

Intradermal exposure of BALB/c strain mice to peanut protein elicits a type 2 cytokine response

Catherine J. Betts^{a,*}, Brian F. Flanagan^b, Helen T. Caddick^a, Rebecca J. Dearman^a, Ian Kimber^a

^a Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK

^b Department of Immunology, Duncan Building, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK

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Abstract

There is a growing need for the development of methods to characterize the allergenic properties of novel proteins, particularly those expressed by transgenic crop plants. Hence, there is considerable interest in the development of suitable animal models for this purpose. The production of specific IgE antibody has been reported following sensitization with food allergen via oral or systemic (intraperitoneal) routes of exposure. We have characterized cytokine profiles induced by intradermal treatment of BALB/c strain mice with a purified peanut allergen, *Arachis hypogaea* lectin. Mice were exposed to peanut lectin by intradermal administration and the cytokine responses in the lymph node draining the site of exposure analyzed at the secreted protein level by enzyme-linked immunosorbent assay (ELISA) and cytokine mRNA level by ribonuclease protection assay (RPA). Exposure to peanut lectin, under conditions that induced robust IgE antibody titers, was found to be associated with a T helper 2 (Th2)-type cytokine expression profile at both the mRNA and secreted protein levels. Culture of naïve lymph node cells with peanut lectin failed to stimulate marked proliferation or cytokine production, confirming this protein is not mitogenic for mouse lymphocytes. Furthermore, the expression of Th2 cytokines was associated with the effector/memory CD62L⁺ cell population. Similar treatment with a non-allergenic protein, potato acid phosphatase, failed to induce Th2 cytokine expression. These data demonstrate that exposure of mice to peanut allergen results in the selective stimulation of a Th2-type response.

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1. Introduction

There has been considerable interest in the development of experimental approaches for the safety assessment of novel foods, including those derived from genetically modified plants. An important issue is whe-

ther the novel protein or proteins have the potential to induce allergic sensitization, or to elicit allergic reactions (FAO/WHO, 2001; Hollingworth et al., 2003; Kuiper et al., 2001; Lack et al., 2002; Metcalfe et al., 1996). Current recommendations for assessment of potential allergenicity incorporate several components, including: (a) consideration of the serological identity of the protein of interest with known human allergens, (b) structural similarity to, or amino acid sequence homology with, allergenic proteins, and (c) resistance to proteolytic digestion in simulated gastric fluid (FAO/WHO, 2001; Metcalfe et al., 1996). Such parameters can provide useful information about the properties of novel proteins, particularly when they are shown to share homology with known allergens. However, in order to provide the basis for a direct evaluation of inherent sensitization potential, there is a need for the development of appropriate animal models (Kimber et al., 2003;

Abbreviations: Antigen presenting cell, APC; Concanvalin A, con A; Enzyme linked immunosorbent assay, ELISA; Fetal calf serum, FCS; Glyceraldehyde 3 phosphate dehydrogenase, GAPDH; Interferon, IFN; Interleukin, IL; Lymph node cells, LNC; ³H-methyl thymidine, ³H-TdR; Ovalbumin, OVA; Passive cutaneous anaphylaxis, PCA; Phosphate buffered saline, PBS; Potato acid phosphatase, PAP; RNase protection assay, RPA; T helper, Th; Tris-borate EDTA buffer, TBE

* Corresponding author. Tel.: +44-1625-514047; fax: +44-1625-585715/590996.

E-mail address: catherine.betts@syngenta.com (C.J. Betts).

Ladics et al., 2003; FAO/WHO, 2001). Several animal species have been considered in this regard, including mice (Dearman et al., 2003b,c; Li et al., 1999; Li and Sampson, 2002), rats (Knippels et al., 1998), dogs (Ermel et al., 1997) and swine (Helm et al., 2002), with protocols employing systemic (intraperitoneal; ip) or oral exposure and the presence or absence of adjuvant.

In general, allergenicity is characterized in such models as a function of the ability of test proteins to provoke immune responses, in particular IgE antibody responses, this being the principal biological effector of food allergic reactions. The production of IgE antibody is regulated by functional subpopulations of CD4⁺ T helper (Th) lymphocytes and cytokines, with the most polarized subsets being designated Th1 and Th2 (Mosmann and Coffman, 1989; Mosmann et al., 1986). The products of Th2 cells favor the elicitation of immediate type hypersensitivity reactions such as asthma and rhinitis, with interleukin (IL)-4 being essential for the induction and maintenance of IgE antibody responses (Finkelman et al., 1988b). In contrast, Th1 cells antagonize allergic responses, with the Th1 cell cytokine interferon (IFN- γ) inhibiting IgE antibody production (Finkelman et al., 1988a). Furthermore, the development in humans of asthma to high molecular weight allergens is commonly associated with a Th2 phenotype (Durham et al., 1992; Robinson et al., 1993; Ying et al., 1997). Similar studies in food allergic individuals are hampered by the very low frequency of circulating allergen-specific lymphocytes. However, using flow cytometric techniques to select these rare cells, it has been shown recently that peanut allergic donors display a Th2 phenotype of cytokine expression, whereas in peanut tolerant or individuals that had outgrown their peanut allergy skewing towards a Th1 profile was observed (Turcanu et al., 2003).

It is hypothesized that proteins that are known to induce IgE antibody responses, and to cause allergic sensitization, will induce in BALB/c strain mice the development of selective immune responses characterized by the preferential production of type 2 cytokines. We have therefore examined cytokine expression profiles associated with the induction of allergic (IgE) antibody responses in BALB/c strain mice in order to provide further mechanistic insight into the regulation of immune responses to protein allergens. A further aim is to consider whether, in principle at least, there may be opportunities to identify and characterize putative protein allergens as a function of their ability to stimulate selective cytokine secretion patterns. There are previous reports that protein or parasite allergens that provoke IgE antibody responses stimulate polarized Th2 type cytokine secretion profiles in various animal models. However, such studies have almost invariably required the co-administration of adjuvants, such as alum, that promote the development of Th2-type cells during sen-

sitization (Bae et al., 1999; Li et al., 1999, 2000; Morafo et al., 2003). In the studies described here, the intrinsic ability of a protein allergen to provoke type 2 cytokine responses, in the absence of adjuvant, has been characterized at the levels of both mRNA expression and protein secretion. Purified peanut agglutinin was selected for these studies, a constituent of one of the most common causes of food allergic reactions in the UK and USA (peanut; Bush and Hefle, 1996), and a protein to which IgE antibody has been reported to be detectable in 20–50% of peanut allergic individuals, (Burks et al., 1994). Cytokine profiles induced by specific stimulation of peanut-primed or naïve lymph node cells (LNC) have been compared with those elicited by culture with the T cell mitogen concanavalin A (con A).

2. Materials and methods

2.1. Animals

Young adult (6–12 weeks old) female BALB/c strain mice (Harlan Seralab, Oxon, UK) were used throughout these studies. Mice were housed in metal cages and food (SDS PCD pelleted diet; Special Diets Services Ltd, UK) and water were available ad libitum. The ambient temperature was maintained at 21 ± 2 °C and relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle. All experiments were conducted under the provisions of the UK Animals (Scientific Procedures) Act, 1986.

2.2. Protein

Peanut agglutinin (*Arachis hypogaea* lectin, 99%) and potato acid phosphatase (PAP, phosphatase, acid, type II from potato) were obtained from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK) and reconstituted in phosphate buffered saline (PBS) at 10–20 and 200 mg/ml, respectively. Endogenous endotoxin content was measured by Limulus Amoebocyte Lysate assay with a chromogenic end point (Cambrex Bio Science, Wokingham, Berkshire, UK) and was <0.02 μ g LPS/mg or <0.25 μ g LPS/mg of protein for peanut agglutinin and PAP, respectively. Aliquots of stock solution were stored at -20 °C and diluted in PBS to between 1 and 2 mg/ml for peanut agglutinin or 20 mg/ml for PAP immediately prior to use.

2.3. Sensitization

Mice ($n = 15$) received an intradermal injection of 30 μ l of 1–2 mg/ml (0.1–0.2%) peanut agglutinin or 20 mg/ml (2%) PAP on the dorsum of each ear on days 0 and 7. Control animals were untreated (naïve) throughout the duration of the experiment. Fourteen days following initiation of exposure all mice were exsanguinated by

cardiac puncture and the draining auricular lymph nodes excised. Serum was prepared and stored at -20°C until analysis.

2.4. Preparation of cells from the draining lymph nodes

Draining auricular lymph nodes were excised and pooled for each experimental group. A single cell suspension was prepared under aseptic conditions by mechanical disaggregation through sterile 200-mesh steel gauze. LNC were cultured in RPMI-1640 growth medium (Invitrogen, Paisley, Scotland) supplemented with 25 mmol/l HEPES, 400 $\mu\text{g/ml}$ streptomycin, 400 $\mu\text{g/ml}$ ampicillin (Sigma) and 10% heat-inactivated fetal calf serum (FCS) (Invitrogen). Cells (1 ml) at $10^7/\text{ml}$ were seeded into 24-well tissue culture plates in the presence of 200 $\mu\text{g/ml}$ peanut agglutinin, 2000 $\mu\text{g/ml}$ PAP, 2 $\mu\text{g/ml}$ of the T cell mitogen concanavalin A (con A), or medium alone, and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture was terminated after 24, 72 and 120 h, the supernatants collected, centrifuged at 100g for 5 min and stored at -70°C until analysis. The cells collected at each time point were resuspended in TRIZOL (Invitrogen) at 1 ml per 3×10^7 cells and the samples stored at -70°C until total RNA extraction according to the manufacturer's instructions. Alternatively, LNC (200 μl) from naïve, peanut-primed or PAP-primed mice were cultured in 96 well plates at a concentration of 2.5×10^6 cells/ml in the presence of 200 $\mu\text{g/ml}$ peanut agglutinin, 2000 $\mu\text{g/ml}$ PAP, 2 $\mu\text{g/ml}$ con A, or medium alone and proliferation measured at 48 h after pulsing for the final 24 h with 2 μCi [^3H]-methyl thymidine ([^3H]-TdR; specific activity 2 Ci/mmol; Amersham International, Amersham, UK). Culture was terminated by cell harvesting and incorporation of ^3H -TdR was measured by β -scintillation counting.

2.5. Cytokine determinations

The cytokine content of culture supernatants derived from LNC following 24, 72 and 120 h in culture was determined using customized sandwich ELISA as described previously (Betts et al., 2002). All assays used plastic microtiter plates (Nunc, Copenhagen, Denmark). For each cytokine-specific ELISA the plates were washed with PBS containing 0.05% Tween 20 (Sigma) between each incubation stage. Optical density at 450 nm was measured using an automated reader (Multiskan, Flow Laboratories, Irvine, Ayrshire, UK). A standard curve derived with murine recombinant cytokine and associated computer software for microplate-based assays (Genesis, Life Sciences International Ltd., Basingstoke, UK) were used to calculate the cytokine concentration in supernatants. Standard errors were less than 10% in most experiments. In cell separation experiments, cytokine protein levels in culture superna-

tants were determined by multiplex bead assay (Bio-plexTM, BioRad, Hemel Hempstead, UK) according to the manufacturer's instructions using bead kits comprising seven Th1/Th2-type cytokines (IFN- γ , IL-4, IL-15, IL-10, IL-12p40, IL-12p70, IL-13).

2.6. Measurement of protein-specific IgE antibody

The presence of IgE antibody in pooled serum samples was detected by homologous passive cutaneous anaphylaxis (PCA) assay as reported previously (Dearman et al., 2003b,c). Pooled serum samples were diluted serially in PBS and injected (30 μl) into the dermis of the ears of naïve recipient mice ($n = 4$). Two days later, 0.25 mg of peanut agglutinin or 0.25 mg PAP, together with Evans Blue dye (1.25 mg, Sigma), in 0.25 ml PBS were injected intravenously via the tail vein. Mice were terminated 30 min following challenge and the diameter of the cutaneous reactions measured. Antibody titer was recorded as the highest dilution of pooled serum resulting in a blue lesion of a diameter greater than 3 mm.

2.7. Magnetic bead separation of cell fractions on the basis of CD62L and CD90 expression

LNC populations were fractionated into CD62L⁺ and CD62L⁻ populations using specific microbeads (Miltenyi Biotec, Bisley, Surrey, UK) as per manufacturer's instructions. The purity of the CD62L⁻ and CD62L⁺ populations was confirmed by FACS analysis on 5×10^5 cells stained with a phycoerythrin-conjugated anti-CD62L monoclonal antibody (Pharmingen, BD Biosciences, Oxford, UK) using a FACSCaliber flow cytometer (BD Biosciences). Fractionated CD62L⁺ and CD62L⁻ cells were cultured as above for 24 h in the presence of 2 $\mu\text{g/ml}$ Con A, harvested and cell pellets resuspended in TRIZOL for subsequent total RNA isolation. In some experiments CD62L⁺ cell fractions were supplemented with CD90-depleted cells from naïve animals in a 5:1 ratio. CD90⁺ cell fractionation was carried out as described above for CD62L using CD90 pan T cell microbeads (Miltenyi Biotec). The purity of the CD90⁻ and CD90⁺ populations was confirmed by FACS analysis on 5×10^5 cells stained with a fluorescein isothiocyanate-conjugated anti-CD4 monoclonal antibody (Pharmingen).

2.8. RNase protection assay

T7 RNA polymerase was used to synthesize ^{32}P radiolabelled antisense RNA probes, using RiboQuant cDNA template mCK-1 (containing IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15, IFN- γ , GAPDH [glyceraldehyde 3 phosphate dehydrogenase] and L32) according to the manufacturer's instructions (Pharmingen). Probe

solutions were hybridized to 20 µg of total RNA at 56 °C overnight followed by digestion with RNase A and T1 (Pharmingen). Samples were then treated with proteinase K to remove RNases, phenol extracted and precipitated in ethanol. Each sample was loaded onto a 6% polyacrylamide urea gel, run at 38 mA in 0.5×TBE (tris-borate EDTA buffer) alongside the undigested probe as size marker. Gels were exposed to autoradiography film (KODAK XAR-5) at –70 °C with intensifying screens. Densitometric analysis was carried out using the “Image” programme (National Institutes of Health, USA).

2.9. Statistics

Cytokine secretion data were evaluated using two-way analysis of variance following a logarithmic transformation, allowing for the replicate structure of the experimental design. For IL-4 data, to allow inclusion of zero values, ten was added to each value prior to logarithmic transformation. Differences between individual group means were compared using two-sided Student's *t*-test based on the error mean square of the analysis of variance. Proliferation data were evaluated using a two-sided Student's *t*-test. Statistical analysis was conducted using the GLM procedure in the statistical analysis system (SAS), version 8.02.

3. Results

3.1. IgE antibody production and proliferative responses induced by exposure to peanut agglutinin

Intradermal administration of peanut agglutinin (0.1%) to BALB/c strain mice resulted in the induction of a specific serum IgE antibody response as measured by homologous PCA assay. In three independent experiments, titers of 1 in 16 (two experiments) or 1 in 32 (one experiment) were achieved. In contrast, id exposure to PAP failed to provoke detectable IgE antibody. This is consistent with previous experience of intraperitoneal exposure to this material in which IgG, but not IgE, antibody responses were stimulated (Dearman et al., 2003c). In subsequent experiments the ability of intradermal treatment with peanut agglutinin or PAP to stimulate proliferative responses in the lymph nodes draining the site of exposure was examined. In order to provide for sufficient tissue to conduct all the analyses, lymph nodes were pooled on a treatment group basis. In kinetic experiments, the optimal time point for analysis of radiolabelled thymidine incorporation was determined (48 h culture of draining LNC; data not shown) and the optimal doses of peanut agglutinin and PAP (200 and 2000 µg/ml, respectively). Only low levels of proliferation (<2000 dpm) were recorded following culture of LNC from naïve mice, or of LNC from pea-

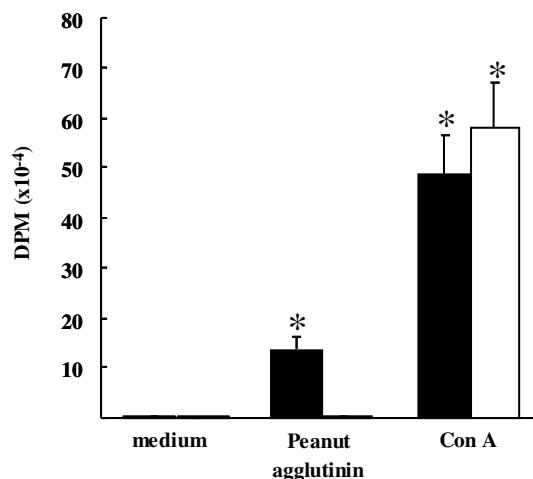


Fig. 1. Proliferative responses of LNC derived from naïve and peanut-primed mice. LNC obtained from peanut-primed (■) or naïve (□) mice ($n = 15$ per group) were pooled on treatment group basis and cultured in vitro with medium alone, peanut agglutinin (200 µg/ml) or con A (2 µg/ml). Tritiated thymidine was added to the culture 24 h prior to cell harvesting at 48 h. Results of $n = 5$ independent experiments are shown (mean and SE disintegrations per minute [dpm]). Statistically significant increases in proliferation were assessed by two-sided Student's *t*-test and are illustrated by * ($0.01 \geq P \geq 0.005$; compared with cells cultured with medium alone).

nut-primed mice in the presence of medium alone (Fig. 1). Restimulation of either cell population with the T cell mitogen con A resulted in a very marked and significant increase in proliferation. Importantly, treatment with peanut agglutinin failed to cause significant effects on thymidine incorporation in naïve LNC, but stimulated vigorous proliferative responses in peanut primed LNC, demonstrating clearly that peanut agglutinin does not act as a nonspecific mitogen for murine LNC. Culture in the presence of PAP induced relatively modest increases in proliferation in LNC derived from PAP-primed animals, approximately 3-fold lower than those observed for specific restimulation of peanut agglutinin-primed LNC. In common with the peanut protein PAP failed to induce a proliferative response in cells derived from naïve animals (data not shown).

3.2. Induction of cytokine protein secretion by peanut allergen

Supernatants were collected from LNC cultured with peanut allergen as described above after 24, 72 and 120 h and the concentrations of IFN-γ, IL-4, IL-5, IL-10, IL-12 and IL-13 were measured by ELISA. Naïve LNC stimulated with peanut agglutinin, or LNC cultured in the presence of medium alone, failed to secrete detectable amounts of any of the cytokines examined over this time course with the exception of IL-12, which was expressed constitutively (Fig. 2(a); representative experiment). However, addition of con A resulted in marked

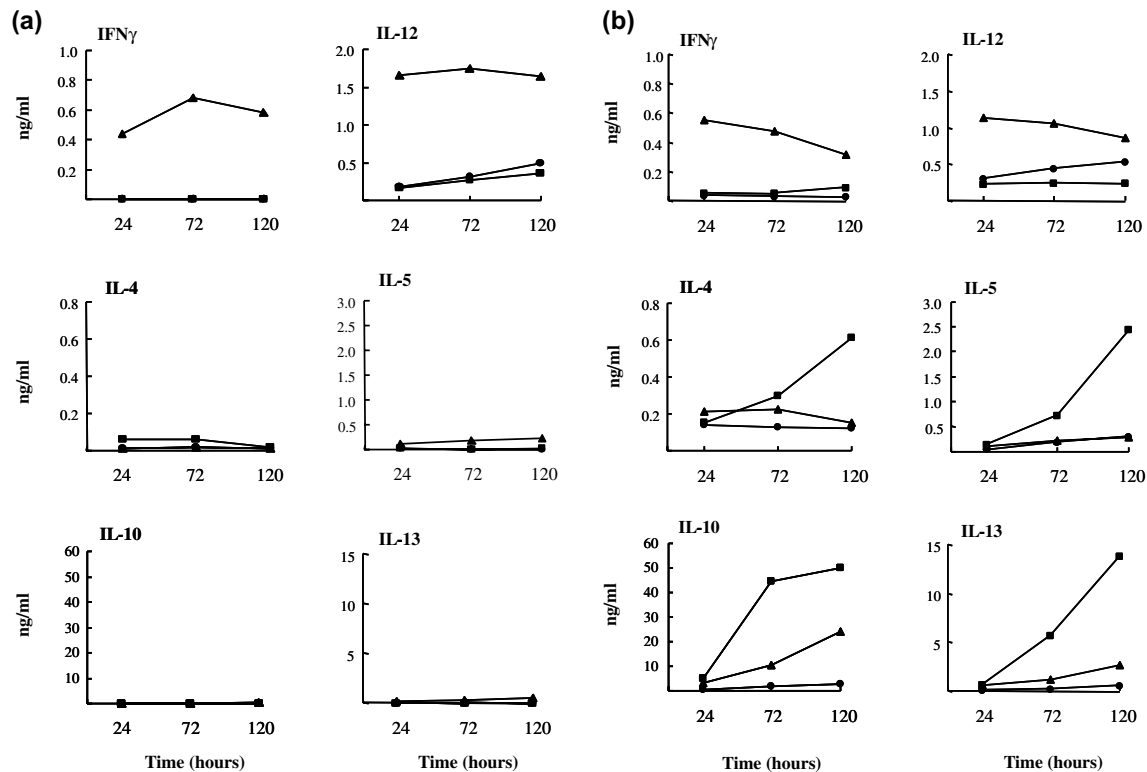


Fig. 2. Cytokine protein secretion profiles induced in naïve and peanut-primed LNC upon secondary restimulation. Naïve (a) or peanut-primed (b) LNC pooled per treatment group ($n = 15$ mice) were cultured in vitro in the presence of medium alone (●), peanut agglutinin (■, 200 μ g/ml) or con A (▲, 2 μ g/ml). Supernatants harvested at 24, 72 and 120 h were analyzed by cytokine-specific ELISA with limits of detection as follows: IFN- γ , 0.078 ng/ml; IL-12, 0.078 ng/ml; IL-4, 0.02 ng/ml; IL-5, 0.02 ng/ml; IL-10, 0.156 ng/ml; IL-13, 0.156 ng/ml. Cytokine protein levels are shown (ng/ml of culture supernatant) for a representative experiment.

increases in IFN- γ and IL-12, along with a modest elevation in IL-10 secretion. These patterns of cytokine secretion were reproducible, with significant con A-induced increases in IFN- γ and IL-12 ($p \leq 0.01$ and $p \leq 0.05$, respectively; $n = 4$) recorded after 120 h culture, compared with cells cultured with peanut agglutinin or medium alone (data not shown).

LNC derived from peanut primed mice also secreted very low levels of most cytokines in the absence of restimulation with con A or peanut agglutinin, again with the exception of IL-12 which was expressed constitutively (Fig. 2(b)). A very different pattern of cytokine secretion was observed when these cells were cultured in the presence of peanut agglutinin (Fig. 2(b); representative experiment). After 72 and 120 h culture with peanut agglutinin, marked increases in the secretion of the type 2 cell products IL-4, IL-5, IL-10 and IL-13 was observed, but no increase in the type 1 cytokines IFN- γ or IL-12. Peak secretion of cytokines was generally observed after 120 h of culture. In contrast, peanut-primed LNC responded to con A by increased secretion of IFN- γ and IL-12, and some increased production of IL-10 and IL-13, compared with cells cultured with medium alone. Although the pattern of cytokine secretion from PAP-primed LNC following 120 h of culture in the

presence of con A was similarly Th1-biased, specific restimulation with PAP failed to induce the Th2 profile of cytokines observed following culture of peanut-primed cells with peanut agglutinin (Fig. 3). Indeed, levels of cytokine production were comparatively low relative to those secreted from peanut-primed LNC.

The inter-experimental variation in cytokine expression from peanut-primed animals was explored (Fig. 4). Five independent experiments in which peanut-primed LNC were cultured for 120 h in the presence of medium alone, peanut agglutinin or con A were conducted, using in each experiment pooled lymph nodes to provide for sufficient cells for all analyses. As observed previously (cf Fig. 2(b)), IL-12 secretion was constitutive, with equivalent expression by cells cultured with medium alone or with the mitogen con A. Interestingly, culture with peanut agglutinin induced a significant reduction in IL-12 expression. There was a modest increase in the very low basal levels of IFN- γ induced by culture of LNC in the presence of peanut agglutinin compared with cells cultured with medium alone. Culture with con A provoked very vigorous secretion of this cytokine. In contrast to the Th1 cytokines, significant increases in IL-4, IL-5, IL-10 and IL-13 production were recorded for LNC cultured with peanut agglutinin compared with

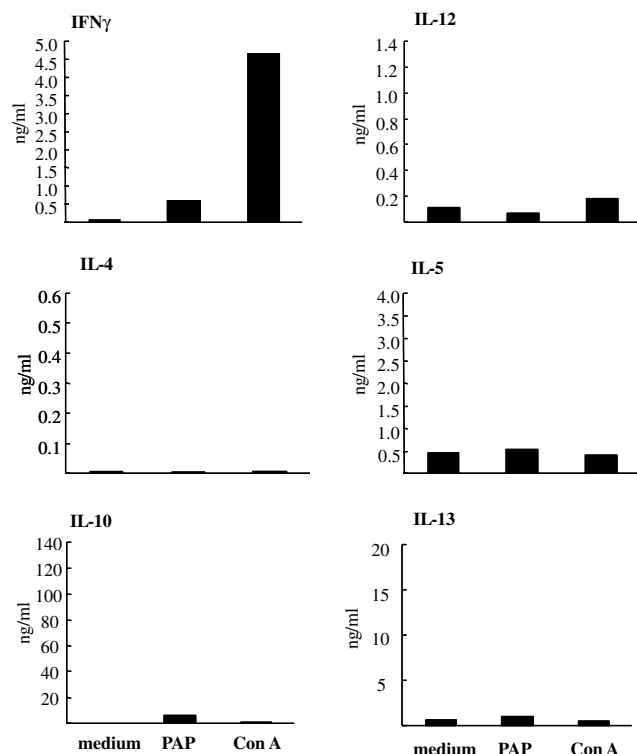


Fig. 3. Cytokine protein secretion profiles of PAP-primed LNC. PAP-primed LNC pooled from $n = 15$ mice were cultured in vitro in the presence of medium alone, potato acid phosphatase (2000 $\mu\text{g/ml}$) or con A (2 $\mu\text{g/ml}$). Supernatants harvested at 120 h were analyzed by cytokine-specific ELISA with limits of detection as follows; IFN- γ , 0.078 ng/ml; IL-12, 0.078 ng/ml; IL-4, 0.02 ng/ml; IL-5, 0.02 ng/ml; IL-10, 0.156 ng/ml; IL-13, 0.156 ng/ml. Cytokine protein levels are shown (ng/ml of culture supernatant) for a representative experiment.

those cultured in the presence of medium or con A. Significant increases in secretion of IL-10 and IL-13 were also observed for con A-stimulated LNC compared with medium controls, although such changes were considerably less marked than those induced by peanut agglutinin.

3.3. Peanut allergen induced changes in cytokine mRNA expression

Following culture for 24, 72 and 120 h as described above, LNC were harvested and total RNA prepared for analysis of cytokine mRNA expression by RPA. Representative acrylamide gels of RPA products obtained following analysis of both naïve and peanut-primed cultured cells are shown in Fig. 5. Culture of naïve LNC with medium or peanut agglutinin did not result in detectable levels of mRNA for any of the cytokines examined over the entire 120 h time course. However, culture of naïve cells with con A resulted in the rapid (24 h) production of IL-2, IL-6 and IFN- γ transcripts, that returned to basal levels at 72 and 120 h. Similarly, peanut-primed LNC cultured with medium

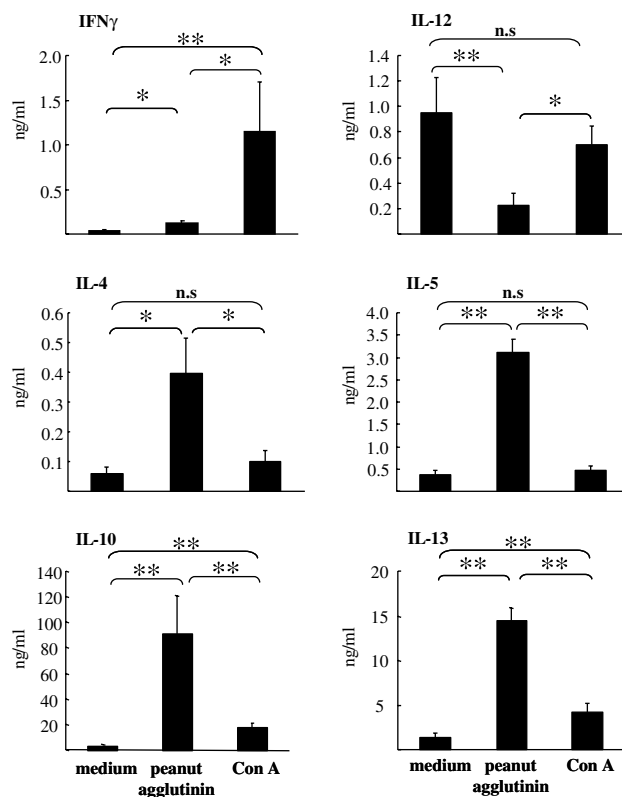


Fig. 4. Cytokine protein secretion profiles of peanut-primed LNC: interexperimental variation. Peanut-primed LNC pooled from $n = 15$ mice were cultured in vitro in the presence of medium alone, peanut agglutinin (200 $\mu\text{g/ml}$) or con A (2 $\mu\text{g/ml}$). Supernatants harvested at 120 h were analyzed by cytokine-specific ELISA with limits of detection as follows; IFN- γ , 0.078 ng/ml; IL-12, 0.078 ng/ml; IL-4, 0.02 ng/ml; IL-5, 0.02 ng/ml; IL-10, 0.156 ng/ml; IL-13, 0.156 ng/ml. Mean and SE of cytokine protein levels in ng/ml of culture supernatant are shown for five replicate experiments. Statistical significance of differences between individual group means was compared using two-sided Student's t -test based on the error mean square of the analysis of variance as described in the materials and method section. Significance is denoted by * ($P \leq 0.05$) and ** ($P \leq 0.01$).

alone did not result in detectable cytokine gene expression. Restimulation with peanut agglutinin resulted in the production of high levels of transcripts for the Th2 cytokines IL-4, IL-5, IL-10 and IL-13, with relatively little Th1 or inflammatory cytokine (IL-2, IL-6 and IFN- γ) expression (Fig. 5(b)). As observed for naïve LNC, con A induced a rapid, but transient, up-regulation of mRNA for the inflammatory/Th1 cytokines IL-2, IL-6 and IFN- γ by peanut primed LNC. Interestingly, culture of peanut-primed LNC with con A also provoked more delayed (72 and 120 h) increases in some type 2 cytokines, with elevated levels of mRNA for IL-10 and IL-13 recorded. These differences in gene expression at 24 h (as detected by RPA) are represented graphically in Fig. 6 (mean and range of two independent experiments) following densitometric analysis and adjustment for housekeeping gene (GAPDH) expres-

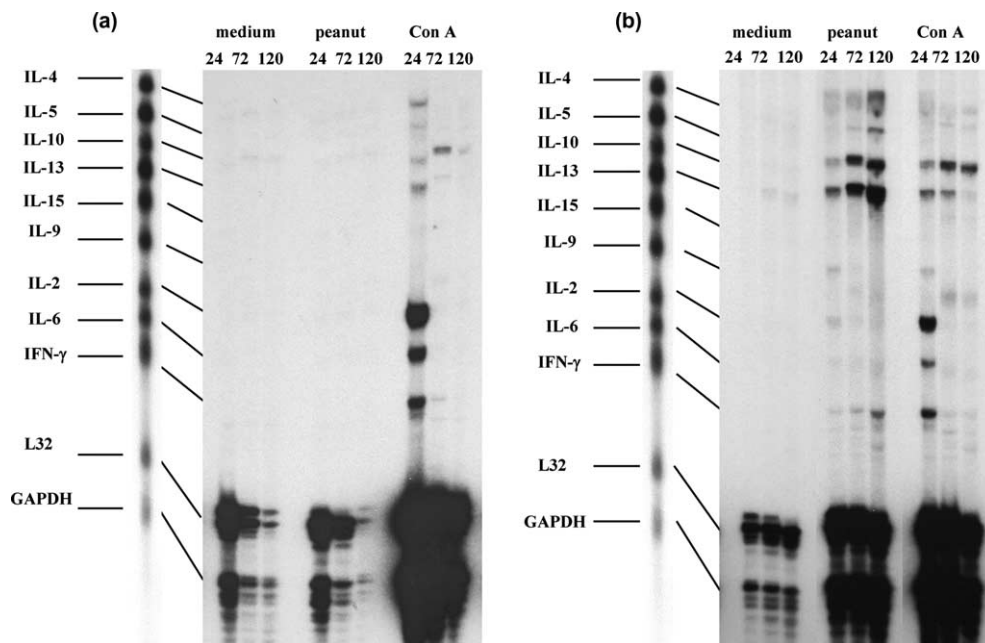


Fig. 5. Cytokine mRNA profiles following in vitro stimulation of naïve and peanut-primed LNC. Lymph nodes excised from naïve (a) or peanut-primed (b) mice pooled per treatment group ($n = 15$ mice) were cultured in vitro in the presence of medium alone, peanut agglutinin (200 $\mu\text{g}/\text{ml}$) or con A (2 $\mu\text{g}/\text{ml}$). Total RNA was isolated from cells harvested at 24, 72 and 120 h and the levels of cytokine and housekeeping gene (L32 and GAPDH) mRNA expression assessed by multiprobe RPA analysis. Representative autoradiographs are shown with concurrent undigested probe samples alongside to provide identification of cytokine fragment sizes.

sion. Consistent with the autoradiograph of the polyacrylamide gel shown in Fig. 5, culture of naïve LNC with peanut agglutinin failed to induce significant expression of any of the cytokines measured. In contrast, culture with con A induced marked up-regulation of IL-2, IL-6 and IFN- γ mRNA. LNC derived from peanut primed mice expressed high levels of transcripts for the type 2 cytokines IL-4, IL-5, IL-10 and IL-13, with relatively low levels of the type 1/inflammatory cytokines IL-2, IL-6 and IFN- γ . Analogous with its effects on naïve LNC, con A activation of peanut primed LNC resulted in high levels of transcripts for IL-2, IL-6 and IFN- γ . In these latter cells, however, con A stimulation also provoked increases in transcripts for the type 2 cytokines, particularly IL-10 and IL-13.

3.4. Cytokine expression in the CD62L⁻ cell fraction of peanut primed LNC

The ability of con A to induce type 2 cytokine up-regulation in peanut primed, but not naïve, LNC populations suggests that the profile of cytokine mRNA and protein expression stimulated by con A differs between nodes comprising naïve cells alone or naïve and activated LNC. It is possible that the LNC population in mice exposed to peanut agglutinin can be segregated into naïve (type 1/inflammatory cytokine) and activated (type 2 cytokine) producing T cells. In order to test this hypothesis we fractionated freshly

isolated LNC from peanut-primed mice on the basis of differential CD62L expression (Assenmacher et al., 1998). CD62L (L-selectin) is a homing receptor preferentially expressed on naïve lymphocytes and is thought to be involved in the trafficking to the peripheral lymph nodes (Bradley et al., 1994; Picker, 1994; Streeter et al., 1988). This receptor is down-regulated upon lymphocyte activation (Camp et al., 1993; Rothman et al., 1991, Varga and Welsh, 1996). Populations of CD62L⁺ and CD62L⁻ cells were supplemented with naïve LNC enriched for antigen presenting cells (APC, T cell depleted, purity greater than 85%) in a ratio of 5:1 to correct for the loss of APC in the CD62L⁺ fraction and cultured for 24 h in the presence of con A. The supernatants from the cell cultures were assayed for cytokine protein secretion. As described for previous experiments, unseparated peanut-primed LNC expressed IL-4, IL-5, IL-10, IL-12p40, IL-13 and IFN- γ upon secondary stimulation with con A (Table 1). However, upon separation the CD62L⁻ fraction (containing activated T cells and APC) was highly enriched for the Th2 cytokines IL-4, IL-5, IL-10 and IL-13 in the absence of a similar increase in the type 1/inflammatory cytokine IFN- γ . The CD62L⁺ fraction (naïve T cells, purity 95–98%) produced relatively low levels of the Th2 cytokines but displayed conserved secretion of IFN- γ and IL-12p40, a pattern similar to that observed following stimulation of unseparated naïve LNC stimulated with con A (Table 1).

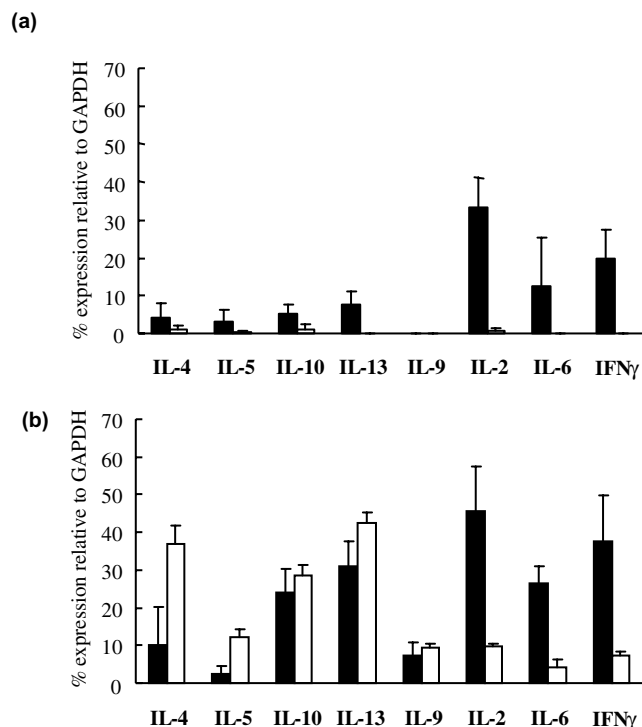


Fig. 6. Expression of cytokine mRNA transcripts in naive (a) and peanut-primed (b) LNC stimulated in vitro with con A (■) or peanut agglutinin (□). Draining lymph nodes excised from naive (a) or peanut-primed (b) mice pooled per treatment group ($n = 15$ mice) were cultured in vitro in the presence of medium alone, peanut agglutinin (200 $\mu\text{g}/\text{ml}$) or con A (2 $\mu\text{g}/\text{ml}$). Total RNA was isolated from cells harvested at 24 h and the levels of cytokine and housekeeping gene (L32 and GAPDH) mRNA expression assessed by multiprobe RPA and quantitated by densitometric analysis. Cytokine mRNA levels are expressed relative to the levels of housekeeping gene, GAPDH, transcripts (mean and range of two independent experiments).

4. Discussion

Intradermal immunization of BALB/c strain mice with peanut agglutinin (0.1%) in the absence of adjuvant resulted in systemic IgE antibody production (titers of 1/16 or 1/32), consistent with the known allergenic potential of this protein (Burks et al., 1994). These titers are similar to those reported previously following intraperitoneal sensitization of the same strain of mice

with the same dose of protein (1/32 or 1/64) (Dearman et al., 2003b,c). Under these conditions of exposure, draining auricular LNC proliferated in response to restimulation in vitro with specific allergen, and expressed a polarized Th2 cytokine profile, at both the protein and mRNA levels. Stimulation of naive auricular LNC with peanut agglutinin did not induce proliferative responses or the production of Th2 type cytokines. These data demonstrate that despite being defined as a lectin, peanut agglutinin does not, under these conditions of exposure, act as a mitogen or in any other way cause the non-specific activation of T lymphocytes.

In contrast, LNC isolated from both peanut-primed and naive animals responded vigorously to restimulation with the T cell mitogen con A, with similar levels of thymidine incorporation recorded. Interestingly, the cytokine responses induced by culture of peanut-primed LNC with con A were markedly different from those observed for LNC derived from naive mice. Naive LNC cultured in the presence of con A expressed primarily transcripts for IFN- γ , IL-2 and IL-6, and IL-12p40 was also detectable at the protein level. In contrast, when cultured in the presence of con A, peanut-primed LNC displayed a more mixed cytokine phenotype, with relatively high levels of the type 2 cytokines IL-4, IL-5, IL-10 and IL-13 and the type 1 cytokines IFN- γ and IL-2. This suggests that the peanut-primed LNC population consists of naive and activated cell populations, that upon activation with con A express Th1/inflammatory type cytokines and Th2 type cytokines, respectively. In order to test this hypothesis, LNC from peanut-primed animals were segregated according to membrane expression of CD62L. CD62L (L-selectin) is a homing receptor preferentially expressed on naive lymphocytes and is thought to be involved in the trafficking of cells to the high endothelial venules within peripheral lymph nodes (Bradley et al., 1994; Picker, 1994; Streeter et al., 1988). Indeed, studies with L-selectin-deficient mice have demonstrated that lymphocyte entry into peripheral LN is predominantly L-selectin dependent (Streeber et al., 1996). This homing receptor is apparently lost upon activation (Camp et al., 1993; Rothman et al., 1991, Varga and Welsh, 1996), and is only expressed at low

Table 1

Cytokine secretion profiles of naive and peanut-primed LNC cultured in the presence of con A

	IFN	IL-12p40	IL-4	IL-5	IL-10	IL-13
Naive	2099	383	4	198	147	308
Peanut-primed	3759	109	12	162	270	534
CD62L ⁺ CD90 ⁻	3261	193	5	33	147	109
CD62L ⁻ CD90 ⁻	2204	128	114	715	3638	3313

Cytokine levels (pg/ml of culture supernatant) are shown for a representative experiment where peanut-primed LNC were cultured in the presence of con A (2 $\mu\text{g}/\text{ml}$) for 24 h following separation on the basis of CD62L expression. Unseparated naive and peanut-primed LNC were also stimulated with con A over a 24 h period and all culture supernatants analyzed by multiplex bead assay technology. CD62L⁺ and CD62L⁻ cell fractions were supplemented with T cell-depleted (CD90⁻) naive cell population in order to reconstitute the APC capacity of the CD62L⁺ cell population and permit cytokine production.

levels on effector T cells. The expression of CD62L might be associated with immune surveillance by naïve lymphocytes whilst loss of this homing receptor is a marker of effector function (Hengel et al., 2003). Peanut-primed LNC populations were separated into CD62L⁺ (naïve) and CD62L⁻ (activated) T cells and con A-induced cytokine secretion profiles were examined. CD62L⁺ cells expressed a cytokine profile similar to that observed in LNC isolated from naïve animals (high levels of IFN- γ , IL-2 and IL-6). The CD62L⁻ (activated) fraction of the peanut-primed LN population was associated with a highly enriched Th2 cytokine profile indicating the presence of a mature population of Th2-type lymphocytes that respond to challenge with specific allergen or mitogen with the expression of type 2 cytokines.

In these studies, cytokine responses have been analyzed at both the transcript and secreted protein level, by RPA and ELISA respectively. The RPA technique in which multiple cytokine transcripts are analyzed in a single reaction using a commercially available multi-probe does not, unlike reverse transcription-polymerase chain reaction (RT-PCR), rely on repeated amplification steps and thus the levels of cytokine measured are quantitative, although at the cost of some reduction in sensitivity compared with RT-PCR. There was a high level of agreement between the methods with respect to both the kinetics and the patterns of the cytokine responses detected. However, both techniques required culture in the presence of allergen or mitogen to stimulate the release or expression of detectable levels of cytokine protein and mRNA, respectively. Neither method was sensitive enough to detect the spontaneous production of cytokine protein and mRNA following sensitization to protein allergen, although both detection systems have been used successfully to investigate cytokine responses induced following exposure to low molecular weight chemical allergens in the absence of restimulation *in vitro* (Betts et al., 2002). Thus, topical exposure of BALB/c strain mice to the chemical contact allergen 2,4-dinitrochlorobenzene results in the selective expansion of LNC that secrete type 1 cytokines whereas similar treatment with the chemical respiratory allergen trimellitic anhydride stimulates a type 2 cytokine profile (Dearman et al., 2001, 2003a). Vigorous proliferative responses and cytokine expression are recorded for auricular LNC derived following exposure *in vivo* to simple chemical allergens without a requirement for secondary stimulation with allergen under conditions where control (vehicle-treated) LNC are relatively quiescent. In addition, cytokine transcripts are readily detected using RPA in freshly isolated tissue from chemical-allergen activated lymph nodes (Betts et al., 2002; Manetz et al., 2001; Plitnick et al., 2002). The differential responsiveness of murine LNC following exposure to low molecular weight chemical allergens

compared with protein allergens is probably related to the fact that for chemical allergens a single allergenic epitope is presented to the immune system at high molar concentrations, resulting in strong and polarized responses, whereas protein allergens are a complex mixture of epitopes that stimulate less vigorous responses overall.

Despite the need for secondary restimulation *in vitro*, we have established the induction of an allergen-specific Th2-type response with high levels of IL-4, IL-5, IL-10 and IL-13 following exposure to peanut allergen in the absence of adjuvant. This pattern of cytokine secretion was not observed following exposure to acid phosphatase from potato (PAP), protein from a foodstuff considered to lack allergenic sensitizing potential. The development of type 2 responses in murine models of protein allergy has been described previously. However, these have generally required exposure to proteins in the presence of type 2 adjuvants such as alum (Adel-Patient et al., 2003; Bae et al., 1999; Iwasaki et al., 2001; Rajagopal et al., 2000) or cholera toxin (Li et al., 1999, 2000, 2001; Morafo et al., 2003). For example, robust increases in specific serum IgE antibody and preferential IL-4 and IL-5 expression by splenocytes have been reported following oral sensitization of C3H/HeJ strain mice with cows' milk or ground peanut in the presence of cholera toxin (Li et al., 1999, 2000). A small number of studies has been reported in which allergen-specific cytokine expression in the absence of adjuvant has been observed. It has been demonstrated that weekly oral exposure of C3H/HeJ strain mice to ground whole peanut (1 mg per application) over a 7 week period could prime spleen cells for the secretion of IL-5, IL-10 and IL-13, although no IL-4 was detected (Bashir et al., 2002). However, concomitant treatment with cholera toxin resulted in more vigorous cytokine expression and specific IgE was only observed when peanut protein was administered in the presence of adjuvant (Bashir et al., 2002). In addition, multiple intraperitoneal injections to BALB/c mice of low doses of the food allergen ovalbumin (OVA) followed by multiple respiratory challenges with OVA resulted in increased expression in the lung of mRNA for IL-5, IL-13 and eotaxin in the absence of elevated transcripts for IL-4 or IFN- γ (measured by RT-PCR) compared with OVA sensitized saline challenged controls (Barends et al., 2002). In both experimental systems it seems that multiple exposures and/or sensitive methods of detection have been necessary to detect allergen-specific cytokine production. In contrast, in the studies described herein, intradermal exposure to peanut allergen over a 14 day protocol has been sufficient to prime draining LNC for type 2 cytokine production, measured at both the mRNA and protein level.

A potentially contentious issue is the selection of route of exposure. Although in the context of food allergy it

may appear appropriate to regard oral administration as the preferred route of exposure, there are compelling reasons to consider alternative approaches. Thus, exposure of rodents to novel proteins in the diet or in drinking water may result in tolerance or immunological hyporesponsiveness rather than sensitization (Knippels et al., 1998). Even gavage exposure of rats or mice to proteins may be rather variable or insensitive with respect to the induction of IgE antibody responses (Dearman et al., 2001; Knippels et al., 1998). The emphasis of the current investigations is to examine end points that may discriminate between proteins that have an inherent potential to provoke allergic responses from those that apparently do not. That is, the emphasis is on the identification of intrinsic hazard. In this context it must be acknowledged that an inherent potential to induce sensitization as defined here will not necessarily translate into a risk of allergy and allergic disease in humans, when other factors, such as the impact of the type of exposure, are taken into account. In addition, the objective of the study was to examine cytokine responses in local lymph nodes draining the site of exposure; we have chosen therefore to expose mice to test proteins using parenteral (intradermal) administration. It is also of some interest that there is increasing reason to believe that the skin might be a relevant route of exposure in some circumstances for the development of sensitization to food proteins (including peanut). For example, an association has reported by the ALSPAC (Avon Longitudinal Study of Pregnancy and Childhood) team between cutaneous exposure to peanut antigens and the subsequent development of food allergy to peanut, with skin exposure to peanut-containing creams being more common in children who became allergic to peanuts than in control infants, either atopic but not peanut allergic, or non-atopic (Lack et al., 2003).

These data demonstrate that intradermal (parenteral) exposure of BALB/c strain mice to protein allergen in the absence of adjuvant, under conditions that stimulate specific IgE antibody production, is associated with the induction of a type 2 cytokine phenotype in mature (CD62L⁺) T cells. Although confirmation of the selectivity and sensitivity of the response is required with other allergens, these preliminary data suggest that it may prove possible in the future to use cytokine expression profiling as an adjunct or alternative to serological analyses.

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