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Failure of SCID mice to generate an oral tolerogen after a feed of ovalbumin: a role for a functioning gut-associated lymphoid system

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SUMMARY

The role of the mucosal immune system in the generation of circulating tolerogenic ovalbumin (OVA) moieties has been investigated after a single feed of the protein. Serum collected from SCID mice 1 hr after a 25-mg feed of OVA was unable to transfer tolerance of delayed-type hypersensitivity (DTH) into naive BALB/c recipients. This is in contrast to serum collected from BALB/c mice which was able to transfer DTH tolerance to naive BALB/c recipients. The levels of circulating OVA detected in the serum of SCID mice 60 min after feeding OVA were approximately half those detected in the serum of BALB/c mice at the same time-point. However even dose adjustment of SCID mouse serum to a level of immunoreactive OVA equivalent to that found in BALB/c serum was unable to induce DTH tolerance in BALB/c recipients. This failure of SCID serum to transfer tolerance was shown to be unrelated to the germ-free conditions under which SCID mice are kept. Serum from OVA-fed germ-free BALB/c mice transferred DTH tolerance at equivalent levels to serum from conventionally reared BALB/c mice. When the intestinal morphology and intraepithelial lymphocyte (IEL) numbers in the duodenum of SCID mice were compared to conventionally reared and germ-free BALB/c controls, SCID mice were characterized by a lower number of IEL with a different morphology from the majority of IEL found in BALB/c mice.

INTRODUCTION

It has previously been reported that serum collected from BALB/c mice 1 hr after a feed of the protein antigen ovalbumin (OVA) can transfer antigen-specific tolerance of systemic delayed-type hypersensitivity (DTH) reactions into naive recipient mice.[1–3] Earlier studies of oral tolerance to OVA and transfer of tolerance had demonstrated that the tolerogenic effect of feeding OVA could be removed if mice received an intravenous injection of anti-OVA antibodies immediately after feeding the OVA.[4] Moreover the tolerogenic activity of serum collected from mice 1 hr after feeding OVA could be abolished if the immunoreactive OVA detected in the serum was affinity adsorbed before injection into recipient mice.[5] Furthermore, tolerance was not induced after transfer of serum from donors which had been parenterally injected with OVA prior to serum collection.[2] These findings suggest that within 1 hr of oral administration the OVA has in some way been modified either by gastrointestinal proteolytic enzymes, by filtration through a biological membrane (the gut epithelium), or by active processing by gut epithelial cells or professional antigen-processing cells of the mucosal or systemic immune system, thereby producing a transferable OVA 'tolerogen'.

The SCID mouse is characterized by a complete lack of all the major serum immunoglobulin classes and an absence of functioning mature T and B cells[6,7] although pre B cells and pre T cells are present.[8,9] Thus the mutation affects T- and B-cell maturation and affected animals generate only 10% of the expected lymphoid tissue (mainly stromal cell elements).[10] Peyer's patches are not normally observed[6] although M cells in low numbers may be present (T. Savidge, personal communication). Macrophage and natural killer cell functions are normal or slightly elevated compared to BALB/c controls[11–13] and SCID mice appear to have a normal tissue distribution of major histocompatibility complex (MHC) class II antigens compared to BALB/c controls.[11] SCID and BALB/c mice are congenic at the immunoglobulin heavy chain locus and therefore display an identical MHC profile. The use of the above strains as serum donors permits investigations into the function of the specific immune system (T and B cells) within 1 hr of an antigen feed using an established serum transfer model. It also facilitates comparisons of the roles of both the adaptive and innate immune systems including macrophages, the intestinal epithelium and gastrointestinal digestion in the generation of a circulating 'tolerogen' after feeding.

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MATERIALS AND METHODS

Animals

Inbred BALB/c (H-$2^{d/d}$) and SCID (H-$2^{d/d}$) mice were bred in the Institute of Child Health.

SCID mice were housed in an isolator (Harlan Olac Ltd, Bicester, UK), received irradiated water *ad libitum* and were maintained, antibiotic free, on a 12-hr light–dark cycle. Inbred germ-free BALB/c (H-$2^{d/d}$) mice were kindly donated by Professor S. Challacombe (Department of Oral Medicine, Guys Hospital, London, UK) and were used immediately on arrival at the Institute of Child Health. All animals were maintained on a diet (Labsure CRM: Labsure Ltd, Manea, UK) previously shown to be free of egg and milk proteins. SCID mice received irradiated food of identical composition. All animals were 6–8 weeks of age.

Antigens

OVA (Fraction V, Sigma Chemical Co., Poole, UK) was dissolved in 0·15 M sterile saline and prepared at a standard concentration (100 mg/ml).

Enzyme-linked immunosorbent assay (ELISA) for OVA detection in serum

Flat-bottomed ELISA plates (Linbro, Flow ICN Biochemicals Ltd, Irvine, UK) were coated (100 μl/well) with affinity-purified rabbit anti-OVA IgG (1 μg/ml) in carbonate coating buffer, pH 9·6 and left for 48 hr at 4°. The plates were washed [phosphate-buffered saline (PBS)–Tween-20] and blocked using 1% normal goat serum (NGS) dissolved in PBS–Tween-20 (200 μl/well) for 1 hr at 37°. The plates were then washed and a range of dilutions of OVA (Fraction V, Sigma Chemical Co.), in 1% NGS, 0·01% normal mouse serum (NMS) in PBS–Tween-20 (100 μl/well) were used in duplicate to give a standard curve over the range 10 μg–1 ng. The test sera were added in duplicate (100 μl/well) neat, diluted 1/10 and 1/100 in 1% NGS, PBS–Tween-20 solution and incubated at 37° for 3 hr. The plates were then washed and a 1 μg/ml dilution of a biotinylated rabbit anti-OVA was added (100 μl/well) and incubated for 2 hr at 37°. After washing, a 1/1000 dilution of streptavidin–horseradish peroxidase (HRP) (Amersham International, Amersham, UK) was added (100 μl/well) and the plates were incubated for 1 hr at 37°. The plates were then washed three times before the addition of substrate solution (100 μl/well) containing 0·5 mg/ml of *O*-phenylenediamine (Sigma) in 0·05% H_2O_2/0·1 M citric acid/0·2 M phosphate buffer. The colour reaction was stopped after incubation at 37° for 20 min by the addition of 25 μl of 4 M H_2SO_4/well. Absorbance values were measured at 490 nm using an ELISA reader (Titertek Multiscan, Flow).

Adoptive transfer of serum

Adult donor mice were fed by gavage with 25 mg of OVA or 0·25 ml of saline alone, using a 20-gauge, 30-mm olive-tipped cannula (Fine Science Tools, Vancouver, Canada). One hour after feeding OVA or saline the mice were bled by cardiac puncture under halothane anaesthesia. The blood was pooled for each group, allowed to clot and then centrifuged at 2500 *g* for 10 min at room temperature. The serum collected from each experimental group was injected intraperitoneally into the recipient mice at a dose of 40 μl/g body weight, equivalent to approximately 15 ng of immunoreactive OVA/g body weight.

Systemic immunization

Seven days after serum transfer the recipient mice were parenterally immunized in the left hind footpad with 100 μg of OVA emulsified in 0·05 ml of complete Freund's adjuvant (Bacto H37Ra, Difco Ltd, Detroit, MI).

Measurement of DTH

The antigen-specific cellular immune response in experimental animals was measured by determining the degree of DTH reaction using the footpad swelling test, 3 weeks after systemic immunization. Mice were injected into the right hind footpad with 100 μg of heat-aggregated OVA in 0·05 ml of sterile saline. Footpad thickness was measured using a dial gauge micro-caliper (Mitutoyo, no. 7301, MFG Co., Tokyo, Japan) both before and 24 hr after antigen challenge. The difference between the means of the two measurements gave an index of footpad swelling in millimetres which was used for group comparisons.

Assessment of specific anti-OVA IgG antibody responses

Flat-bottomed 96-well ELISA plates (Flow ICN Biochemicals Ltd) were coated (100 μl/well) with OVA (100 μg/ml in carbonate coating buffer pH 9·6) and left for 24 hr at 4°. The plates were washed three times with PBS–Tween-20 and blotted dry. The free sites were then blocked using 1% NGS in PBS–Tween-20 (200 μl/well) for 1 hr at 37°. After washing the plates, aliquots of both the test sera and the standard affinity-purified mouse anti-OVA IgG for the standard curve were diluted in 1% NGS, 0·01% NMS in PBS–Tween-20 and were added to the plates (100 μl/well). After a 90-min incubation at 37°, the samples were discarded and the plates washed three times in PBS–Tween-20 before blotting dry. Alkaline phosphatase-conjugated goat anti-mouse IgG Fc-specific antibody (Sigma) was diluted to 1/3000 in 1% NGS in PBS–Tween-20. The wells were filled with 100 μl of this solution and incubated for a further 90 min at 37°; the plates were then washed and dried as before. The wells were then filled with 100 μl of a solution containing 1 mg/ml *p*-nitrophenyl phosphate (PNPP) in a solution of carbonate coating buffer containing 0·1 mM magnesium chloride. The plates were incubated at 37° and the colour reaction stopped after 40 min by the addition of 25 μl of 3 M sodium hydroxide. Absorbance values were measured at 410 nm using an ELISA reader (Titertek Multiscan, Flow).

General histological techniques

Immediately following death under halothane anaesthesia, pieces of duodenum (10 cm beyond the ligament of Treitz) were removed, placed on a card, cut open and immersed in fixative (10% buffered formalin), villus surface upwards, embedded in paraffin wax and sectioned (5 microns). The sections were then stained with haematoxylin and eosin. Specimens were examined under an Olympus BO61 microscope at × 1000 (oil immersion) magnification and only well-cut sections with a single epithelial cell layer were counted. Intraepithelial lymphocyte counts were expressed as the number of IEL/1000 villus epithelial cells.[14] Counts were obtained by enumerating epithelial and lymphoid cell nuclei lying unequivocally above the basement membrane and a total of 1000 cells were counted in each specimen.

Statistical comparisons

The measurements of IgG antibody, systemic DTH and OVA levels in serum were expressed as means ± 1 SD and all group comparisons were made using unpaired two-tailed Student's *t*-tests.

RESULTS

Immunological effect of adoptive transfer of gut-processed OVA from SCID mice into BALB/c recipients

Three weeks after systemic immunization serum anti-OVA antibodies and systemic DTH responses were assessed and these results are shown in Fig. 1.

Serum from OVA-fed BALB/c donor mice suppressed the systemic DTH responses in syngeneic BALB/c recipients compared with recipients of serum from saline-fed SCID mice ($P < 0{\cdot}0005$; Fig. 1). Serum from OVA-fed SCID mice did not induce significant suppression of systemic DTH in BALB/c recipients compared to recipients of serum from saline-fed SCID mice or compared to recipients of OVA-spiked serum

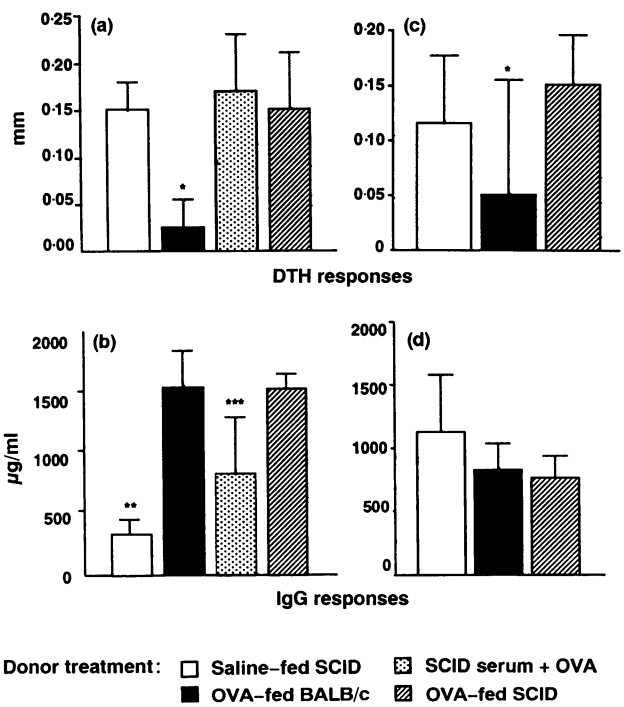

Figure 1. (a,b) Lack of tolerogenic effect of serum from OVA-fed SCID mice in naive BALB/c mice. Recipient groups ($n = 8$) received serum from saline-fed SCID mice, OVA-fed BALB/c mice, SCID serum plus OVA 'spike' and OVA-fed SCID mice. The systemic immune responses [(a) DTH; (b) antibody)] to OVA were assessed in BALB/c recipients and expressed as means ± 1 SD. *$P < 0{\cdot}0005$ compared with saline-fed group; ** and ***$P < 0{\cdot}01$ compared with the OVA-fed BALB/c group. (c,d) Effect of dose adjustment of serum from OVA-fed SCID mice to give equivalent levels of immunoreative OVA as in BALB/c mice 60 min after a single 25-mg feed. BALB/c recipients ($n = 8$) received serum from saline-fed SCID mice, OVA-fed BALB/c mice, OVA-fed SCID mice (dose adjusted to normal BALB/c levels). The systemic immune responses [(c) DTH; (d) antibody] are presented as means ± 1 SD. *$P < 0{\cdot}002$ compared with the OVA-fed SCID group.

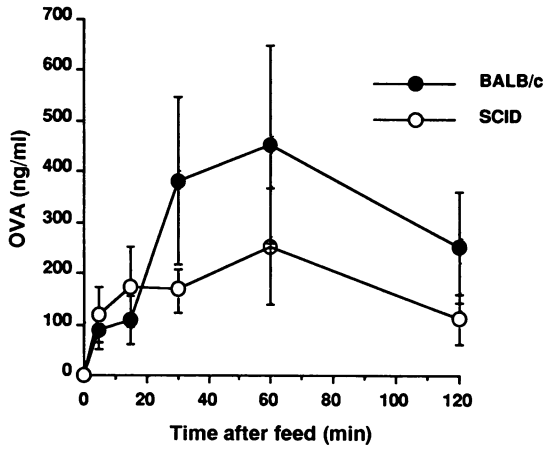

Figure 2. Serum concentrations of OVA measured at different times after feeding 25 mg of OVA to groups ($n = 5$) of BALB/c and SCID mice. The results are presented as means ± 1 SD (OVA levels at 30 min, $P < 0{\cdot}005$).

(Fig. 1). The systemic antibody response in recipients of serum from OVA-fed BALB/c mice or in recipients of OVA-fed SCID mice was significantly primed compared to recipients of OVA-spiked SCID serum ($0{\cdot}01 < P < 0{\cdot}001$; Fig. 1) or, compared to recipients of serum from saline-fed SCID mice ($P < 0{\cdot}0005$; Fig. 1).

Concentration of circulating immunoreactive OVA after feeding SCID and BALB/c mice

OVA was detected in the serum of both groups of mice 5 min after feeding and levels of the protein peaked in both groups after 60 min (Fig. 2). The profile of circulating OVA was similar in both groups until 30 min after the feed when the levels in the SCID mice began to rise less steeply. In contrast, the BALB/c mice continued to show a rapid increase in circulating OVA levels [180 ± 15 ng/ml in SCID mice compared to 390 ± 120 ng/ml in BALB/c mice at 30 min post-feeding ($P < 0{\cdot}005$; Fig. 2)]. Both groups showed decreasing levels of circulating OVA 120 min after feeding.

Transfer of serum from SCID and BALB/c mice after OVA feeding (adjusted to comparable OVA levels)

Serum IgG antibody and systemic DTH responses of these animals are shown in Fig. 1c,d.

Serum from OVA-fed BALB/c mice induced tolerance of DTH in syngeneic recipients compared to recipients of serum from saline-fed SCID mice ($0{\cdot}02 < P < 0{\cdot}01$; Fig. 1). In contrast, even with dose adjustment of the SCID serum there was no demonstrable suppression of systemic DTH responses in BALB/c recipients and these values were indistinguishable from those observed in saline-fed SCID mice (Fig. 1). There was no significant difference in the systemic antibody responses between the three experimental groups (Fig. 1).

Adoptive transfer of serum from OVA-fed germ-free BALB/c mice into conventionally reared BALB/c recipients

In these experiments germ-free BALB/c mice were used as serum donors in order to investigate the possible effect of gut

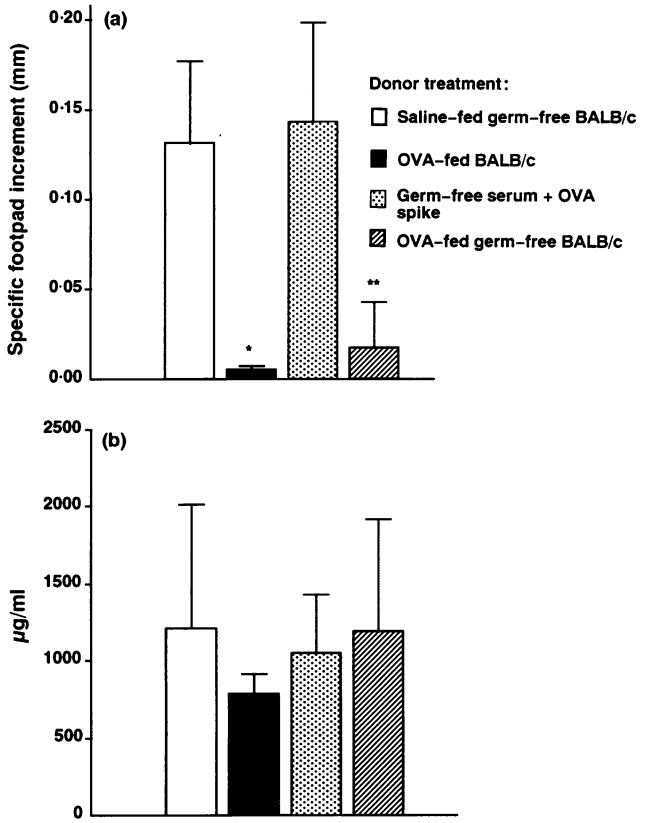

Figure 3. Effect of serum transfer of gut-processed OVA from germ-free BALB/c mice into conventionally reared BALB/c recipients. Recipient mice (n = 8) received either serum from saline-fed germ-free BALB/c mice, OVA-fed BALB/c mice, germ-free BALB/c serum plus OVA or OVA-fed germ-free BALB/c mice. The systemic immune responses [(a) DTH; (b) antibody] were measured in the BALB/c recipients and presented as mean ± 1 SD. * and **$P < 0{\cdot}0005$ compared with group receiving germ-free serum spiked with OVA.

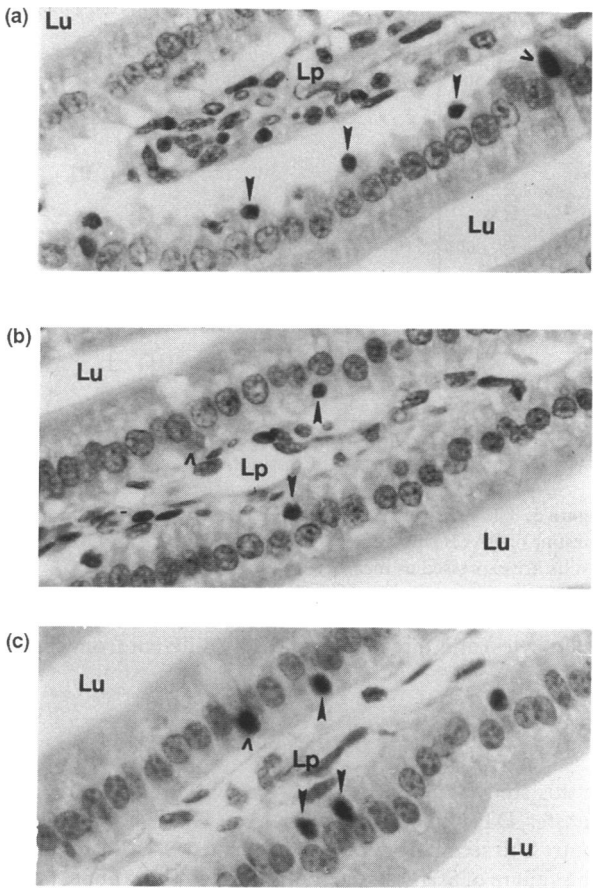

Figure 4. Haematoxylin and eosin-stained murine intestine, under a ×400 magnification demonstrating typical IEL (arrowheads) in (a) BALB/c mouse jejunum, (b) germ-free BALB/c mouse jejunum and (c) SCID mouse jejunum, ∧ goblet cells, Lu = lumen of the gut, Lp = lamina propria.

flora on the generation of an OVA tolerogen. Serum from OVA-fed, conventionally reared, BALB/c mice significantly suppressed the systemic DTH responses in syngeneic recipient mice when compared to (1) recipients of serum from saline-fed germ-free BALB/c mice or (2) recipients of OVA-spiked serum ($P < 0{\cdot}0005$ for both comparisons; Fig. 3). Serum from OVA-fed germ-free BALB/c donor mice also significantly suppressed the systemic DTH responses to OVA in recipient mice when compared to responses in recipients of serum from saline-fed donors or in comparison to recipients of OVA-spiked serum ($P < 0{\cdot}0005$ for both; Fig. 3). There was no significant difference in the systemic antibody responses against OVA between any of the four experimental groups (Fig. 3).

Intestinal morphology and intraepithelial cell numbers in SCID, BALB/c and germ-free BALB/c mice

In order to determine whether there are major differences in morphological features which could underlie the observed immunological differences, the morphology of SCID mouse small intestine was compared with the small intestine of age-matched BALB/c mice (villus length, crypt depths and IEL numbers). The gross morphology of each section was examined (Fig. 4). All three mouse types had recognizable villi and crypts with no gross differences apparent in either crypt depth or villus length. On dissection the SCID mouse gut appeared to be more fragile than the BALB/c mouse gut suggesting a thinner mucosa. On examination of the lamina propria of the three mouse types it was apparent that the SCID mouse gut had fewer cells in the lamina propria when compared to the other two mouse types.

IEL morphology. In general the IEL observed in the sections obtained from conventionally reared BALB/c mice and germ-free BALB/c mice presented as round cells with a large dense nucleus and very little cytoplasm, i.e. similar to that of small lymphocytes in the blood and lymphoid organs. In contrast, the majority of IEL observed in the sections from the SCID mice were larger than the IEL seen in the BALB/c mice and had a rough 'angry' morphology with more cytoplasm which was often granulated (Fig. 4).

IEL counts. Conventionally reared BALB/c mice had 99·5 ± 2·5 IEL/1000 enterocytes compared to germ-free BALB/c mice which had a mean IEL count of 53 ± 17/1000 enterocytes. SCID mice had even fewer IEL (34 ± 7/1000

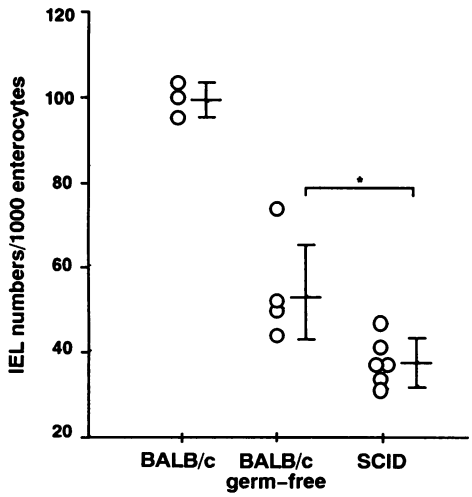

Figure 5. The relative frequency of IEL/1000 enterocytes in the small intestine of BALB/c mice, germ-free BALB/c mice and SCID mice. The results are expressed as means ± 1 SD. $*P < 0{\cdot}025$.

enterocytes) and this was significantly different from germ-free BALB/c mice ($P < 0{\cdot}025$; Fig. 5).

DISCUSSION

In this study serum from OVA-fed SCID mice was unable to transfer DTH tolerance to OVA into BALB/c recipients in contrast to serum from identically treated BALB/c donor mice. This failure of SCID serum to transfer tolerance appears to be a qualitative rather than a quantitative difference as dose adjustment of SCID serum for adoptive transfer to levels of OVA equivalent to those in tolerogenic BALB/c serum had no effect on its inability to transfer DTH tolerance. Furthermore, this absence of tolerogenic activity in SCID serum appeared not to result from microbiological colonization as the use of germ-free BALB/c mice as serum donors provided a tolerogenic signal which was indistinguishable from that given by the serum of conventionally reared BALB/c mice. Compared to conventionally reared BALB/c and germ-free BALB/c mice, SCID mice were found to have fewer IEL and these were characterized by a different morphology but it would be premature at this stage to ascribe our observations to this fact alone.

On the basis of these novel findings two working hypotheses can be proposed: (1) specific immune cells (T and/or B cells) are directly involved in the 'processing' of fed OVA in order to generate a tolerogenic form of OVA; or (2) specific immune cells are a necessary component in the development of normal intestinal function which ultimately results in the generation of the OVA 'tolerogen'.

Additional evidence suggesting a key role for a functioning immune system in the generation of an OVA tolerogen was obtained by Bruce *et al.*,[15] who demonstrated that irradiated mice lost the capacity to produce such a tolerogen. Furthermore, this function was only restored following reconstitution of these mice with normal spleen cells. However, the same authors reported that irradiation severely damaged the mucosal architecture and influenced the capacity to generate an OVA tolerogen. Both the present studies and those reported by Bruce *et al.*[15] provide evidence for a requirement of immune cells in the generation of a tolerogen. However, the results do not permit a distinction to be made between the role of the IEL in the normal development of the mucosal system and a more direct role in the generation of a tolerogenic moiety.

Evidence for the direct involvement of specific immune cells in antigen processing and presentation, especially for tolerance induction, has recently been published.[16,17] These groups proposed that small resting B cells can process and present soluble protein antigens to T cells. They postulate that if the T cells which encounter antigen on small resting B cells are virgin T cells, the presentation of antigen results in specific tolerance to that particular antigen and not the usual antigen-specific activation.

In the case of oral tolerance to OVA the mice are immunologically naive to OVA and, therefore, any OVA is likely to be presented to virgin T cells. As the main immune cells of the Peyer's patches are B cells[18] the above mechanism of tolerance induction seems to be an attractive model for DTH suppression after serum transfer.

In this model, it may be envisaged that small resting B cells in the Peyer's patches of the small intestine could capture and process OVA from the lumen of the gut and present it to virgin T cells, thereby inducing OVA-specific tolerance in the T-cell population. In the serum transfer model, oral tolerance to OVA may be induced by a soluble factor which is probably a processed form of OVA. Furthermore, on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of affinity-absorbed OVA from tolerogenic BALB/c serum and non-tolerogenic SCID serum there appears to be a fragment of OVA-like material in the tolerogenic serum which is not present in the non-tolerogenic SCID serum (our unpublished observations, manuscript in preparation). In this situation, for the small resting B-cell hypothesis to be tenable one would have to propose that antigen processed by B cells would also reach the circulation, thereby permitting transfer to naive recipients.

Although B cells can be very efficient antigen-presenting cells, they do not behave like dendritic cells and macrophages, which are apparently able to present antigens without selection. The array of peptides presented by antigen-presenting cells other than B cells is determined by the relative concentrations of peptides in the MHC assembly vesicles. Therefore the first hypothesis is unlikely, because, in order for B cells to take up antigen non-specifically rather than via the surface immunoglobulin receptor, there would need to be a great excess of antigen extracellularly. Although it is known that B cells can process protein antigens into small peptides for insertion into the groove of the MHC molecule there is no evidence to support the proposal that B cells are able to process and release protein antigens.

IEL have been previously implicated in immunoregulation of the immune response after feeding of protein antigens.[19] However, in our studies the relatively short interaction time of 60 min would appear to be insufficient for IEL to recognize and respond to the protein antigen, thereby providing an antigen-specific signal which could be transferred in serum. On the other hand, any loss of integrity of the mucosal immune system early in development could dramatically affect the function of the gut and consequently its capacity to generate a transferable OVA tolerogen. However, there is no evidence for

any deficiency in the spectrum of gastrointestinal enzymes in SCID mice and it is not clear what might be the effect of absent B and T cells in the developing mucosae on the morphology and phenotype of the epithelial cells of the gut. Nevertheless intestinal epithelial cells have been implicated in the processing of orally administered antigen for tolerance induction.[20,21] The limited studies reported here on intestinal morphology and histology suggest that SCID mice have a different IEL morphology, which might be expected to have different cytokine products and requirements which could directly affect the development and function of the surrounding mucosal cell types. Previous reports have speculated that the IEL present in SCID mice may be very early precursors of T lymphocytes before the rearrangement of the T-cell receptors.[22] This proposal suggests that these cells would be unable to produce the array of cytokines that would normally be produced by the heterogeneous population of IEL normally present in a fully functioning intestinal mucosa.[23–25] Although a comprehensive biological explanation for the mechanisms involved in oral tolerance and in the production of a transferable OVA tolerogen remains elusive, a functioning intestinal immune system (GALT) does appear to be essential.

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