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Immune modulation in suckling rat pups by a growth factor extract derived from milk whey

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Summary. Oral tolerance to foreign enteral antigens is not fully developed in early neonatal life. Epidemiological evidence supports a role for maternal milk in the development of immune responses, including oral tolerance. Formula fed infants have an increased susceptibility to food allergy and the later development of autoimmune disease. This may relate to the lack in infant formula of growth factors found in maternal milk. Bovine milk contains proteins, growth factors and cytokines. Various studies have outlined the immune modulating potential of bovine milk-derived products. Fractionated whey extracts have therapeutic potential in disease states where there is an excessive inflammatory reaction, and disease preventive potential for infants who are not breast-fed. We have shown that daily oral administration of a growth factor-enriched fraction from milk whey to naturally suckling rat pups between days 4–9 postnatal can down-regulate immune activation to a specific orally administered food antigen, ovalbumin, assessed by lymphocyte proliferation. In addition, non-specific down-regulation in the intestine was observed as assessed by the expression of MHC I. Treatment of rat pups with whey extract at the time of oral sensitisation to ovalbumin also resulted in an increased secretion of TGF-β into the culture supernatant of spleen cells incubated with specific antigen. TGF-β is an immuno-down-regulatory cytokine involved in tolerance induction. Immune modulation by extracts derived from milk whey could be of potential benefit for formula-fed and pre-term infants in reducing susceptibility to inappropriate activation to food antigens.

Keywords: Infant, immune-modulation, whey, TGF-β.

After birth, the gastrointestinal tract of the neonate is exposed to food, bacterial and environmental antigens. This antigenic challenge requires that the mucosal immune system adapt to distinguish between potential pathogens, which require a protective immune response, and food or self-antigens, where the immune response must be regulated. Indirect evidence suggests that maternal milk components play a role in regulation of mucosal immune activity to luminal antigens (Carver *et al.* 1991; Enomoto *et al.* 1993; Pabst *et al.* 1997). For example, breast fed infants are reported to produce higher antibody responses to oral and parenteral vaccines, and

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better cell-mediated responses to *Bacillus Calmette-Guerin* when compared to formula-fed infants. (Pabst *et al.* 1989, 1997; Pickering *et al.* 1998). In addition-autoimmune diseases such as Insulin-Dependent Diabetes and Crohns's disease show an inverse correlation with breast-feeding (Mayer *et al.* 1988; Koletzko *et al.* 1989; Saukkonen *et al.* 1998).

Maternal milk may regulate immune responses in the neonate until weaning and provide a cytokine environment for antigen priming (Ellis *et al.* 1997). A number of cytokines are reported to be present in milk, including IL-4, IL-10, IL-1rA and TGF-β (Rudloff *et al.* 1993; Saito *et al.* 1993; Garofalo *et al.* 1995); these cytokines have the potential to regulate mucosal immune activation. IL-4 has been shown to down-regulate the pro-inflammatory cytokine IFN gamma. IL-10 null mice develop enteritis with increased levels of IFN gamma (Sellon *et al.* 1998). TGF-β is one of the major cytokines present in milk. TGF-β is involved in IgA production and tolerance development. The evidence for a role for milk TGF-β in infant mucosal function is particularly strong. TGF-β null mice suckled on TGF-β +/− or TGF-β +/+ mothers survive for only a few weeks post weaning (Letterio *et al.* 1994; Nakabayashi *et al.* 1997). Oral administration of human milk or TGF-β to adult mice inhibited the antibody response to sheep red blood cells (Ishizaka *et al.* 1994).

Bovine milk contains proteins, growth factors and cytokines. In the past, various studies have outlined the immune-modulating potential of bovine milk-derived products (Stoeck *et al.* 1989; Mincheva Nilsson *et al.* 1990; Wong *et al.* 1997, 1998; Cross & Gill, 1999). Purified whey extracts have the potential to be of benefit as a therapeutic agent particularly in disease states where there is an excessive inflammatory reaction (Beattie *et al.* 1998; Murch & Walker Smith, 1998), or in the case of infants who have a compromised gut immune function (Carver *et al.* 1991). Bovine milk proteins also have the potential to regulate immune development in infants. In this study we show that oral administration of a growth factor-enriched fraction from milk whey down-regulated the peripheral immune response to oral antigen in the suckling period. A non-specific down-regulation of immune activation was also seen in the intestine.

materials and methods

Animals

Pregnant Hooded Wistar rats were obtained from the University of Adelaide breeding colony. The study was carried out with the approval of the Women's and Children's Hospital Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

Whey protein extract

Whey protein extract was prepared as described by Francis *et al.* (1995). Briefly, clarified bovine whey was fractionated on cation-exchange resin, and acidified to release latent bioactive proteins. The material was then passed through a 0·2-μm filter and bottled aseptically. The whey protein extract used had a protein content of 47 mg/ml (whey extracts contained 880 g protein/kg). Typical analysis of the major proteins present in the whey extracts is as follows; lactoperoxidase 65–75%, immunoglobulin 10–25%, insulin-like growth factor-I (13·4 ng/ml), insulin-like growth factor-II (19·14 ng/ml), and TGF-β (100–200 ng/ml). The insulin-like growth factors in the whey extract were assayed by radio immuno-assay and TGF-β levels assayed by Mv1Lu bioassay as described by Owens *et al.* (1990) and Belford *et al.*

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(1997). Whey extracts were negative for endotoxin (< 10 ng/mg protein) as measured by the LAL assay.

Regulation of immune responses by whey protein extract

Rat pups were orally gavaged with ovalbumin (900 μg) (Sigma, St Louis, MO, USA) in 0·1 ml phosphate buffered saline (PBS, pH 7·4) on days 4 and 5 of life. The ovalbumin gavaged pups were then divided into three groups, A, B and C, respectively (6 pups per group). Group A animals were gavaged orally with whey protein extract diluted into PBS at 200 μg/pup daily from day 4 to day 9. An initial dose-finding study was carried out where 200 μg whey protein extract/d per pup was shown to be optimal for modulating intestinal inflammation (data not shown). Group B were gavaged with PBS (0·1 ml/pup) from day 4 to day 9 and Group C were gavaged with casein at 200 μg/d per pup. Another two groups of six animals received a systemic dose of ovalbumin (i.p.) (50 μg/pup) on days 4 and 5. These animals were divided into two groups: group D were gavaged orally with whey protein extract diluted into PBS at 200 μg /pup from day 4 to day 9 and group E were gavaged with PBS 0·1 ml/pup from day 4 to day 9. A mother-reared unchallenged reference group was also included. At age 10 d, rat pups were killed by decapitation and the gastrointestinal tract rapidly excised and placed onto an ice cold glass slab. The small intestine was isolated and two 1-cm segments were collected from the ileum. Tissue was cooled by isopentane surrounded by liquid nitrogen and embedded in OCT (Miles Inc. Elkhart, IN, USA).

To assess the response to ovalbumin challenge, mesenteric lymph node and spleen cells were collected at 10 d and pooled from each group. Single cell suspensions were made by pushing the cells through a fine wire grid. Cells were then resuspended in RPMI 1640 medium containing 10% FCS (Life Technologies, Rockville, Maryland, USA), and mononuclear cells were purified on Lymphoprep (Nycomed Pharma AS, Oslo, Norway). The cells were washed twice in RPMI 1640 containing 10% FCS, 2 mM-L-glutamine, and 100 IU/ml benzylpenicillin, 0·1 mM-streptomycin/ml. Antigen specific proliferation was assessed by ^{3}H thymidine (Amersham Life Science, Buckinghamshire, UK) uptake. Cells (1×10^6 cells/ml) were cultured in triplicate in wells of a 96-well tray with ovalbumin (20 or 60 μg/ml) and an unrelated control antigen KLH 60 μg/ml (Sigma) for 5 d. Mitogen Con A, 1 μg/ml, (Sigma) was also used as a control to show that the cells were capable of a proliferative response. The peak of Con A responsiveness is normally taken at 3 d; in the case of neonatal rat cells, 5 d was optimal for responses to ovalbumin and therefore Con A was assessed at the same time even though it was not at the peak of the response. Cells were pulsed for the last 18 h of culture with 1 μCi ^{3}H thymidine/well and harvested onto fibreglass filters with a 96-well cell harvester (Shaktron, Lier, Norway) for analysis in a Beta Counter (Packard, Canberra, Australia). Unstimulated cells always showed a minimal background proliferative response (^{3}H thymidine uptake) in all experiments and this was consistent across all the animal groups.

TGF-β Bioassay: cell growth inhibition of Mv1Lu cells

TGF-β concentration in spleen cell culture supernatant was measured by the standard method of Meager (1991) using Mv1Lu epithelial cells (American Type Culture Collection CCL64). Cells were cultured for 24 h then changed to medium containing various dilutions of recombinant rhTGF-β (R&D Systems, MN, USA) (murine, and human reactive) to generate a standard curve, or supernatant from spleen cell cultures was assayed as described above. TGF-β concentration in the

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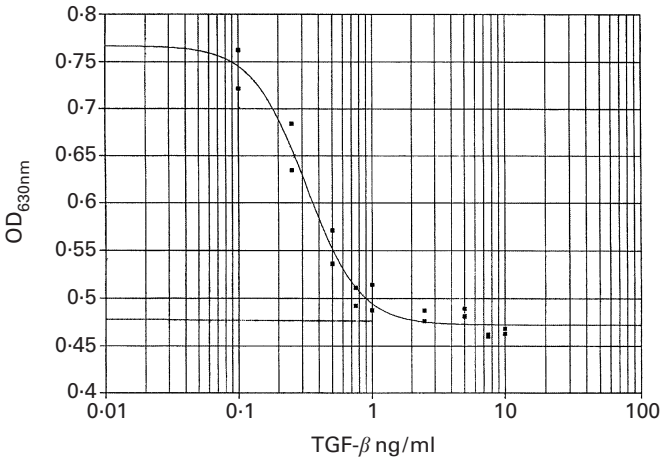


Fig. 1. TGF-β Mv1Lu bioassay: standard curve of recombinant TGF-β. Recombinant TGF-β1 (100 ng/ml) was serially diluted and the curve was generated by logistic regression. The measured optical density (OD) of test samples were converted into ng/ml according to the standard curve on each plate.

culture supernatant was determined by reference to a TGF-β standard curve (Fig. 1).

Immunofluorescence

Cryostat sections (8 μm) of rat intestinal tissue (ileum) were cut and mounted onto gelatin-coated slides. The sections were air dried, fixed in acetone for 10 min at room temperature, then dried and stored at -20 °C.

Tissue sections were washed with 10 mM-Tris buffered saline (TBS; pH 7·4), and preincubated with 3% (v/v) bovine serum albumin and 1% (v/v) normal mouse serum (Sigma) for 30 min to block non-specific binding. The sections were then incubated with mouse-anti rat MHC class I or class II antibody (Ox3 and Ox17 respectively; antibodies from Serotec). After incubation for 60 min, the sections were washed in TBS containing 0·5 g polyxyethelene 20 sorbitan monolurate (tween 20)/l (BDH Lab Supplies, Pool, UK). The sections were then incubated with biotinylated horse antimouse antibody (Vector Labs, Burlingame, CA, USA) for a further 60 min; after washing (as above), the sections were incubated with streptavidin Cy3 (Jackson Laboratories, PA, USA) for 30 min. Control sections were incubated without primary antibodies, or with mouse IgG at the same concentration as the primary antibody. Sections were mounted in non-fluorescent mounting media (Dako, Carpenteria, CA, USA). The intensity of labelling was assessed using a fluorescence microscope (Leica, Wetzlar, Germany) at 400× magnification. Four to six animals per group were assessed, and only intact villi were measured.

In vitro *regulation of MHC I expression on IEC-6 cells after interferon gamma stimulation*

Rat IEC-6 cells were cultured in 10 ml DMEM (Cell Image, Adelaide, Australia) with 10% FCS + penicillin/streptomycin + L-glutamine (Life Technologies) in T-75 flasks. The cells were passaged when approximately 80% confluent, counted and replated in T-25 flasks at 1×10^6 cells per flask in 5 ml DMEM containing 10% FCS. Flasks were incubated for 24 h at 37 °C, the culture medium was aspirated and cell monolayers were washed once with 5 ml PBS. Medium was replaced with

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DMEM containing 1% FCS. Murine Interferon gamma (100 U/ml) (Life Technologies) was added to each flask, as well as whey protein extract at concentrations ranging from 1 mg/ml to 0·001 mg/ml. Flasks were incubated for 48 h at 37 °C, before cells were collected and resuspended in 1 ml culture medium and counted. Cells were incubated for a further 2 h in polyheme-coated wells at 37 °C, then washed at 400 ***g*** for 5 min, and resuspended at 1×10^7 cells/ml in cold PBS containing 0·1 g Na azide/l. For flow cytometry, the IEC-6 cells (5×10^5/50 μl) were aliquoted in 5-ml tubes on ice and mixed with 5 μl of either Ox18 (mouse anti rat MHC I) antibody (Serotec) or X-63 control mouse myeloma supernatant. After 30 min on ice, cells were washed twice with 3 ml cold PBS/azide by centrifuging at 400 ***g*** for 5 min at 4 °C. Fifty microlitres of cold fluorescein-labelled anti-mouse immunoglobulin (Silenus) diluted 1/50 with PBS/azide was added and the cells incubated on ice for 30 min. The cells were then washed twice as above and fixed in 40 g paraformaldehyde/l and analysed by flow cytometry.

Statistics

Statistical analysis was carried out by the non-parametric method of Mann–Whitney (significance: $P < 0{\cdot}05$).

RESULTS

Whey protein extract regulation of splenic and mesenteric node cell proliferation after oral antigen sensitisation

We studied the *in vitro* immune response to ovalbumin of spleen and mesenteric node cells after oral ovalbumin sensitisation and gavage with whey protein extract. Spleen cells from rat pups which had received whey protein extract showed a significant decrease in thymidine uptake (Fig. 2*a*; $P < 0{\cdot}05$ for ovalbumin at 20 and 60 μg/ml in culture) compared with animals gavaged with PBS or an equivalent concentration of casein. The proliferative response to an unrelated antigen to which the animals had not had prior oral exposure, KLH, was not down-regulated. Spleen cells from animals gavaged with PBS had a stimulation index of $6{\cdot}2 \pm 2{\cdot}7$ after incubation with 60 μg/ml KLH, whereas spleen cells from animals gavaged with whey protein extract had a stimulation index of 14 ± 3 after incubation with 60 μg/ml KLH. Mesenteric node cells showed a similar down-regulation of proliferation to ovalbumin after gavage with whey extract, although this was significant only for the higher ovalbumin concentration (data not shown).

Systemic sensitisation with ovalbumin had no suppressive effect on the proliferative response to ovalbumin *in vitro* (Fig. 2*b*). Oral treatment with either PBS or whey protein extract at the time of oral ovalbumin gavage resulted in no significant difference in the proliferative response to a mitogen (Con A), as demonstrated by uptake of ^{3}H thymidine (Fig. 2*c*). In contrast, when Con A was co-cultured with ovalbumin, the spleen cells from animals that had received an oral gavage with whey protein extract and oral ovalbumin exposure had a significant reduction in the proliferative response *in vitro*. No down-regulation was seen in the response of spleen cells from the PBS-treated group when cells were cultured with Con A in conjunction with ovalbumin. Since the spleen cells were able to proliferate strongly in culture with Con A alone, but the response was down-regulated when ovalbumin was added to the culture with cells from the whey gavaged animals, cytokine secretion into the culture supernatant by ovalbumin-sensitised cells may be down-regulating the response to Con A (bystander suppression).

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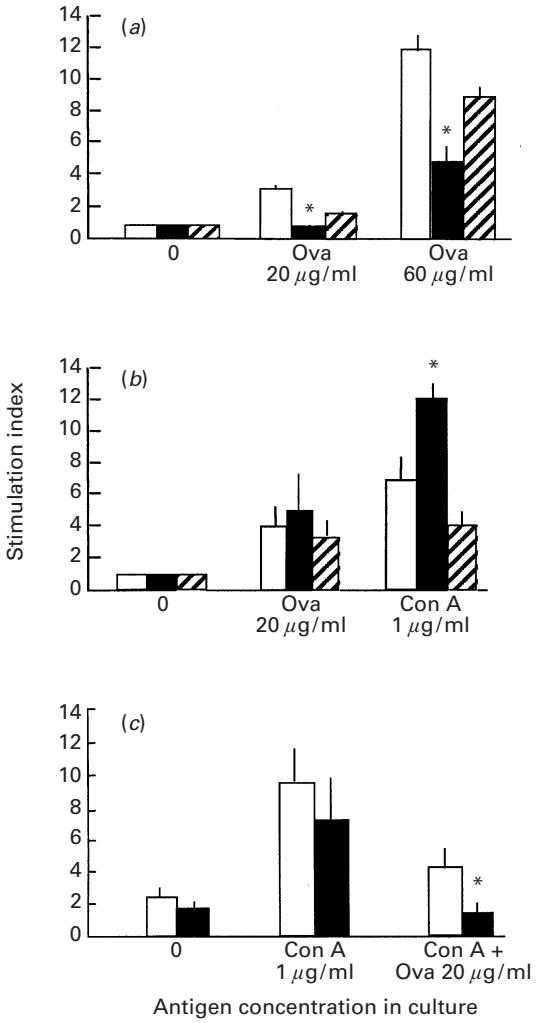


Fig. 2. Stimulation index of spleen cells from rat pups gavaged with PBS (open bars), casein (diagonal bars) or whey protein extract (filled bars) incubated with ovalbumin (Ova; 20 or 60 μg/ml) or Concanavalin A (Con A; 1 μg/ml) or a combination. ^{3}H thymidine uptake measured in spleen cells from (*a*) rat pups (age 10 d) which were gavaged days 4 to 9, and sensitised orally with ovalbumin at days 4 and 5; (*b*) gavaged rat pups sensitised systemically to ovalbumin; (*c*) rat pups gavaged with saline or whey protein extract and orally sensitised to ovalbumin. Values are means $\pm$ SD for $n = 4$ experiments. Significant difference between the PBS and whey protein extract treatment groups is indicated with asterisks (* $P < 0{\cdot}05$).

Whey protein extract and local mucosal immune regulation

To assess the general immune activation status of the intestine, we measured MHC I and MHC II expression on epithelial cells in sections of ileum from pups orally sensitised to ovalbumin and gavaged daily with whey protein extract or PBS (Fig. 3). For comparison, a group of naturally suckled rat pups with no treatment was included.

MHC I labelling of epithelial cells was strong in the ileum of animals sensitised with oral ovalbumin and gavaged with PBS (Fig. 3*a*). MHC I labelling was reduced in intensity in ovalbumin-sensitised rat pups gavaged daily with 200 μg/pup of whey protein extract (Fig. 3*b*). The level of labelling was reduced almost to that seen in

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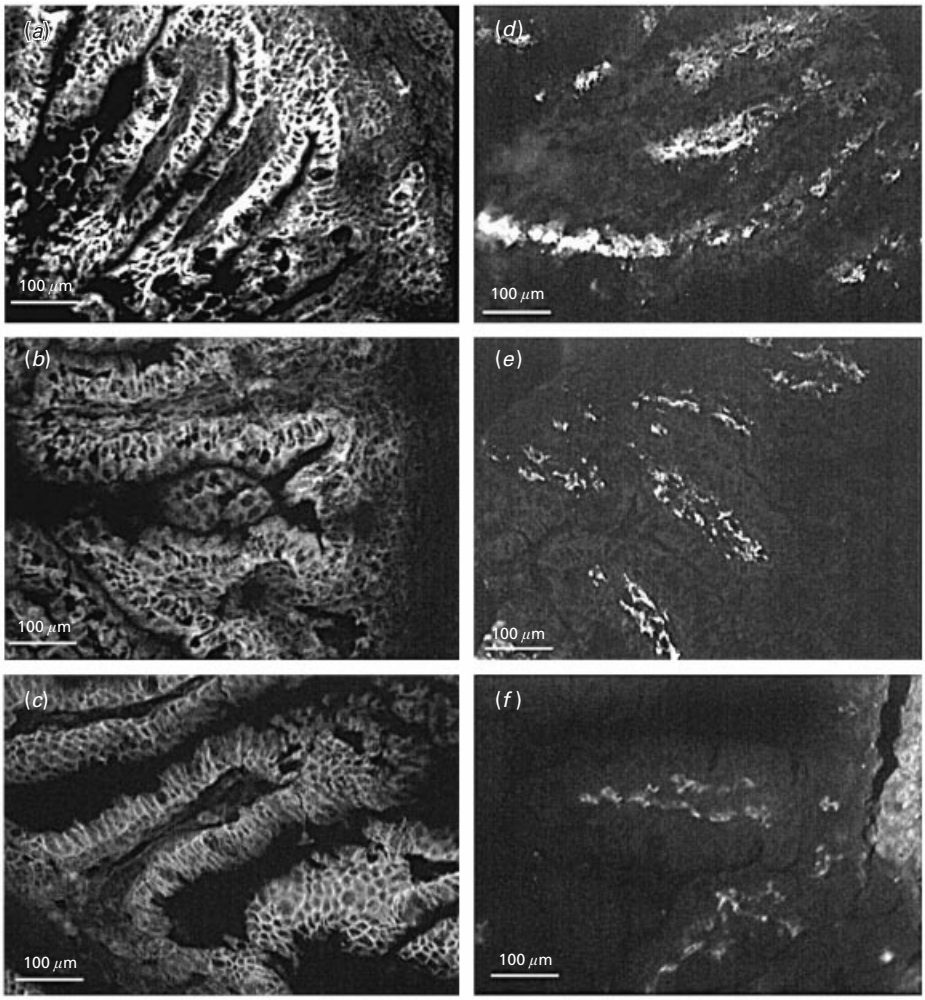


Fig. 3. MHC I (*a*, *b*, *c*) and MHC II (*d*, *e*, *f*) expression is shown in ileum after oral ovalbumin sensitisation in rat pups gavaged with PBS (*a*, *d*) and 200 μg/d whey protein extract (*b*, *e*). Expression in ileum from naturally suckled, unchallenged animals is shown for comparison (*c*, *f*). 400× magnification.

unsensitised (no treatment) rat pups which have a constitutive expression of MHC I (Fig. 3*c*). When the intensity of labelling was quantified, a dose of 200 μg/d per pup was considered optimal for down-regulation of MHC I expression (data not shown). There was a decrease in MHC II expression after oral whey protein extract treatment compared to the saline group (Fig. 3*d*, *e*, respectively), however, the difference was not statistically significant.

TGF-β concentration in cell culture supernatant

To assess whether the above cell growth inhibitory effects might be due to stimulation of TGF-β secretion by the cultured cells, TGF-β concentration was measured in cell culture supernatants. Spleen cells from whey extract gavaged animals showed five-fold higher TGF-β concentrations in the supernatant after culture with ovalbumin, compared with spleen cells from saline-gavaged animals

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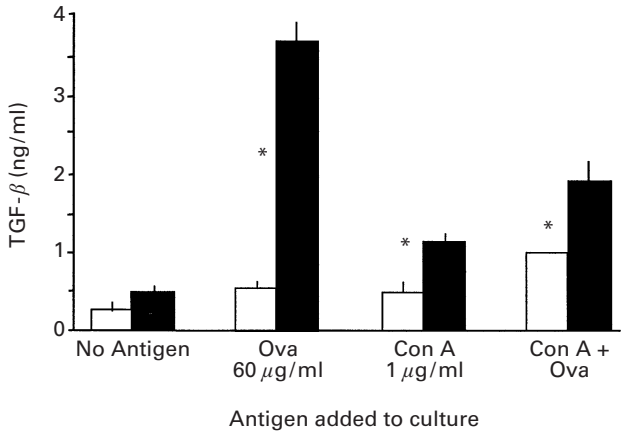


Fig. 4. TGF-β1 concentration (ng/ml$\pm$SD) in culture supernatant of spleen cells from rat pups gavaged with PBS (open bars) or whey protein extract (filled bars) and orally sensitised to ovalbumin. 1×10^6 spleen cells were cultured with ovalbumin 60 μg/ml alone or Con A 1 μg/ml alone, or a combination of ovalbumin and Con A. Significant difference between treatment groups is indicated with asterisks (* $P<0{\cdot}05$).

(Fig. 4). Whey protein extract gavage also induced a two-fold increase in TGF-β secretion in mitogen-stimulated cultures when compared with PBS gavage. TGF-β1 concentration was also assessed in the cell culture supernatant, where there was a down-regulation of the Con A response after co-culture with ovalbumin. TGF-β1 ($1{\cdot}93\pm0{\cdot}24$ ng/ml) was detected in the supernatant of spleen cells from whey extract-treated animals incubated with ovalbumin + Con A, in contrast to $<1{\cdot}0$ ng/ml of TGF-β1 detected in the culture supernatant of spleen cells from PBS-treated animals.

Whey protein extract and MHC I expression on intestinal epithelial cells in vitro

In view of the observation that whey protein extract inhibited MHC I expression on intestinal epithelial cells *in vivo*, we assessed inhibition of interferon-stimulated MHC I expression in IEC-6 cells cultured *in vitro* with IFN gamma. Whey protein extract at 0·01 mg/ml in culture inhibited MHC I expression as measured by mean fluorescence intensity by $41{\cdot}5\pm8\%$ (Fig. 5*a*). This inhibition in MHC I expression was dose dependent (Fig. 5*b*), suggesting that the whey protein extract is able to directly modulate intestinal epithelial immune function.

DISCUSSION

The whey protein extract in this study has immuno-down-regulatory activity both *in vivo* and *in vitro*. We have shown that oral administration of a whey protein extract to naturally suckling rat pups can down-regulate immune activation of spleen cells to an orally administered food antigen, ovalbumin. Immune down-regulation required prior exposure to oral antigen, and was not seen after peripheral antigen sensitisation. Immune down-regulation was not seen when whey protein extract was replaced by an equivalent protein concentration of casein, or PBS. Treatment of rat pups with oral whey protein extract when ovalbumin was given by gavage resulted in an increased secretion of TGF-β into the culture supernatant of

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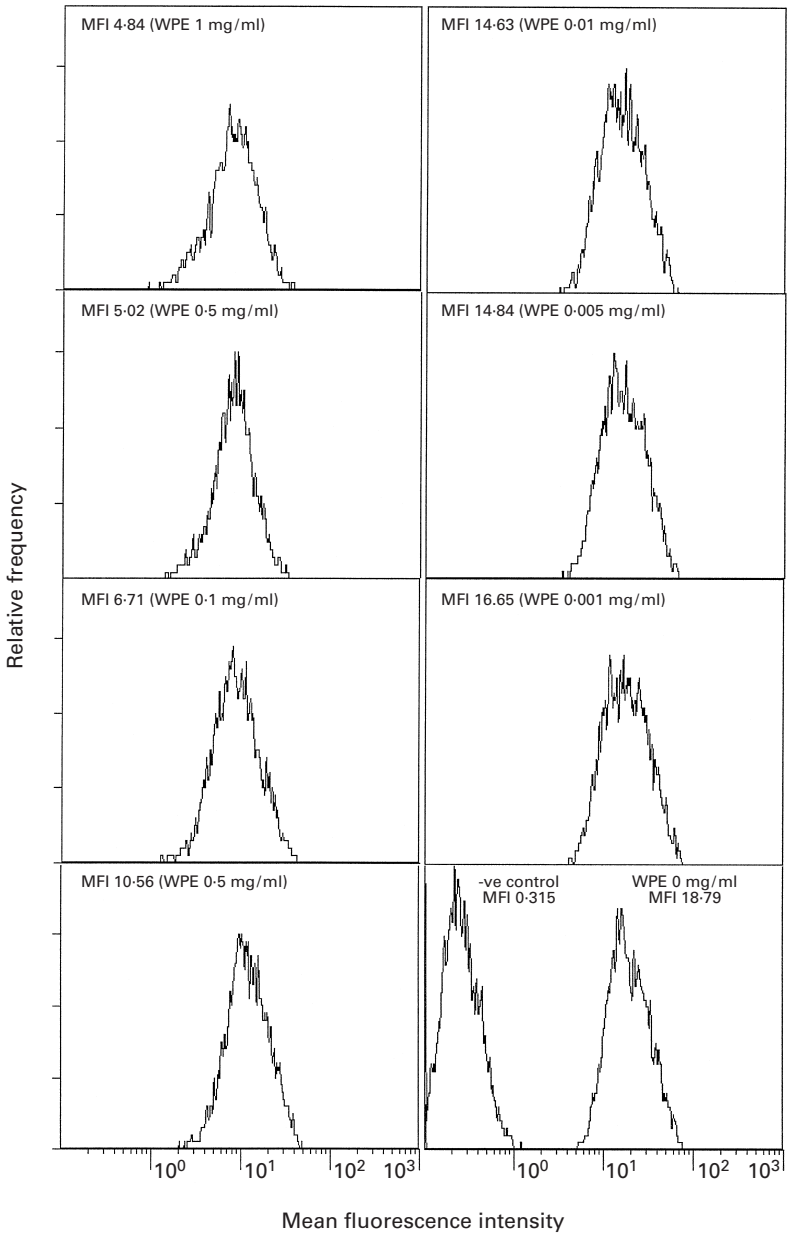


Fig. 5. A representative dose response histogram of interferon gamma-induced MHC I expression on IEC-6 cells. Mean fluorescence intensity (MFI) is increased as concentrations of whey protein extract decrease.

spleen cells stimulated with ovalbumin *in vitro*. These results suggest that oral whey protein extract may induce TGF-β secreting cells after oral sensitisation with ovalbumin. The role of TGF-β requires confirmation, but TGF-β is known to be secreted by lymphocytes during tolerance induction and to down-regulate immune responses (Miller *et al.* 1991; Hancock *et al.* 1995; Strober *et al.* 1998). *In vitro* TGF-β in the culture supernatant could down-regulate the specific proliferative response

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Fig. 6. Inhibition of interferon gamma-induced MHC I expression on IEC-6 cells after treatment with 0·01 mg/ml whey protein extract. Values are mean fluorescence intensity ± SD for $n = 7$.

to the antigen and also induce bystander suppression as seen when Con A was co-cultured with the antigen.

There are various reports in the literature of the immuno-modulatory effects of milk proteins, (Stoeck *et al.* 1989; Mincheva Nilsson *et al.* 1990; Wong *et al.* 1997, 1998). Studies have usually concentrated on isolated proteins rather than mixtures of extracted proteins. Recently, Cross & Gill (1999) reported on immuno-modulation by a whey protein mixture. The individual components mediating the immune modulation in whey extracts remains unknown. We report on the immuno-modulatory activity of our whey protein extract in the context of using milk-derived products as a potential therapeutic agent. Milk-derived proteins and their immune down-regulatory activity is thought to be important in infant gut immune development (Cummins & Thompson, 1997). Oral gavage with whey protein extract resulted in an increase in TGF-β secretion into the culture supernatant after incubation with ovalbumin. TGF-β, which is present in high concentrations in maternal milk, is an immuno-down-regulatory cytokine involved in tolerance induction, and TGF-β knock-out mice do not survive post weaning (Miller *et al.* 1991; Letterio *et al.* 1994; Garside & Mowat, 1997; Penttila *et al.* 1998).

Oral tolerance describes the state of systemic hyporesponsiveness, which follows systemic challenge with a previously fed protein (Weiner, 1997). Oral antigen exposure, particularly low dose antigen, can induce lymphocytes with regulatory function (T helper 3 cells) that secrete TGF-β and other down-regulatory cytokines, (Miller *et al.* 1991; Hancock *et al.* 1995; Strober *et al.* 1997, 1998), which can suppress or tolerise responses to other unrelated antigens (bystander suppression). High dose antigen induces anergy and deletion of reactive T cells (Chen *et al.* 1994; Dahlman Hoglund *et al.* 1995, 1997; Garside & Mowat, 1997; Gilbert *et al.* 1997). The capacity to develop systemic tolerance in response to oral antigen is not fully developed in neonates (Strobel & Ferguson, 1984; Enomoto *et al.* 1993). Most evidence suggests that oral tolerance development in humans and rodents is an age-dependent phenomenon. Feeding antigens such as ovalbumin or myelin basic protein to newborn animals may result in immune priming for humoral as well as cell-mediated immune responses, whereas oral tolerance occurs when the same antigen is fed to adult animals (Strobel & Ferguson, 1984; Enomoto *et al.* 1993; Miller *et al.* 1994).

Gut immune function and the cytokine environment that an antigen-presenting cell encounters are key factors in determining the outcome of exposure to dietary

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antigens, i.e. tolerance or immune activation. Individuals susceptible to autoimmune disease may respond to dietary antigens, with inappropriate activation and immune responses different from a healthy gut. Breast milk or whey protein extract as we describe above have the potential to regulate inappropriate immune responses to oral antigen by providing a cytokine environment for antigen presentation.

Immune response development is influenced by the antigen-presenting cell type as well as the cytokine environment that antigen presentation occurs in. After birth, the intestine is exposed to a large influx of antigens, which stimulate the gut-associated lymphoid tissue (Thompson *et al*. 1996, 1998). Enterocytes have been shown to be antigen-presenting cells. Enterocytes express classical (MHC I, MHC II) and non-classical MHC antigens (CD1d) (Reyes *et al*. 1997; Arosa *et al*. 1998). Enterocytes are not as efficient at presenting the antigen when assessed for proliferation and induction of lymphokines as classical antigen-presenting cells. However, antigen presentation by enterocytes may result in activation of T cells with suppressive function (Li *et al*. 1995), and presentation of non-conventional antigens such as lipid moieties to intraepithelial lymphocytes via MHC I (Schild & Chien, 1994; Schild *et al*. 1994; Tanaka *et al*. 1994). Intra-epithelial lymphocytes are activated by presentation of the antigen via MHC I, and activated intraepithelial cells can abrogate oral tolerance after transfer to recipient-tolerant mice (Takahashi *et al*. 1995; Guehler *et al*. 1999). Down-regulation of MHC1 and antigen presentation with whey protein extract could result in a down-regulation of inflammatory immune responses. MHC II expression was down-regulated by treatment with oral whey protein extract at the time of antigen challenge *in vivo*, but the difference was not statistically significant when assessed by image analysis. After oral antigen encounter, MHC II would be required for antigen presentation regardless of the final outcome of the immune response, i.e. down-regulatory or pro-inflammatory cytokine release.

Since infant formula is deficient in growth factors found in maternal milk, potential additives such as the whey protein extract described above may assist in directing the development of appropriate immune responses in the suckling period.

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