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(54) Title: IMPROVEMENT OF ORAL VACCINES

(57) Abstract: The present invention relates to a method for preparing and improving oral vaccines by modifying polypeptides which comprise at least one immunogenic epitope, particularly at least one immunogenic T cell opitope.

Improvement of oral vaccines

Description

The present invention relates to a method for preparing and improving oral vaccines by modifying polypeptides which comprise at least one immunogenic epitope, particularly at least one immunogenic T cell epitope.

An orally administered soluble antigen can induce a specific peripheral immune tolerance ("oral tolerance") which prevents systemic immune responses against food-derived antigen and antigens of commensal microorganisms (Weiner, Immunol.Today 18 (1997), 335-343). On the other hand, soluble factors produced by enteric pathogens can induce active mucosal and systemic immune responses that protect against a subsequent infection by the same or a related pathogen. Pre-clinical studies using rodent models have shown that both types of immune responses can be induced experimentally, and that oral tolerance can be used to suppress autoimmune diseases (Weiner (1997), supra), whereas oral vaccination with soluble antigens mixed with a mucosal adjuvant protects against various infectious diseases (Shalaby, Clin.Immunol.Immunopathol.74 (1995), 127-134). Unfortunately, in human clinical trials testing the oral delivery of soluble antigens for the induction of specific immune responses, rather low efficacies have been obtained for essential unknown reasons indicating the need to further optimize oral antigen delivery (MacDonald, Curr.Opin.Immunol.10 (1998), 620-627) (Michetti et al., Gastroenterology 116 (1999), 804-812). A better understanding of the induction of specific immune responses against oral antigens could also help to predict the allergenic potential of "novel" transgenic food (Lehrer et al., Crit.Rev. Food Sci.Nutr.36 (1996), 553-564).

Amount, purity (Benson et al., J.Immunol.162 (1999), 6247-6254), conformation (Peng et al., Scand.J.Immunol.48 (1998), 491-496) and chemical modification (Peng et al., Scand.J.Immunol.47 (1998), 475-480) of an oral antigen influence its immunogenicity. All these parameters could be severely altered as a result of luminal degradation by digestive enzymes, indicating that differential digestion could provide a major contribution to the variance of immune responses (Shalaby (1995), supra). The experimental evidence for such a role of digestion in oral immune responses, however, is controversial.

For the most commonly used model antigen, ovalbumin, it is unknown to what extent gastrointestinal digestion occurs. Based on its very slow in vitro digestion by pepsin and trypsin it has been assumed that most oral ovalbumin remains essentially intact during its luminal passage through the stomach and the small intestine (Furrie et al., Immunology 86 (1995), 480-486) but this has not been experimentally verified. Shortly after feeding, significant amounts of intact ovalbumin appears in the serum of mice but only very low amounts of smaller fragments (Bruce & Ferguson, Immunology 59 (1986), 295-300; Furrie et al. (1995), supra) which seems to support a minor role of luminal digestion. However, free serum levels might underrepresent the intestinal absorption of small fragments because of their rapid clearance (Bloch et al., Gastroenterology 95 (1988), 1272-1278).

It is controversial, whether luminal digestion (if it occurs at all) alters the net immunogenicity of an oral antigen. Several investigators reported that previous administration of protease inhibitors diminished the immune responses against an oral antigen which was interpreted as evidence for luminal digestion playing an important role in the induction of an immune response. However, these data could not be reproduced (Furrie et al. (1995), supra) and another report indicated that protease inhibition actually enhances the antigen uptake (Ziv et al., Biochem.Pharmacol.36 (1987),

1035-1039) which could lead to a stronger immune response. Protease inhibitor studies could be difficult to interpret because of their multiple physiological effects. Other studies showed that intragastric but not rectal administration led to significant immune responses and this was taken as evidence for an important role of intestinal digestion, but again the data could not be reproduced (Furrie et al. (1995), supra) and rectal administration might severely alter the gut physiology.

If luminal digestion of an oral antigen to small fragments occurs, this could influence the entry sites and permeation rates for oral antigens. The intestinal epithelium has rather low permeation rates for macromolecules like intact ovalbumin. Some protein is endocytozed by enterocytes (Bijlsma et al., Am.J.Physiol.271 (1996), G147-G155; Kaiserlian & Etchart, Semin.Immunol.11 (1999), 217-224; Zimmer et al., Gastroenterology 118 (2000), 128-137). Some dendritic cells extend their processes between intestinal epithelial cells and might thus be able to directly capture luminal antigens. Specialized M-cells of the follicle-associated epithelium of Peyer's patches transcytose particles and macromolecules and this is generally assumed to be the immunologically most important uptake route for protein antigens (MacDonald (1998), supra) (Mowat & Viney, Immunol.Rev.156 (1997), 145-166). In contrast to these various, rather inefficient permeation pathways for macromolecules, short peptides can very efficiently permeate the intestinal epithelium presumably by a paracellular pathway (Pappenheimer et al., PNAS USA 91 (1994), 1942-1945; Pappenheimer et al., J.Pharmacol.Exp.Ther.280(1997), 292-300). Until now, however, there is no evidence that luminal digestion to small immunogenic fragments might enable an oral antigen to penetrate the permeability barrier of the large intestinal epithelium at greatly increased rates. Further, it is difficult to directly test this hypothesis because of the experimental difficulties to detect the uptake of small immunogenic peptides in situ.

In summary, the role of luminal digestion in the induction of an immune response against an oral antigen is little understood and will need to be more clearly defined in order to rationally improve oral antigen administration.

The present application is based on a study, wherein the extent, localization and kinetics of luminal digestion of orally administered ovalbumin in mice was analyzed by western blot of small intestinal contents. To detect the mucosal uptake of immunogenic fragments in situ, a new method involving adoptive transfer of ovalbumin-specific transgenic T effector cells was developed. In addition, the induction site of ovalbumin-specific naive T helper cells was determined. By these methods it was found that luminal antigen digestion plays a central role in oral antigen delivery. These results are the basis for the optimization of this easy, safe, and well-accepted route of antigen delivery.

Thus, in a first aspect the present invention relates to a method of preparing oral vaccines comprising the steps

(a) providing a polypeptide comprising at least one immunogenic epitope sequence and

(b) modifying said polypeptide sequence by

(i) removing cleavage sites for proteolytic digestion enzymes from the epitope sequence and/or

(ii) introducing cleavage sites for proteolytic digestion enzymes adjacent the epitope sequence.

A further aspect of the present invention are novel polypeptides comprising at least one immunogenic epitope sequence which has been modified by

(i) removing cleavage sites for proteolytic digestion enzymes from the epitope sequence and/or

(ii) introducing cleavage sites for proteolytic digestion enzymes adjacent the epitope sequence.

Further, oral vaccines based on these polypeptides are provided.

Still a further aspect of the present invention is a method for improving the efficacy of oral vaccines comprising the steps:

(a) providing a polypeptide comprising at least one immunogenic epitope sequence and

(b) modifying said polypeptide sequence by

(i) removing cleavage sites for proteolytic digestion enzymes from the epitope sequence and/or

(ii) introducing cleavage sites for proteolytic digestion enzymes adjacent the epitope sequence.

The method of the present invention is particularly suitable for preparing T cell epitopes, e.g. peptide sequences which are capable of stimulating the cellular immune response. These peptide sequences preferably are capable of binding to MHC molecules such as mammalian MHC class I or class II molecules, particularly human HLA alleles such as HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ. The immunogenic epitope sequence preferably has a length of from 10-30 amino acids, more preferably from 15-25 amino acids and most preferably from 17-22 amino acids.

The immune response may comprise an enhanced immunoreactivity directed against the peptide sequence which is administered, or alternatively an enhanced immunotolerance against the peptide which is administered. The nature of the immune response depends on the peptide sequence and the type of administration. For example, oral administration of the immunogenic epitope sequence without an adjuvant may enhance the oral tolerance. In contrast thereto, oral administration with an adjuvant may lead to enhanced immunogenicity.

Thus, a further subject matter of the invention is a modified allergen which can be administered orally to hyposensibilize against this allergen during an

existing allergy by inducing peripherial tolerance. Current approaches induce peripherial tolerance by intravenous injection of allergens. In our approach, improved epitopes can induce a sufficient peripherial tolerance against the allergen to specifically downregulate allergic immune responses which help to improve atopic (=allergic) conditions. Oral administration greatly facilitates the treatment and increases compliance of patients. Epitope improvement can be based on the extensive characterization of allergic epitopes.

The epitope sequence is included in a polypeptide carrier sequence. The polypeptide carrier sequence may be a sequence which is homologous to the epitope sequence, i.e. the polypeptide sequence which naturally contains the immunogenic epitope sequence. Alternatively, the polypeptide sequence may be heterologous to the immunogenic epitope sequence, i.e. the polypeptide carrier may be a naturally occurring or synthetic polypeptide sequence which is foreign to the epitope sequence.

The immunogenic epitope sequence may be derived from a pathogen-associated polypeptide, e.g. a polypeptide which is derived from a virus, a bacterium, a protozoan or a fungi. Examples for viruses are HIV (proteins nef, mag). Examples for bacteria are Helicobacter, Mycobacterium, Chlamydia, Streptococcus and Pseudomonas. Among the Helicobacter polypeptides, pathogenic and virulent factors such as urease A, urease B, catalase, vacA, cagA, HP0231, HP0410 and HP1098 are especially preferred. Examples of protozoan organisms are Plasmodium falciparum. Candida albicans is an example for a fungi.

The immunogenic epitope sequence may also be derived from an autoimmune disease-associated polypeptide such as collagen II being associated with rheumatoid arthritis. Furthermore, the immunogenic epitope may be derived from a tumor-associated polypeptide such as MAGE1, MAGE2 being associated with melanoma.

Possible allergens include pollen allergens (e.g. birch pollen allergen). Allergens that normally enter the body through the respiratory tract are preferred, since local adverse reaction in the gastrointestinal tract are less likely to occur following oral administration for such allergens.

The polypeptide which is used for preparing an oral vaccine and which contains at least one immunogenic epitope sequence has preferably a length of at least 50 amino acids and more preferably of at least 100 amino acids. It should be noted that the polypeptide sequence is modified in a way that (i) cleavage sites for proteolytic digestion enzymes are removed from within the epitope sequence, if necessary, and (ii) cleavage sites for proteolytic digestion enzymes are introduced adjacent the epitope sequence. The cleavage sites are recognized by proteolytic digestion enzymes which occur in the intestinal tract of the animal to be treated. Preferred examples for proteolytic digestion enzymes are pepsin, in particular pepsin A and pepsin C, gastro-intestinal proteases such as trypsin and chymotrypsin. Cleavage sites for these enzymes are for pepsin A and pepsin C at both sides of Tyr, Phe, for trypsin at C-side of Arg, Lys and for chymotrypsin at C-side of Tyr, Trp, Phe, Leu.

The polypeptide of the present invention may be prepared by recombinant DNA techniques, e.g. by expressing a nucleic acid encoding the polypeptide in a suitable host cell. The nucleic acid encoding the polypeptide is preferably operatively linked to an expression control sequence which is active in a desired host cell, e.g. a bacterium such as E.coli or a eukaryotic cell such a yeast cell, an insect cell or a mammalian cell. Suitable expression control sequences for desired host cells are known and described in numerous publications and textbooks such as Sambrook et al., Molecular Cloning, Second Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor. More preferably, the nucleic acid of the present invention is located on a recombinant vector. The recombinant vector may be an extrachromosomal or chromosomal vector. Examples of suitable

vectors are e.g. plasmids, cosmids and viral, e.g. phage vectors. The recombinant vector may be used for transforming a suitable host cell by known methods, whereby a recombinant cell containing a nucleic acid encoding the polypeptide of the invention is obtained. The recombinant cell, e.g. a prokaryotic cell or a eukaryotic cell such as a yeast cell, an insect cell and, particularly, a mammalian cell, may be cultured under suitable conditions, wherein the polypeptide is expressed and the polypeptide may be obtained from the cell and/or the culture medium.

The polypeptide of the present invention may be formulated into an oral vaccine. The oral vaccine may further comprise a pharmaceutically acceptable carrier or diluent and depending on the intended use an adjuvant. Particularly, when administration of the oral vaccine is intended to enhance immunoreactivity against the epitope sequence, the oral vaccine may comprise an adjuvant such as aluminum oxide or cholera toxin, particularly, detoxified cholera toxin. Particularly suitable are chemically or genetically detoxified variants of heat-labile toxin from E. coli (LT) or Choleratoxin (from Vibrio cholerae).

The oral vaccine may be administered in any convenient form such as a liquid, e.g. a solution or dispersion, or as a solid such as tablet, coated tablet, capsule, powder, as it is known in the pharmacological art. The oral vaccine may be administered together with other nutrients, or separately. The administration protocol and the dosage greatly depend on the effect which is to be achieved. Suitable administration protocols are described, for example, in Michetti, P. et al., Gastroenterology 116 (1999), 804, disclosing a study with Helicobacter urease using LT as adjuvants.

According to the present invention an oral vaccine is provided comprising as an active ingredient at least one polypeptide as described above. The oral vaccine may further comprise a pharmaceutically acceptable and particularly orally acceptable carrier or diluent. The oral vaccine may be in the form of

a drinkable liquid or a powder which is soluble or dispersible in a liquid. Alternatively, the oral vaccine may be in the form of a tablet, a capsule, a coated tablet or an effervescent.

The oral vaccine is suitable for any application involving a stimulation of an immune response, particularly a cellular immune response. The oral vaccine may be used for immunization against diseases mediated by pathogens such as viruses, bacteria or protozoans, against autoimmune diseases or against tumor diseases. The dosage of the oral vaccine mainly depends on the type and severity of the disease to be prevented or treated. Typically a dosage of 1 μg to 1 mg per kilogram body weight is administered daily. The administration protocol is e.g. as follows: peptide/protein is dissolved in 30 ml PBS and 3 doses are administered at weekly intervals.

By tailoring the presentation of the immuogenic epitope as described above the efficacy of oral vaccines may be greatly increased. Particularly, the mucosal uptake of immunogenic polypeptide fragments, particularly epitope sequences as described above is improved.

Finally, the present invention relates to a vaccination method, wherein a subject in need thereof, particularly a human, is immunized by orally administering a polypeptide in an amount sufficient to induce an immune response in said subject against an immunogenic epitope sequence.

Further, the present invention is explained in more detail in the following examples and figures.

Figure 1

Figure 1 shows the progressive in vivo digestion of ovalbumin in the small intestine of mice.

Figure 2

Figure 2 shows the mucosal uptake of orally administered ovalbumin in the distal jejunum as revealed by an ovalbumin-specific accumulation of pre-stimulated transgenic T cells.

Figure 3

Figure 3 shows the mucosal uptake of orally administered ovalbumin along the intestine. (A) Local accumuluation of ovalbumin-specific T cells. (B) Mucosal distribution of transgenic ovalbumin-specific T cells after immunization.

Figure 4

Figure 4 shows the blast formation by naive ovalbumin-specific T helper cells in the mesenteric lymph nodes after an oral immunization with ovalbumin and the adjuvant cholera toxin.

Figure 5

Figure 5 shows the division and differentiation of in vivo stimulated transgenic ovalbumin-specific cells after oral immunization with ovalbumin.

Figure 6

Figure 6 shows the variation of specific blast formation after oral immunization for individual mesenteric lymph nodes.

Example

1. Materials and Methods

1.1 Mice and adoptive transfer

Balb/c mice and DO11.10 mice (Murphy et al., Science 250 (1990), 1720-1723) were bred in the Bundesamt für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany, and were kept at specific pathogen

free conditions in full accordance to the german guidelines for animal care. All experiments were approved by the Berlin Animal Welfare Comittee.

Ammonium chloride-treated splenocytes from 8-12 weeks old female Do11.10 x Balb/c crosses were either directly transferred to sex- and age-matched Balb/c recipient mice (4×10^6 TCR-transgenic $CD4^+$ T cells per recipient mouse) or first cultured in vitro in the presence of 1 FM ovalbumin peptide fragment 323-339 (ISQAVHAAHAEINEAGR) for 4 days in 24 well plates at 3×10^6 cells per well, washed, and then transferred into Balb/c mice (5×10^6 transgenic ovalbumin-specific $CD4^+$ T cells per recipient mouse).

Recipients of unstimulated cells were immunized one day after transfer while recipients of prestimulated T cells were immunized four days after transfer.

1.2 Oral immunization

After overnight fasting, chimeric mice or normal Balb/c mice were intragastrically immunized with 5 mg ovalbumin in 100 µl phosphate-buffered saline containing 3% sodium bicarbonate with or without 10.000 U cholera toxin (equivalent to 10 µg fully active toxin). After immunization, the mice were given free access to drinking water and food pellets. Control mice were immunized with 5 mg bovine serum albumin instead of ovalbumin.

1.3 Western blot analysis of intestinal contents

At various time intervals after immunization, Balb/c mice were sacrificed under anesthesia and various small intestinal segments were flushed with ice-cold phosphate-buffered saline. The samples were separated on 16.5 % polyacrylamid gels using a tricine-buffer system with high resolution for small peptides (Schagger & von Jagow, Anal.Biochem.166 (1987), 368-

379), blotted onto nitrocellulose, and detected with a polyclonal rabbit antibody to ovalbumin followed by a peroxidase chemoluminescence assay.

1.4 Immunohistochemistry

Recipients of prestimulated ovalbumin-specific T cells were immunized with 5 mg ovalbumin or 5 mg bovine serum albumin and sacrificed 6 h later. Samples from various small intestinal regions were embedded in freezing medium and frozen on dry ice. Cryostat cross sections (7 μm) were treated with 0.3 % hydrogen peroxide in methanol to block endogenous peroxidase activity, stained with biotinylated KJ1-26 clonotypic monoclonal antibody (Haskins et al., J.Exp.Med.157 (1983), 1149-1169) followed by avidin-biotin-complex, developed with diaminobenzidine, and counterstained with hematoxylin. The number of KJ1-26 positive cells per intestinal cross section was determined.

1.5 Flow cytometry

At various time intervals after immunization, chimeric mice were sacrificed and single cell suspensions of Peyer's patches, mesenteric lymph nodes, and the spleen were prepared. Cells were stained with biotinylated anti-tgTCR antibody KJ1-26, anti-CD4-fluorescein, anti-CD69-phycoerythrin, and anti-B220-allophycocyanin, followed by streptavidin-FarRed (Gibco), and analyzed with a Calibur flow cytometer (Beckton&Dickinson). $CD4^+$ $tgTCR^+$ $B220^-$ lymphocytes were analyzed for their forward scatter and CD69-expression. Alternatively, cells were stained with biotinylated anti-tgTCR antibody KJ1-26, anti-CD4-fluorescein, anti-CD69-phycoerythrin, and anti-B220-allophycocyanin, followed by streptavidin-FarRed (Gibco), and $CD4^+$ $tgTCR^+$ $B220^-$ lymphocytes were analyzed for CD45RB-expression.

1.6 Data Analysis and Statistical Analysis

Statistical significance of differences was determined using the t-test.

2. Results

2.1 Ovalbumin digestion in the small intestine

To characterize the intestinal digestion of orally administered ovalbumin, mice were orally immunized with ovalbumin and small intestinal luminal contents were analyzed after various time intervals by protein immunoblotting with a polyclonal antibody to ovalbumin. In contrast to what was previously assumed (Furrie et al. (1995), supra), most ovalbumin was rapidly digested along its passage through the small intestine (Fig. 1). Undigested ovalbumin and large fragments appeared mainly in the proximal small intestine, whereas ovalbumin-derived peptides in the size range of a few kDa were most abundant in the distal part. The actual amounts of the small peptides were probably higher than suggested by the intensity of staining because small peptides have a low blotting efficacy and not all peptides might contain an epitope that is recognized by the antibody to ovalbumin. In conclusion, the size distribution of ovalbumin digestion products varied considerably along the small intestine.

Fig. 1 demonstrates that progressive in vivo digestion of ovalbumin in the small intestine of mice rapidly generates ovalbumin fragments with a regionally varying size distribution. Intact ovalbumin and large fragments are most abundant in the proximal small intestine whereas small ovalbumin-derived peptides are most prominent in the distal small intestine. Protein immunoblots of luminal contents of proximal, medium, and distal segments of the small intestine sampled at 10 (a), 20 (b), or 30 min (c) after immunization with ovalbumin and cholera toxin (3) are shown. No ovalbumin fragments were detected at later time points (data not shown). Similar results were obtained in 3 independent experiments. Similar results were also obtained for mice immunized with ovalbumin without cholera toxin (data not shown). Luminal contents of control mice immunized with bovine serum albumin showed no detectable immunostaining at any time point (data not shown).

2.2 Mucosal uptake of immunogenic ovalbumin fragments

To determine where immunogenic ovalbumin species penetrate the mucosa along the small intestine, a new method was developed. This method is based on the rapid accumulation of ovalbumin-specific T effector cells in tissues in which immunogenic ovalbumin or ovalbumin-derived peptides are present (Hurst & Barrett, Semin.Gastrointest.Dis.7 (1996), 118-123) (Kearney et al., Immunity 1 (1994), 327-339). Do11.10 mice that are transgenic for a class II-restricted T cell receptor specific for ovalbumin (Murphy et al. (1990), supra) were used as a source of ovalbumin-specific T helper cells. The transgenic T cells were first pre-stimulated in vitro by co-incubation with the recognized ovalbumin epitope which enabled them to populate non-lymphoid tissues including the intestinal mucosa (Hurst et al., J.Immunol.163 (1999), 5937-5945). After stimulation, a few transgenic effector cells were transferred into non-transgenic syngenic Balb/c mice (Pape et al., Immunol.Rev.156 (1997), 67-78) and traced in vivo using a clonotypic monoclonal antibody (Haskins et al. (1983), supra). The chimeric mice were orally immunized and sacrificed 6 h later. Various small intestinal regions were sectioned and immunostained for ovalbumin-specific transgenic T cells. In mice that had been orally immunized with ovalbumin but not in control mice, many pre-stimulated ovalbumin-specific T cells accumulated in the small intestinal mucosa (Fig. 2, Fig. 3A) indicating mucosal uptake of ovalbumin or immunogenic ovalbumin-derived peptides that contain the recognized epitope.

Fig. 2 shows the mucosal uptake of orally administered ovalbumin in the distal jejunum as revealed by an ovalbumin-specific accumulation of pre-stimulated transgenic T cells. Most ovalbumin-specific cells accumulate in the crypt area. Ovalbumin-specific transgenic T cells were pre-stimulated in vitro and then transferred into non-transgenic mice. The chimeric mice were orally immunized with ovalbumin and sacrificed 6 h later. Sections from the distal jejunum were immunostained with a clonotypic monoclonal antibody that specifically recognizes the transgenic cells. Similar results

were obtained for 4 more mice immunized with ovalbumin while in sections from 5 control mice immunized with bovine serum albumin transgenic only a few ovalbumin-specific T cells were detected (see Fig. 3). Similar results were obtained in an independent experiment.

There was a marked proximal-distal accumulation gradient with no detectable ovalbumin-specific T cell accumulation in the duodenum, little accumulation in the proximal jejunum, and strong accumulation in the distal jejunum and the ileum (Fig. 3A) indicating a corresponding proximal-distal gradient in ovalbumin uptake. A comparison of this gradient with the size distribution of ovalbumin digestion fragments along the small intestine (Fig. 1) shows that most ovalbumin uptake occurred in regions with abundant small ovalbumin fragments whereas regions exposed mainly to undigested ovalbumin or large fragments showed little uptake. This could indicate that small fragments of ovalbumin permeate the intestinal mucosa in contrast to undigested ovalbumin.

Fig. 3 shows a variation of the mucosal uptake of orally administered ovalbumin along the small intestine and is restricted to the crypt area. Ovalbumin uptake was indirectly detected by the accumulation of transgenic ovalbumin-specific T cells as shown in Fig. 2. (A) Accumulation of ovalbumin-specific T cells in the duodenum (D), the proximal jejunum (pJ), the distal jejunum (dJ), and the ileum (I) (proximal-distal direction) at 6 h after oral immunization with bovine serum albumin (+) or ovalbumin (o). The data represent averages and SEMs of the number of ovalbumin-specific T cells per cross section of 5 mice per group. Differences between the ovalbumin group and the bovine serum albumin-control group were analyzed with the t test (*: $p < 0.05$, **: $p < 0.01$). (B) Mucosal distribution of transgenic ovalbumin-specific T cells 6 h after immunization with bovine serum albumin (open columns) or ovalbumin (shadowed columns). Ovalbumin-specific cells were localized in the villi (vil) or crypt (cry) area and values were pooled for one section each from the duodenum (D), the

proximal jejunum (pJ), the distal jejunum (dJ), and the ileum (I) of a single mouse. The data represent averages and SEMs for 5 mice per group. Differences between villi and crypt area were analyzed with the t test (**: $p < 0.01$).

The intestinal epithelium which has low permeation rates for macromolecules has recently been shown to have high permeation rates for small peptides up to a molecular weight of a few kDa (Pappenheimer et al. (1997), supra) which is compatible with the proposed differential uptake of ovalbumin fragments depending on their size. Permeation of molecules in the size range of a few kDa has been proposed to occur mainly through a paracellular pathway between the immature crypt epithelial cells (Bjarnason et al., Gastroenterology 108 (1995), 1566-1581; Ma et al., J.Lab.Clin. Med.120 (1992), 329-341). Interestingly, the accumulation of ovalbumin-specific T cells in the intestinal mucosa was largely restricted to the crypt area with very few ovalbumin-specific T cells directly interacting with the enterocytes of the villus epithelium (Fig. 2, Fig. 3B) indicating a preferential uptake of ovalbumin-derived peptides in the crypts. This is in agreement with the proposed permeation pathway for small immunogenic ovalbumin fragments. On the other hand, ovalbumin that might have been endocytosed by the enterocytes is apparently not directly presented to cognate T cells in vivo as has been proposed previously (Kaiserlian & Etchart (1999), supra) since the necessary physical cell-cell interaction is not detectable. This supports that gastrointestinal digestion generates small immunogenic ovalbumin fragments that penetrate the intestinal mucosa whereas intact ovalbumin and large fragments are largely excluded by the intestinal epithelium.

2.4 Ovalbumin-specific T cell activation in mesenteric lymph nodes and in Peyer's patches

To induce an immune response against ovalbumin, ovalbumin-derived peptides must be presented to immune cells. The activation of specific T

helper cells is especially important for protective immune responses against many pathogens and for the induction of oral tolerance (McGhee et al., Vaccine 10 (1992), 75-88; Mowat & Viney (1997), supra; Weiner (1997), supra). The accumulation of pre-stimulated T effector cells (Fig. 2, Fig. 3) indicates that presentation of orally administered ovalbumin and cognate interactions can occur directly in the intestinal mucosa. However, the important naive T helper cells rarely migrate through the intestinal mucosa (Hurst et al. (1999), supra), so they must be initially activated elsewhere at some accessible inductive lymphoid tissue. It is difficult to study the initial activation of naive T helper cells in normal animals because of the very low precursor frequency of ovalbumin-specific T cells and the lack of specific tools to detect such rare cells. Therefore, the Do11.10 T cell-receptor transgenic mouse model (Pape et al. (1997), supra) was again used to determine the inductive site for ovalbumin-specific naive T helper cells. In this case, a few non-stimulated ovalbumin-specific transgenic T helper were directly transferred into non-transgenic mice and their in vivo activation was followed using flow cytometry. Within 6 h after an oral immunization with ovalbumin and cholera toxin, transgenic ovalbumin-specific T helper cells in the Peyer's patches, mesenteric lymph nodes, and the spleen upregulated the very early activation marker CD69 (Fig. 4) in agreement with previous studies (Gutgemann et al., Immunity 8 (1998), 667-673; Sun et al., J.Immunol.162 (1999), 5868-5875; Williamson et al., Immunology 97 (1999), 565-572). Subsequently, many of the activated ovalbumin-specific cells in the mesenteric lymph nodes and some in the Peyer's patches became larger indicating blast formation. In the spleen, little blast formation was observed but large cells that had already downregulated CD69 migrated through the spleen at day 3.

Fig. 4 shows that many naive ovalbumin-specific T helper cells form blasts in the mesenteric lymph nodes after an oral immunization with ovalbumin and the adjuvant cholera toxin. Logarithmic probability plots (50% levels) indicating early activation (measured as CD69 upregulation) and blast

formation (measured as an increase in forward scatter) of transgenic ovalbumin-specific T helper cells at various time intervals after oral immunization in Peyer's patches (PP), mesenteric lymph nodes (mLN), and the spleen are shown. A few non-stimulated ovalbumin-specific transgenic T helper cells were transferred from T cell receptor-transgenic mice to non-transgenic recipient mice and followed in vivo in the chimerical mice by flow cytometry with a clonotypic monoclonal antibody specifically recognizing the transgenic cells. Similar results were obtained in 5 independent experiments. Weaker but qualitatively similar responses were obtained after immunization with ovalbumin without coadministered cholera toxin (data not shown).

By day 7 after oral immunization, many ovalbumin-specific cells in the mesenteric lymph nodes expressed low levels of the surface marker CD45RB (Fig. 5A) indicating cell division (Lee & Pelletier, Cell Immunol.188 (1998), 1-11; Sun et al. (1999), supra) and phenotypic differentiation whereas ovalbumin-specific T cells in control chimeric mice retained a CD45RB^{hi} phenotype. Oral immunization with ovalbumin activated only ovalbumin-specific T cells but not detectable numbers of non-transgenic bystander T helper cells (data not shown) and immunization with cholera toxin alone led to no detectable activation of ovalbumin-specific T cells (data not shown).

Fig. 5 demonstrates that in vivo stimulated transgenic ovalbumin-specific cells divide and differentiate after oral immunization with ovalbumin as indicated by a downregulation of CD45RB. Transgenic cells were analyzed with flow cytometry 7 days after oral immunization of chimerical mice with ovalbumin and cholera toxin (solid line) or bovine serum albumin and cholera toxin (shadowed).

2.5 Differences in T cell activation for individual mesenteric lymph nodes

After oral immunization with ovalbumin and cholera toxin, blast formation by ovalbumin-specific T helper cells occurred mainly in the mesenteric lymph nodes (Fig. 4). Mice like other mammals have several mesenteric lymph nodes that drain sequential parts of the small intestine. In previous mucosal immunology studies, the different mesenteric lymph nodes have generally been pooled (Chen et al., Cell Immunol.178 (1997), 62-68; Gutgemann et al. (1998), supra; Sun et al. (1999), supra; Williamson et al. (1999), supra) which neglects regional differences among the various parts of the small intestine. However, some regional variation in the immune response might be expected because of the observed regional differences in ovalbumin digestion and uptake (Fig. 1, Fig. 3). To test this, 4 individual mesenteric lymph nodes that could be reproducibly identified were separately analyzed for ovalbumin-specific blast formation (Fig. 6). Interestingly, individual mesenteric lymph nodes that drained various small intestinal regions differed considerably in the extent of ovalbumin-specific T cell blast formation (Fig. 6A) indicating a previously unknown regional variation of the specific immune response along the small intestine. The strongest responses occurred in the distal nodes which drain the distal small intestinal regions where efficient mucosal uptake of small ovalbumin-derived peptides had occurred. In contrast, the proximal regions with little ovalbumin uptake had only weak responses. This suggests that digestion of ovalbumin to small fragments enabling their subsequent mucosal uptake is relevant for the induction of a specific immune response.

Other factors might also contribute to a regional variation in the immune response. The coadministered mucosal adjuvant cholera toxin enhanced the immune response but had little influence on the regional pattern as oral immunization with ovalbumin alone led to a weaker but qualitatively similar regional response pattern (Fig. 6A). This indicates that the uneven distribution along the small intestine of the cholera toxin - receptor GM1-ganglioside (Lindner et al., Scand.J.Immunol.40 (1994), 564-572) was

not relevant for the regional immune response. However, simply shortening the pre-immunization fasting period from overnight to only 4h shifted the regional response pattern in the proximal direction with the strongest response now occurring in the medium mesenteric lymph nodes (Fig. 6B). This indicates that intrinsic differences between various intestinal regions can not explain the regional response pattern. Interestingly, with 4h fasting prior to oral immunization, small ovalbumin-derived peptides were most abundant in the medium small intestine during digestion because of a slower gut passage (data not shown). Hence, regions with abundant small peptides and strong regional immune response could be shifted together by a simple change in pre-immunizations treatments. This further supports that intestinal digestion strongly influences the immunogenicity of orally administered ovalbumin.

Fig. 6 shows that specific blast formation after oral immunization varies for individual mesenteric lymph nodes that drain different small intestinal regions. This regional heterogeneity is not due to the mucosal adjuvant cholera toxin or an intrinsically different immune responsiveness of various intestinal regions. (A) Regional responses after overnight fasting before immunization. Ovalbumin-specific transgenic T cells in individual mesenteric lymph nodes along the small intestine (numbers 1-4 in the proximal-distal direction) of chimeric mice were analyzed for blast formation 2 days after oral immunization with buffer (+), ovalbumin (o), or ovalbumin and cholera toxin (●). The data represent averages and SEMs for groups of 3-7 mice. Differences to the buffer control group were analyzed with the t test (*: $p < 0.05$, **: $p < 0.01$). (B) Regional responses after short fasting (4 h) prior to oral immunization with ovalbumin and cholera toxin.

3. Discussion

An orally administered antigen might be degraded by gastrointestinal digestive enzymes and this might affect its immunogenicity (Shalaby (1995), supra). Despite numerous studies of immune responses against

orally administered ovalbumin, its intestinal digestion has remained uncharacterized. Here it is shown for the first time that most ovalbumin is rapidly digested during its small intestinal passage in mice in contrast to a previous assumption (Furrie et al. (1995), supra).

The extensive intestinal digestion of ovalbumin could have important consequences for the induction of an ovalbumin-specific immune response. Cleavage of important epitopes would decrease the immunogenicity but digestion could also generate small immunogenic fragments that efficiently penetrate the intestinal epithelium. If epitope destruction is the dominant effect of digestion, the ovalbumin-specific immune response should regionally decrease with progressive digestion along the small intestine. In contrast, if generation of small immunogenic fragments is more important, antigen uptake and immune responses should increase with the progressive generation of small peptides along the small intestine.

Previous methods to determine antigen uptake across the intestinal epithelium have severe drawbacks. Since small peptides are difficult to fix, most detection methods strongly bias for large molecules. Moreover, common labeling methods including fluorophores and radioactive tags can not distinguish between immunogenic and non-immunogenic fragments and are more sensitive for large molecules. Antigen-specific antibodies detect B cell epitopes of the antigen but for both oral tolerance and oral vaccination, T cell epitopes are more important (MacDonald (1998), supra). Small peptides containing a functional T cell epitope can escape antibody detection if they lack a recognized B cell epitope again introducing a bias for large molecules. To avoid these disadvantages, a new method was developed to detect antigen uptake in situ using ovalbumin-specific T effector cells that accumulate in tissues where the recognized epitope is present. Although this method is indirect, it focuses directly on the physiologically relevant specific T cell interaction and is free of any bias against small peptides. With the new method, a previously unknown striking

gradient of antigen uptake along the small intestine was observed indicating that progressive intestinal digestion indeed greatly increases the permeation rates of immunogenic ovalbumin fragments.

Previous evidence from the analysis of serum samples by immunoblotting indicated that very little small fragments of ovalbumin are absorbed in the intestine (Bruce & Ferguson (1986), supra; Furrie et al. (1995), supra). However, small fragments with a size in the range of few kDa as detected here, would have escaped detection in the earlier studies because the electrophoresis buffer systems used can not resolve such small peptides (Schagger & von Jagow (1987), supra). Also, small peptides are rapidly cleared from the serum (Bloch et al. (1988), supra).

To test if the striking gradient in ovalbumin uptake along the small intestine is relevant for the induction of specific T cell responses, unstimulated ovalbumin-specific T cells were adoptively transferred and traced in the recipient mice following oral administration of ovalbumin. Mesenteric lymph nodes draining the distal small intestine were the most important inductive site for a ovalbumin specific T cell induction and the proximal-distal response gradient closely correlated with the differential uptake of ovalbumin. In conclusion, the combined data indicate that gastrointestinal digestion yields small immunogenic ovalbumin-fragments that efficiently permeate the epithelial barrier of the small intestinal mucosa and are transported by afferent lymphatic drainage to the regional mesenteric lymph nodes where they induce strong T helper cell responses.

This model differs from various previous models regarding oral antigen uptake and the induction of specific immune responses (Kaiserlian & Etchart (1999), supra; Mowat & Viney (1997), supra). The most widely accepted model assumes that an orally administered antigen is preferentially taken up by active transcytosis by the M-cells in the follicle-associated epithelium of the Peyer's patches. The antigen would then be presented to cognate naive

T cells in the interfollicular T cell area of the Peyer's patches. Shortly after this initial presentation, activated T cells might migrate to the mesenteric lymph nodes where the form blasts and further differentiate (Schaffeler et al., Intern.Immunol.9 (1997), 1555-1562). This model has been generally accepted because of the low macromolecular permeability of the normal intestinal epithelium in contrast to the M-cells with their active transcytosis of luminal macromolecules and particles (Gebert et al., Int.Rev.Cytol.167 (1996), 91-159; Toy & Mayer, Semin.Gastrointest.Dis.7 (1996), 2-11). However, there is little direct evidence supporting this model for soluble protein antigens (Kaiserlian & Etchart (1999), supra). Indeed, the data obtained here suggest that small immunogenic peptide fragments instead of macromolecular antigens enter the mucosa and that extended epithelial surfaces of the distal small intestine participate in this process (Fig. 2, Fig. 3a). The surface area involved is several orders of magnitude larger than the epithelial area of all Peyer's patches combined which might explain why only little specific T cell blast formation occurs in the Peyer's patches as compared to the mesenteric lymph nodes (Fig. 4). The M-cells of Peyer's patches may, however, be the predominant route of entry for particulate antigens.

Enterocytes have been shown to take up oral ovalbumin and to target it to MHC class II-containing vesicles suggesting that enterocytes themselves present ovalbumin-derived epitopes to cognate T helper cells (Kaiserlian & Etchart (1999), supra; Zimmer et al. (2000), supra). However, the data obtained here indicate that presentation by enterocytes to cognate T helper cells does rarely occur in vivo (Fig. 2, 3b). Transcytosis by enterocytes could participate in oral antigen entry but probably makes only a weak contribution since it would not explain the observed strong proximal-distal uptake gradient as large fragments are at least as likely to be endocytozed as compared to small fragments. The same argument applies to the apparently weak involvement of dendritic cells that directly sample the intestinal lumen. Dendritic cells might, however, be involved in presentation

to cognate T effector cells in the mucosa and the transport from the mucosa to the draining mesenteric lymph nodes (Liu & MacPherson, J.Exp.Med.177 (1993), 1299-1307; Williamson et al. (1999), supra). In summary, passive permeation of small peptides is the most likely entry pathway for immunogenic ovalbumin fragments.

In contrast to previous common belief, the new model suggests that gastrointestinal digestion of an antigen to small fragments is not merely a destructive and unwanted physiological barrier but instead a key event in the induction of an immune response against orally administered antigen. Hence, the previously neglected digestion physiology of oral antigens could be an important optimization parameter for oral antigen formulations. Obviously, a delicate balance is necessary between the productive generation of immunogenic peptide that are small enough to cross the epithelial barrier, but yet large enough to contain functional T cell epitopes. Depending on the particular sequence of an antigen, digestion might yield small fragments that retain relevant T cell epitopes or mainly non-immunogenic fragments with destroyed epitopes. This could explain the greatly varying efficacy of oral immunizations for different antigens which is difficult to reconcile otherwise. To optimize oral vaccines for productive gastrointestinal digestion, one could use in vitro digestion systems (Savoie, Con.J.Physiol.Pharmacol.72 (1994), 407-414) and polarized crypt epithelial cell monolayer cultures that have permeability properties similar to intestinal crypts (Ma et al. (1992) supra). Additionally, site-directed mutagenesis of the antigen could be used to introduce additional protease cleavage sites around protective epitopes that would facilitate the productive digestion to appropriate small peptides, and conversely, to delete existing protease cleavage sites within major T cell epitopes which would inhibit non-productive digestion. Improved vaccines could thus be obtained that yield high efficacies using the attractive oral delivery route.

Oral administration of antigen without an adjuvant usually leads to specific oral tolerance instead of an active immune response against the antigen. This effect is currently under intense preclinical and clinical evaluation as a therapy against autoimmune diseases. Interestingly, the digestion patterns and regional immune responses that were observed after oral immunization with ovalbumin were qualitatively similar independent of whether or not the adjuvant cholera toxin was coadministered. Hence, the digestive generation of small immunogenic peptides that induce a immune response and its implications might also apply to oral tolerance and its malfunction causing food allergy.

Claims

1. A method of preparing oral vaccines comprising the steps:
   (a) providing a polypeptide comprising at least one immunogenic epitope sequence
   (b) modifying said polypeptide sequence by
      (i) removing cleavage sites for proteolytic digestion enzymes from the epitope sequence and/or
      (ii) introducing cleavage sites for proteolytic digestion enzymes adjacent the epitope sequence.

2. The method of claim 1 wherein said immunogenic epitope sequence comprises a T cell epitope and/or an allergen.

3. The method of claim 2 wherein said immunogenic epitope sequence comprises a mammalian MHC allele-specific epitope sequence.

4. The method of claim 3 wherein said immunogenic epitope sequence is a human MHC class I or class II sequence.

5. The method of claim 4 wherein said immunogenic epitope sequence is selected from HLA-A, HLA-B, HLA-C, HLA-DR or HLA-DQ sequences.

6. The method of any one of claims 1-5 wherein said immunogenic epitope is derived from a pathogen-associated polypeptide, an autoimmune disease-associated polypeptide, a tumour-associated polypeptide or an allergen.

7. The method of claim 6 wherein said immuogenic epitope is derived from a Helicobacter polypeptide.

8. The method of claim 7 wherein said Helicobacter polypeptide is selected from urease A, urease B, catalase, vac A, cag A, HP0231, HP0410 or HP1098.

9. The method of any one one claims 1-8 wherein said proteolytic digestion enzymes are selected from pepsin, trypsin or chymotrypsin.

10. The method of any one of claims 1-9 wherein the length of epitope sequence between the cleavage sites is from 15-25 amino acids.

11. The method of claim 10 wherein the length is from 17-22 amino acids.

12. A polypeptide comprising at least one immunogenic epitope sequence which has been modified by

(i) removing cleavage sites for proteolytic digestion enzymes from the epitope sequence and/or

(ii) introducing cleavage sites for proteolytic digestion enzymes adjacent the epitope sequence.

13. The polypeptide of claim 12 wherein said immunogenic epitope sequence comprises a T cell epitope.

14. The polypeptide of claim 13 wherein said immunogenic epitope sequence comprises a mammalian MHC allele-specific epitope sequence.

15. The polypeptide of claim 14 wherein said immunogenic epitope sequence comprises a human MHC class I or class II epitope sequence.

16. The polypeptide of any one of claims 12-16 wherein said proteolytic digestion enzymes are selected from pepsin, trypsin or chymotrypsin.

17. The polypeptide of any one of claims 12-17 wherein the length of epitope sequence between the cleavage sites is from 15-25 amino acids.

18. The polypeptide of claim 17 wherein the length is from 17-22 amino acids.

19. A nucleic acid encoding the polypeptide of any one of claims 12-18.

20. The nucleic acid of claim 19 which is operatively linked to an expression control sequence.

21. The nucleic acid of claim 19 or 20 which is located on a recombinant vector.

22. A recombinant cell which is transformed with a nucleic acid of any one of claims 19-21.

23. An oral vaccine comprising as an active ingredient a polypeptide of any one of claims 12-18.

24. The oral vaccine of claims 23 further comprising a pharmaceutically acceptable carrier or diluent.

25. A method of improving the efficacy of oral vaccines comprising the steps:

(a) providing a polypeptide comprising at least one immunogenic epitope sequence

(b) modifying said polypeptide sequence by

(i) removing cleavage sites for proteolytic digestion *enzymes from the epitope* sequence and/or

(ii) introducing cleavage sites for proteolytic digestion *enzymes adjacent the epitope* sequence.

26. The method of claim 25 wherein the improvement comprises an improvement of the mucosal uptake of immunogenic polypeptide fragments.

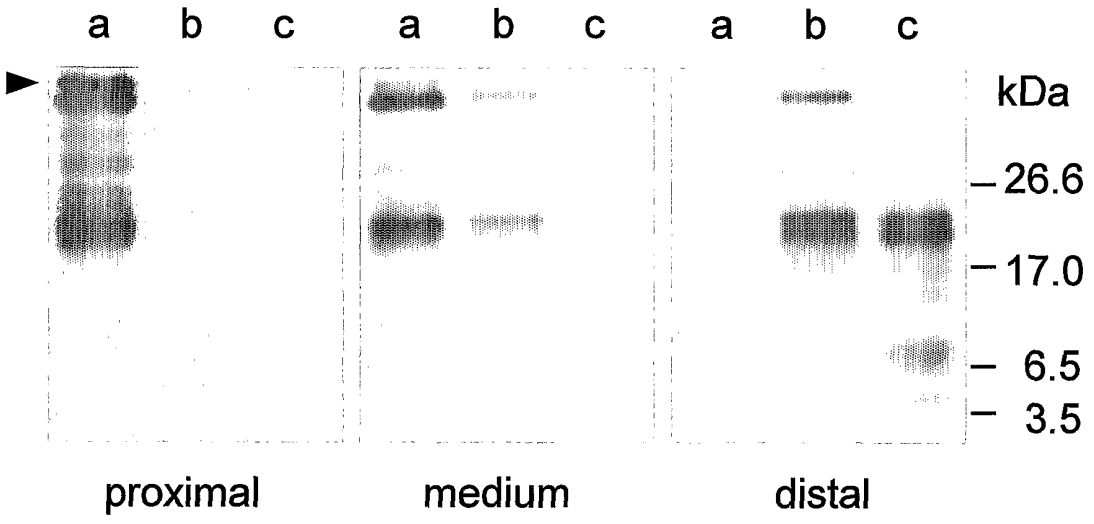
27. A method for vaccinating a subject in need thereof comprising orally administering polypeptide comprising at least one immunogenic epitope sequence which has been modified by

(i) removing cleavage sites for proteolytic digestion enzymes from the epitope sequence and/or

(ii) introducing cleavage sites for proteolytic digestion enzymes adjacent the epitope sequence in an amount sufficient to induce an immune response in said subject against said at least one immunogenic eptitope sequence.

28. The method of claim 27 wherein said subject is a human.

Fig. 1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6.5
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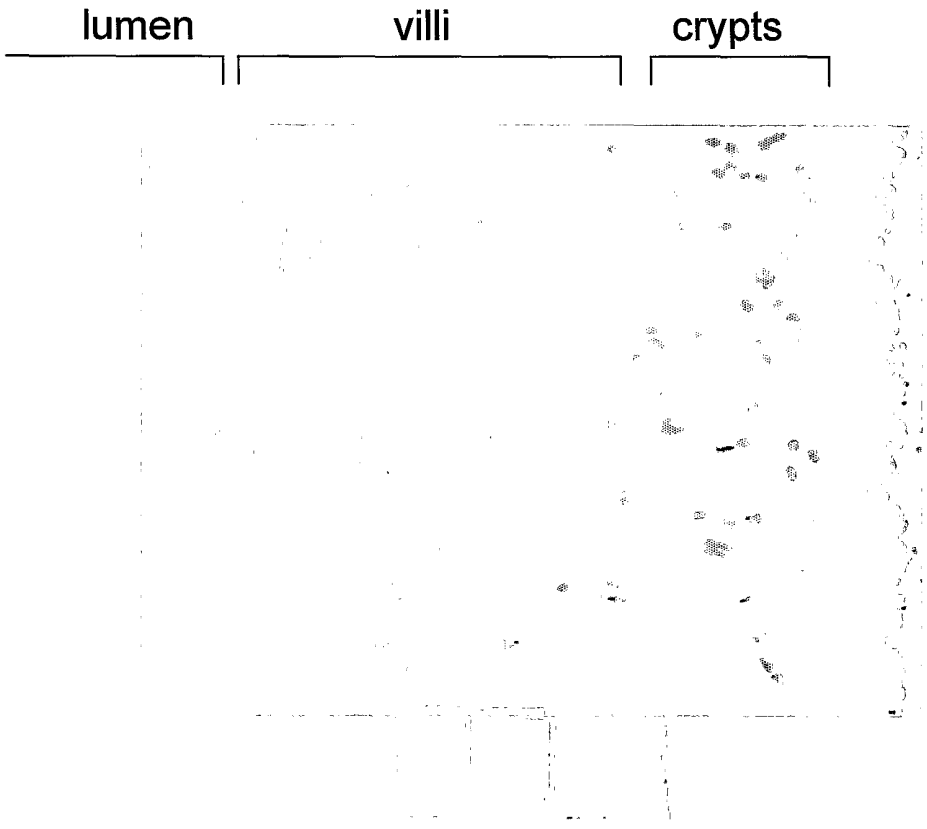
proximal
medium
distal

Fig. 2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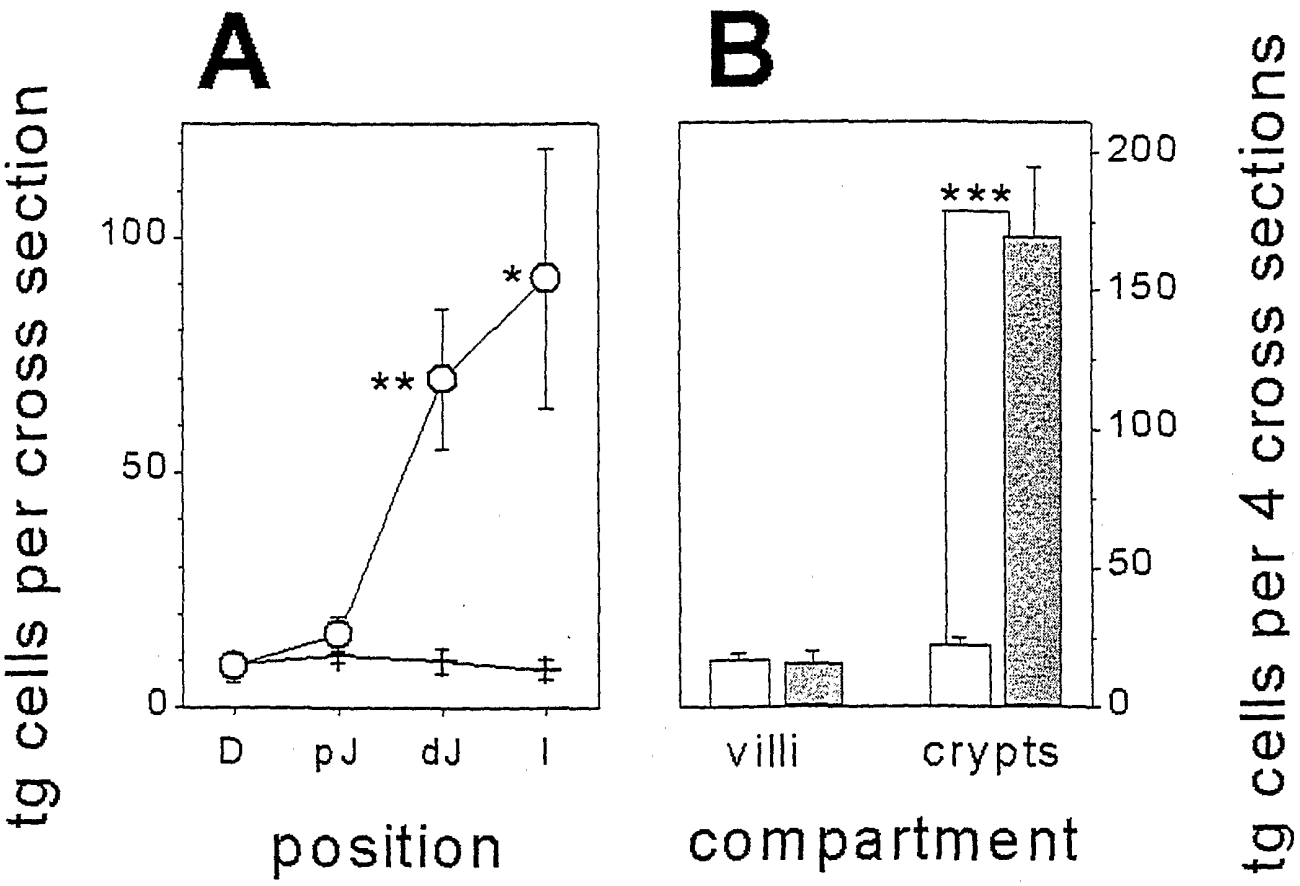
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B
tg cells per cross section
100
50
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D
pJ
dJ
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Fig. 3

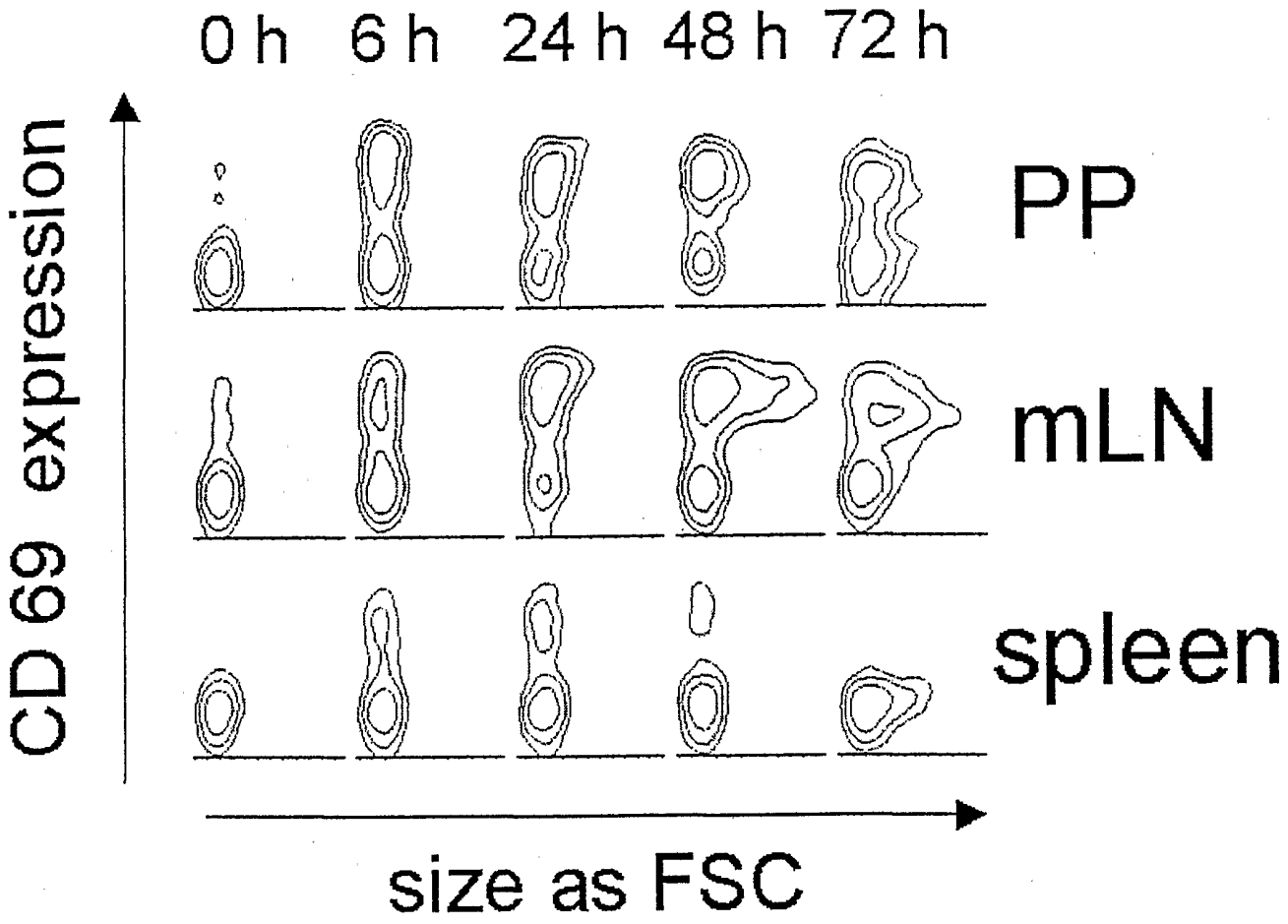

Fig. 4

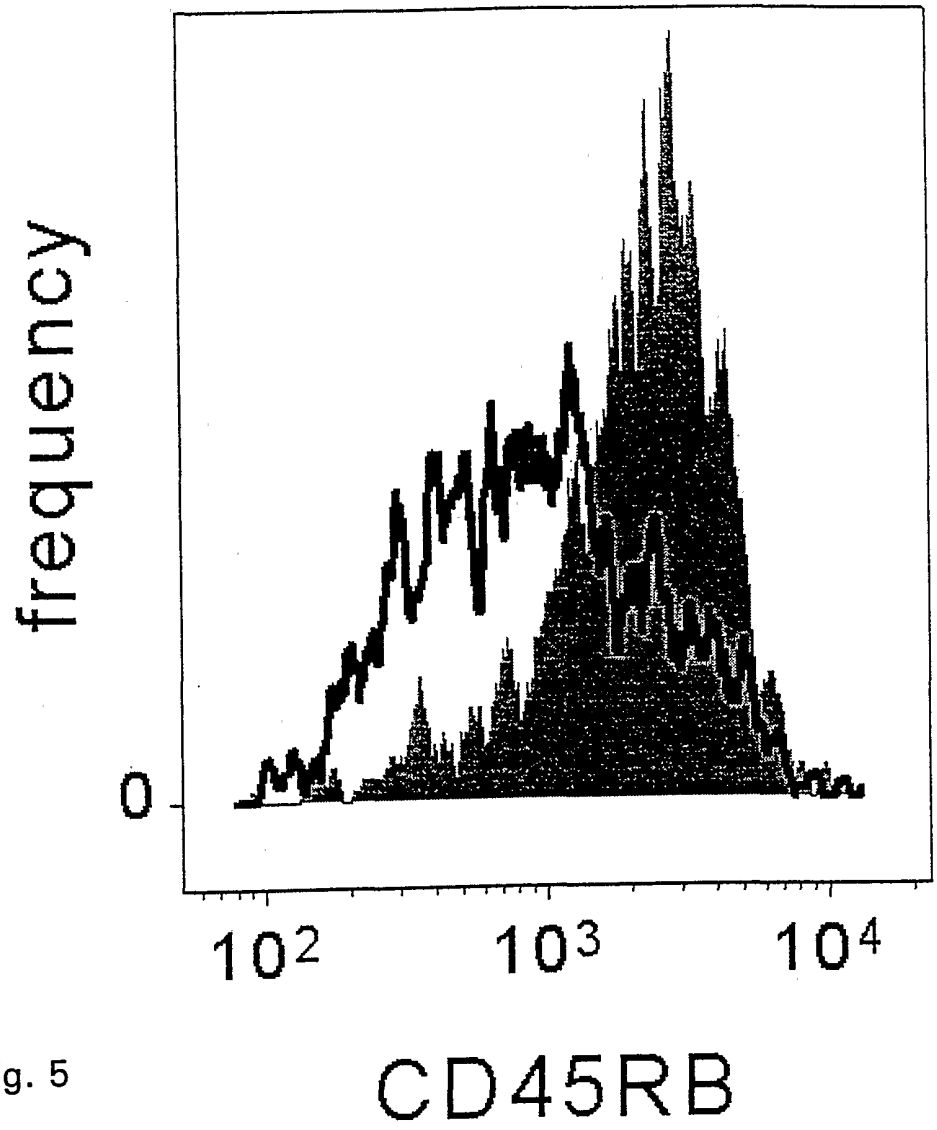

Fig. 5

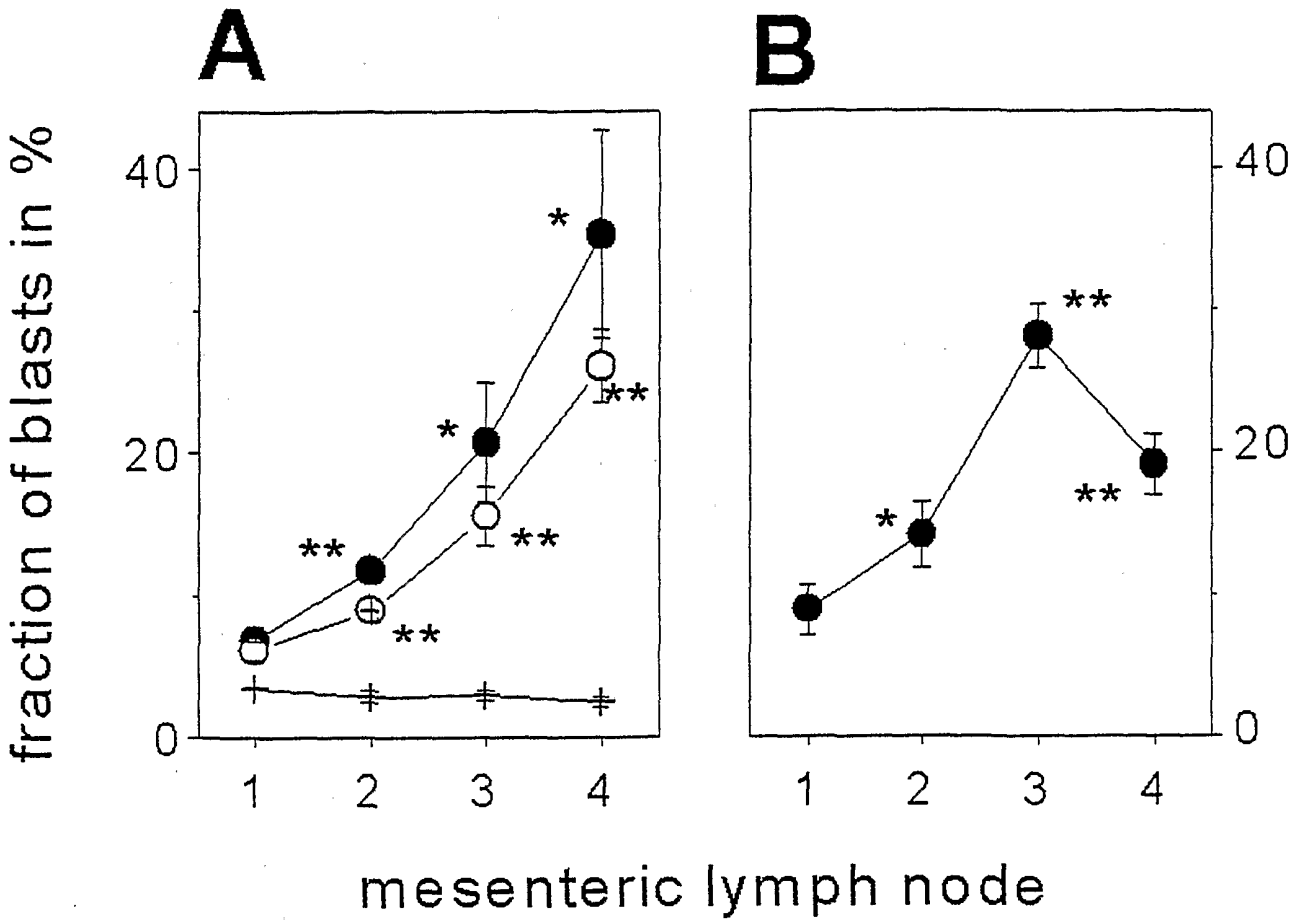

Fig. 6