsive state and other accompanying phenomena after the two very different challenges 16 days later. The systemic challenge is the method used in classical oral tolerance experiments, but the oral challenge is clearly the most physiologically relevant one. However, an effect on the latter has been difficult to demonstrate as the oral challenge itself leads to a rapid loss of response, thus depriving the researcher of an important readout: antigen-specific cellular proliferation and antibody response.

The dosages per body weight used in this experiment as well as the antigen type were chosen to be as similar as possible to those used routinely in rodents as a sensitisation

protocol. In older rodents, a single dose of OVA corresponding to our high (1,000 mg) dose lies at the lower end of a tolerogenic protocol, reported to be >0.5 mg/g body weight [8], yet would sensitise neonates. A dose corresponding to our low (10 mg) dose would be expected to sensitise even older rodents and would certainly be expected to sensitise neonatal mice. Another factor which might account for the observed species differences could be the amount of intact IgG transported across the gut wall. However, very similar values for this have been reported for pigs and rodents [32, 33]. Thus, we conclude that the colostrum-deprived status, the dosage and the timing of our experiment appear to fulfill the conditions which in rodents would lead to sensitisation.

To generalise our results, we could show that this work was similar to previously published work on the effect of

soya protein given to piglets at birth, where such neonatal exposure reduced IgG responses in response to a soya-containing diet [15]. However, the response to a systemic intraperitoneal challenge with soya had failed to reach significance in the previous experiment [15]. The difference between the extent of tolerance induction may be due to different antigenicities of soya and OVA. Alternatively, there may have been confounding effects of antibody/antigen derived via colostrum and milk: in the earlier experiment, piglets had been reared conventionally on the sow, and thus had obtained both soya protein and soya antibody via the sow's colostrum and milk, whereas the present experiment dealt with piglets reared without any maternally derived factors. Nevertheless, in both experiments, giving relatively low doses of antigen orally to neonatal piglets reduced rather than augmented secondary responses.

It may also be of interest to mention that anti-OVA titres in the donor animals fed an egg-white-containing diet had continued to rise over the whole experimental period of 4 weeks, in contrast to a complete lack of IgG antibody rise in the experimental neonatal animals after the dietary challenge 16 days later. Thus, in spite of demonstrating tolerance defined by a lack of IgG response to intraperitoneal injection of OVA, these donor animals had increased their IgG titre in response to dietary OVA up to this point in time. This difference may be due to the different ages or different immunological maturity: a single OVA dose given to the OVA-naïve neonatal animals appeared to have a more profound tolerising effect than the continuous dietary exposure of 3-week-old weaner pigs.

A more detailed look at the cellular mechanisms involved revealed that the classical definition of 'oral tolerance', i.e. reduced IgG antibody responses to a later systemic challenge, was accompanied by other differences, compatible with previously described phenomena reported to accompany oral tolerance. The tertiary antigen-specific *in vitro* proliferative response (generally accepted to be due to antigen-specific memory T cells) also showed evidence of tolerance induction. However, this differed depending on the route of the antigenic challenge: only T cells from piglets challenged by intraperitoneal OVA proliferated after a subsequent *in vitro* antigenic stimulation. This proliferation was reduced/abrogated by OVA exposure at birth. The tissue which showed this effect most clearly was the MLN. Both 10 and 1,000 mg of OVA at birth resulted in an almost total lack of antigen-specific cellular responses in MLN, whereas spleen cell proliferation was only reduced by the higher OVA

dose. Thus, the reduced systemic antibody response was paralleled by reduced proliferative memory T responses in MLN and to a lesser extent in the spleen.

The piglets receiving oral OVA at birth or later egg white in the diet showed no consistent OVA-specific T-cell proliferation in any of the tissues studied. Nevertheless, 26-day-old piglets which had experienced the novel diet for 10 days did show phenotypic evidence of T-cell activation and progression towards a memory status. This was partially abrogated for those given OVA at birth. No polyclonal phenotypic differences in T cells have been reported previously in animals challenged systemically. We assume that the measurable increase in memory Th-cell numbers prevented by the single low dose of OVA at birth seen in this experiment is due to the prolonged nature of the dietary challenge, as well as to a relatively low background number of other memory cells in these animals which were totally antigen-naïve at birth and had been reared in a sterile environment. Interestingly, whereas both Tc and Th showed evidence of memory induction by diet, only Th-cell phenotypes were affected by the experience of OVA at birth. This suggests that the exposure to OVA-containing egg white in the diet resulted in both memory Tc and Th cells in the local lymph nodes, but neither of them capable of OVA-specific *in vitro* proliferative recall responses 10 days later. Cytotoxic T cells are generally thought to be involved in responses to intracellular viral pathogens and not to dietary antigens, their involvement in this system investigating the immune response to dietary antigen is therefore surprising and not clear. It is however possible that these memory Tc cells may not be specific for OVA.

The role of CD4+ cells in the induction and maintenance of oral tolerance has been demonstrated [34–36]; our work supports the view that these cells are mainly induced in the lymph nodes draining the mucosa. This work also confirms that they do not respond with antigen-specific *in vitro* proliferation 10 days later. The phenotypic evidence further suggests that they do not respond *in vivo* to a second oral challenge either. We may postulate that the single dose of OVA given orally at birth had led to the production of similar activated/memory but unresponsive CD4+ cells in MLN, just as it had in the older pigs fed with the egg-white-containing diet, but that this change had been too small or too transient to be detectable in the 26-day-old animals. It may have been followed by the permanent loss by apoptosis or emigration into 'anergic sites' such as the mucosa. The parallel reduced proliferation in spleen caused by the high but not the low dose suggests that this anergy or cellular loss is

system wide but the extent dose dependent. It is most clearly seen in local lymph nodes. The lack of OVA-responsive CD4+ cells almost certainly accounted for the lack of systemic IgG responses, too.

Early but transient responses followed by tolerance in response to oral antigen have been shown before [37]. There is considerable evidence that memory cells specific for dietary antigen are induced in MLN by dendritic cells from the mucosa [38, 39], become memory cells [40] and return to and are sequestered in the diffuse mucosal tissues, but in an anergic state which responds to in vitro challenge by apoptosis [20, 41, 42]. We failed to find supporting phenotypic evidence for their appearance in the mucosa; there were no obvious changes in cellular composition in the jejunum. However, given the size of the mucosal cell compartment, it is perhaps not surprising that measurable changes in the small local lymph nodes are blurred in this much larger cell pool. Given the lack of OVA-specific cell proliferation in either mucosal tissue, we can state that active (responsive) memory cells were not found in mucosal sites in either group.

In contrast to the clear and profound effects of antigenic experience immediately after birth, the effect of antibody uptake from hyperimmune or tolerised serum was much less obvious. Neither of the functional tests in the form of serum IgG responses or of in vitro T-cell proliferation showed differences between experimental groups. The phenotypic differences found when comparing the proportion of memory CD4+ cells between the three groups of piglets challenged by diet showed fewer memory CD4+ cells in the presence of tolerised and more such cells in the presence of hyperimmune serum. However, without supporting functional evidence, we cannot exclude at this stage that this may have been due to chance.

It appears that the effect of giving the sera at birth had already caused a degree of immunological activation, as

all memory CD4+ cells in the systemically challenged animals appeared more numerous in the second experiment, even in the controls. This may have made it more difficult to detect phenotypic effects due to the experimental variables. In this context, it also seems likely that the extremely naïve status of the colostrum-deprived animals in experiment 1 had enabled us to detect an effect on an OVA-specific CD4+ cell subset; as yet such gross changes in cellular composition due to a single antigen have not been demonstrated in rodents.

In conclusion, the finding of the tolerising effect of low antigenic exposure in the neonatal pig may have implications for human health, suggesting that there are species differences and that in some species at least, small amounts of dietary antigen do not sensitise a neonatal antigen-naïve animal, but rather predispose to tolerance, as they do in older animals. The presence of antibody alone appears to have no effect.

Many questions remain to be answered. It is vital to establish if the human response is more like that observed in rodents or in pigs. It is also important to know if other antigens, such as for example peanuts, can also induce oral tolerance in neonatal pigs and, most importantly, we need to know if the phenomenon of oral tolerance parallels a lack of atopic reactions. The latter two questions could be answered by repeating the above experiments with the pig model for peanut allergy used by Helm et al. [43].

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