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Diabetes-prone BioBreeding rats do not have a normal immune response when weaned to a diet containing fermentable fibre

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Diet is known to modulate the development of diabetes in diabetes-prone BioBreeding (BBdp) rats. The objective of the present study was to determine the effect of fermentable fibre (FF) on immune function in BBdp and diabetes-resistant BioBreeding (BBdr) rats after weaning. Weanling BBdp (thirty-six to thirty-eight per diet) and BBdr rats (thirty to thirty-two per diet) were fed a nutritionally complete, semi-purified, casein-based diet containing either cellulose (control diet, 8 % w/w) or FF (3·2 % cellulose + 4·8 % w/w inulin). At 35 d, the small intestine was excised and lymphocytes isolated from spleen, mesenteric lymph nodes and Peyer's patches. Feeding FF to both BBdr and BBdp rats affected the production of anti-inflammatory cytokines ($P=0·02$). In BBdr rats, feeding FF compared with cellulose resulted in an increased small intestinal length ($P=0·0031$), higher proliferative (stimulation) index from both splenocytes ($P=0·001$) and mesenteric lymph nodes ($P=0·04$), and an increased proportion of CD8 + T-cells in the Peyer's patches ($P=0·003$). We did not observe an effect of diet on the number of IgA-bearing cells in the jejunum from BBdr rats. Feeding FF to BBdp rats did not affect the same parameters. BBdp rats had both a higher proportion of B-cells in the Peyer's patches ($P=0·01$) and a higher number of IgA + cells in the jejunum ($P=0·0036$) when fed a diet containing FF, a response not observed in BBdr rats. We demonstrate that several aspects of the BBdp immune system respond differently than that of BBdr rats when challenged at weaning with FF.

BB rat: Diabetes: Dietary fibre: Immune function: Weaning

The exact role of early diet in the pathogenesis of type 1 diabetes remains unknown. Studies of dietary intake during early life and subsequent development of diabetes in man are under way, such as the DAISY (Norris et al. 2003) and BABY DIAB (Ziegler et al. 2003) studies, although identification of precise dietary triggers are not clear at this point in time. One recent hypothesis suggests that the timing of the introduction of grains and cereals may play a role in increasing the risk of diabetes among children who are genetically at risk for this disease (Norris et al. 2003). Modifying the diet, and thereby altering exposure to potential dietary antigens in the diabetes-prone BioBreeding (BBdp) rat and non-obese diabetic mouse, two models of autoimmune diabetes, has been shown to significantly delay or prevent the onset of diabetes (Scott et al. 1985; Elliott et al. 1988). The mechanism by which diet is protective or diabetogenic has not been clearly defined, although the link between diet and development of diabetes strongly implicates a role for the gastrointestinal tract in its pathogenesis.

There is evidence from our laboratory and others that BBdp rats, compared to diabetes-resistant BioBreeding (BBdr) rats, may not be responding appropriately to a change in diet (dam's milk to cereal-based diet) at weaning. BBdr rats fed a non-purified cereal-based diet, as opposed to a semi-purified diet, had an increased small intestinal length, lower intestinal proglucagon mRNA (Reimer et al. 1998) and a greater [^{3}H]thymidine incorporation in mitogen-stimulated lymphocytes (Field et al. 1999a) whereas diet did not affect these measures in BBdp rats. Recently, the activity of intestinal enzyme invertase was reported to be increased in BBdr rats fed a cereal- or wheat gluten-based compared to a casein-based diet, while diet did not affect activity in BBdp rats (Courtois et al. 2004a).

Components of the standard cereal-based diet, such as fibres that are degraded in the large intestine by intestinal bacteria (fermentable fibres, FF), have received limited attention in this model (Perrin et al. 2003). The cereal-based diet contains some FF, while semi-purified diets usually contain cellulose, a compound not considerably metabolised by the gut microflora. In non-diabetic animals and human subjects, changing the type of fibre in the diet alters immune function in the gastrointestinal tract and periphery (reviewed by Schley & Field, 2002).

Early introduction of cereals to an infant at weaning (0–3 months) has recently been associated with increased islet cell autoimmunity (Norris et al. 2003). There is an abundant amount of evidence in BioBreeding rats (MacFarlane et al. 2003) and non-obese diabetic mice (Beales et al. 2003; Schmid et al. 2003) that wheat proteins, such as gluten, are diabetogenic. Cereals such as wheat contain FF, for example, β-glucans. Since FF can affect the immune system, the effects of this dietary component on BBdp rats at weaning warrants further investigation. We hypothesised that the gastrointestinal and peripheral immune system of BBdp rats would respond differently than

Abbreviations: BBdp, diabetes-prone BioBreeding; BBdr, diabetes-resistant BioBreeding; ConA, concanavalin A; FF, fermentable fibre; IFN-γ, interferon-γ; MLN, mesenteric lymph nodes; PMA, phorbol myristate acetate; TGF-β, transforming growth factor-β.

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BBdr rats when animals were weaned to a diet containing FF. The specific objective of this study was to determine the effects of adding the FF, inulin, to a semi-purified, casein-based diet (FF diet), on parameters of immune function in both BBdp and BBdr rats after weaning.

Experimental methods

Experimental design and diets

Male and female BBdr and BBdp rats were obtained from a colony maintained in the Department of Agricultural, Food and Nutritional Science at the University of Alberta. The breeding stock for this colony originated from the Animal Resources Division, Health Protection Branch, Health Canada, Ottawa, ON, Canada. BBdr rats have a rate of diabetes of less than 1 % (Like et al. 1982) and are used as a control strain. The incidence rate of diabetes in the BBdp rats from this colony was approximately 60 % on a cereal-based diet (unpublished results). This study was conducted in accordance with the Canadian Council of Animal Care Guidelines and the protocol was reviewed and approved by the Animal Policy and Welfare Committee in the Faculty of Agriculture, Forestry and Home Economics at the University of Alberta.

All animals in the present study were housed in plastic shoebox cages with woodchip bedding in a temperature- and humidity-controlled environment, on a 12 h light/dark cycle. Pregnant BBdr and BBdp dams were fed the control diet (Table 1). At delivery, litters were culled (when necessary) to a maximum of nine pups per litter. Pups were housed with their mother until 21 d old when they were weaned and randomised within a litter to one of two nutritionally complete, casein-based, semi-purified diets. The diets were identical except for the composition of the dietary fibre (80 g/kg; Table 1). The fat content and composition of the two diets was based on that of breast milk and infant formula. Rats were fed ad libitum on the diets for 14 d and were weighed regularly during this time. Food intake during these days was estimated by measuring the total amount of food given to each cage of weaned pups and dividing by the number of rats per cage (three rats per cage). At the end of the 14 d, rats were anaesthetised using halothane and a blood sample was taken by cardiac puncture. Rats were killed by cervical dislocation before tissue samples were collected.

Table 1. Composition of experimental diets (g/kg)

Ingredient	Control diet	Fermentable fibre diet
Corn starch*	378	378
Casein*	270	270
L-Methionine*	2·5	2·5
Stearate†	136	136
Safflower oil‡	62	62
Flaxseed oil§	7·2	7·2
Cellulose*	80	32
Inulin‖	0	48
AOAC Vitamin Mix*	10	10
Bernhart-Tomerelli Mineral Mix*	50	50
Inositol*	6·25	6·25
Choline bitartrate*	2·75	2·75

*Harlan-Teklad, Madison, WI, USA.
†CanAmara, Edmonton, AB, Canada.
‡Tosca Foods.
§Gold Top, Edmonton, AB, Canada.
‖Quadra Chemicals Ltd, Oreye, Belgium.
For details of diets and procedures, see p. 646

Lymphocyte isolation

At necropsy, the spleen and mesenteric lymph nodes (MLN) were removed under sterile conditions and the immune cells were isolated as previously described (Field et al. 1999a). Briefly, lymphocytes isolated from the spleen and MLN were washed with a Krebs–Ringer–HEPES buffer, pelleted and reconstituted in a complete culture media (RPMI 1640 with antibiotic/antimycotic, 5 % (v/v) fetal calf serum (Gibco, Burlington, ON, Canada), 25 mM-HEPES and 2·5 mM-2-mercaptoethanol, pH 7·4). Lymphocyte viability was assessed with trypan blue and was estimated to be >95 % (Sigma Chemical Co., St Louis, MO, USA) for all groups. Spleen cells were subjected to an additional treatment to lyse erythrocytes with a buffer containing 155 mM-NH_4Cl, 0·1 mM-disodium EDTA and 10 mM-$KHCO_3$.

The small intestine was excised from the body cavity between the stomach and the caecum and flushed with approximately 20 ml cold, sterile Hank's balanced salt solution with 4 mM-dithiothreitol. Peyer's patches were located visually, removed, placed in Hank's balanced salt solution containing 5 % (w/v) bovine serum albumin and 4 mM-dithiothreitol, and pressed through a sterile nylon mesh before being processed in the same manner as cells from the MLN.

Quantification of IgA+ cells in the lamina propria

A small piece of the jejunum from each rat was fixed in formalin, embedded in paraffin and later sectioned for determination of IgA by immunohistochemistry. Sections were cleared and rehydrated using xylenes and a graded series of ethanol dilutions, respectively. Endogenous peroxidase activity was blocked by covering the tissue section with 0·3 % (v/v) H_2O_2 for 12 min. Sections were then covered with citrate buffer (2 mM-citrate buffer, pH 6·0, 95 °C, 30 min) to facilitate antigen retrieval. Non-specific binding was blocked with 20 % (v/v) goat serum made in PBS. The primary antibody (monoclonal mouse anti-rat IgA; Sigma) was applied overnight at 4 °C. After washing, the sections were exposed to the secondary antibody (goat, anti-mouse IgG; Vector Laboratories, Burlingame, CA, USA; 20 min, room temperature). Peroxidase reagents, avidin/biotin complex kit and a diaminobenzidine kit were used for colour development. Slides were counterstained with Harris's haematoxylin (Sigma). Control slides were subjected to the same treatment as described earlier, without addition of the primary antibody. Isotype controls for IgA were performed using mouse anti-pig CD45RA IgG1 antibodies processed in the same manner as earlier. Non-specific staining was not observed in any of the controls. All samples were randomised and counted in a blinded manner by the same individual on a light microscope (Dialux 20; Leitz Wetzlar, Germany) at a magnification of 500 ×. Cells found in the lamina propria region that were positive for IgA were counted from twelve villi per sample (at four predetermined positions in three different sections).

Phenotype distribution

Lymphocyte subsets from the spleen, MLN and Peyer's patches were identified by one- and two-colour immunofluorescence

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using fluorescence-activated cell sorting as previously described (Field et al. 1999a). Briefly, mouse anti-rat monoclonal antibodies (IgG), specific for the following determinants: Ox8 (CD8 + T-cells), w325 (CD4 + T-cells), Ox42 (Macrophages), w313 (Pan T-cells), Ox12 (B-cells), 3.2.3 (Natural Killer cells), Ox19 (CD5 + T-cells) were kindly provided by Dr Rabinovitch, University of Alberta, Edmonton, AB, Canada. CD3-fluorescein isothiocyanate-conjugated mouse anti-rat was purchased from PharMingen (Mississauga, ON, Canada). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cedarlane, Hornby, ON, Canada) was used as a secondary antibody. For samples labelled with two colours, the second fluorescent label was anti-mouse phycoerythrocyn (Cedarlane). All antibodies were diluted in PBS with 4 % (v/v) heat-inactivated rat serum prior to use. All samples were fixed in 1 % (w/v) paraformaldehyde (Anachemia Science, Montreal, PQ, Canada) in PBS prior to acquisition and analysis (Field et al. 1999a).

Mitogen stimulation

Mitogen-stimulated proliferation of isolated lymphocytes was estimated by incubating spleen and MLN lymphocytes ($1{\cdot}25 \times 10^6$ cells/ml) for 68 h in the presence or absence of concanavalin A (ConA; 5 μg/ml; ICN, Montreal, PQ, Canada) or phorbol myristate acetate (PMA; 20 ng/ml; ICN) plus ionomycin (0·5 nmol/ml; Sigma) as previously described (Field et al. 1999a). Eighteen hours prior to harvesting, cells were pulsed with 37·5 kBq (1 μCi) [^{3}H]thymidine (Amersham Canada, Oakville, ON, Canada). Cells were harvested (Filter Mate® harvester; Canberra Packard Canada, Mississauga, ON, Canada) and radioactivity was counted (TopCount NXT® plate reader, Microscint scintillation fluid; Canberra Packard Canada). A stimulation index was calculated for each sample as: amount of [^{3}H]thymidine incorporation by stimulated cells per amount of incorporation by unstimulated cells.

Cytokine production

Lymphocytes isolated from the MLN, spleen and Peyer's patches ($1{\cdot}0 \times 10^6$ cells/ml) were incubated in the presence or absence of ConA or PMA + ionomycin (same concentrations as the proliferation assay) in 1 and 2 ml volumes. After 72 h of incubation, cell cultures were centrifuged (250 g for 5 min) and the supernatants collected and stored (−70 °C) until they were analysed for T_h1 (IL-2, TNF-α, interferon-γ (IFN-γ)), T_h2 (IL-10, IL-4) and T_h3 (transforming growth factor-β (TGF-β)) cytokines. Cytokine concentrations were determined in duplicate using commercially available ELISA kits (BD Biosciences, Mississauga, ON, Canada), according to manufacturer's instructions. The absorbance was read at 405 nm using the SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). For each cytokine, the concentration of the cytokine in each sample was quantified by comparison to a standard curve using recombinant cytokine standards. Cytokine detection limits were 31·3–2000 pg/ml for IL-2, TNF-α and IFN-γ, 1·6–100 pg/ml for IL-4, 15·6–1000 pg/ml for IL-10 and 15·6–1000 pg/ml for TGF-β. Duplicate samples with a CV of 15 % or less were used in the statistical analysis. Due to a limited amount of supernatant we were unable to run all the assays on each rat, the number of samples for which cytokines were assayed is indicated in Table 4.

Statistical analysis

All statistical analyses were conducted using the SAS (Statistical Analysis System) statistical software (version 7.0; SAS Institute Inc., Cary, NC, USA). Data are expressed as mean with the standard error of the mean and n, which represents the number of rats from at least three different litters. Statistical significance was set at $P \leq 0{\cdot}05$. Data were first examined to determine if there were any effects of sex using a three-way ANOVA for diet, sex and strain. Animals were blocked by litter to control for litter effects. In data sets showing no sex or litter effects, data were combined and analysed using a two-way ANOVA for main effects of diet and strain. Effects of sex were only observed in the body weights and weight change of BioBreeding rats, and did not affect any immunological parameters. Differences between groups were identified using least square means when an interaction occurred between the main effects.

Results

Rat characteristics

BBdp rats weighed significantly ($P = 0{\cdot}005$) more than BBdr (47 (SEM 1) g, n 73 v. 43 (SEM 1) g, n 63). However, weight change between 21 and 35 d of age did not differ between diet groups and rat strains. Male rats gained significantly more weight (67 (SEM 1) g, n 47) than females (57 (SEM 1) g, n 53) in all groups ($P < 0{\cdot}0001$) except for the BBdp rats fed the control diet, where weight change did not differ significantly between males (64 (SEM 3) g, n 14) and females (61 (SEM 2) g, n 21). Food intake per rat for the 14 d period did not differ significantly among groups (159 (SEM 5) g per 14 d), calculated per cage (thirty-three total cages). Sex did not significantly affect any immunological parameters tested. The percentages of females and males were for BBdr control, 55 and 45, BBdr FF, 45 and 55, BBdp control, 61 and 39, and BBdp FF 55 and 45, respectively.

The small intestine was longer ($P = 0{\cdot}0031$) for BBdr rats fed the FF diet (Table 2) compared with BBdr rats fed the control diet. Diet did not affect small intestinal length in BBdp rats. Spleen weight was not affected by diet in either strain, but BBdr rats had a significantly greater ($P < 0{\cdot}0001$) spleen weight than BBdp animals (Table 2). BBdr rats had a significantly higher ($P < 0{\cdot}0001$) spleen weight (5·4 (SEM 0·1) mg/g, n 47) when expressed per gram body weight than did BBdp rats (3·7 (SEM 0·2) mg/g, n 45). Consistent with the lymphopenia in these animals, significantly fewer ($P < 0{\cdot}001$) lymphocytes were isolated from the spleen and MLN of BBdp compared to BBdr rats. Both BBdr and BBdp rats fed the FF diet had significantly fewer splenocytes than the strain-matched rats fed the control diet ($P = 0{\cdot}026$). The number of immune cells isolated from the Peyer's patches did not differ between diet groups or rat strains (Table 2).

IgA+ cells in the jejunal lamina propria

In BBdr rats (twelve per group), the number of IgA+ cells within the lamina propria did not differ significantly between rats fed the control diet (48 (SEM 5) per twelve villi) v. the FF diet (58 (SEM 6) per twelve villi) (Fig. 1). However, BBdp rats fed the FF diet had a significantly higher ($P = 0{\cdot}0036$) number of IgA+ cells (88 (SEM

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Table 2. Characteristics of diabetes-resistant BioBreeding (BBdr) and diabetes-prone BioBreeding (BBdp) rats fed fermentable fibre (FF) and control diets

	BBdr						BBdp						Significance*		
	Control			FF			Control			FF					
Characteristic	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Diet	Strain	Diet × strain
Small intestinal length (cm)	80[a]	1	26	84[b]	1	26	79[a]	1	24	78[a]	1	25	NS	NS	P=0·0031
Spleen weight (mg)	531	18	24	537	30	23	400	24	22	397	14	24	NS	P<0·0001	NS
No. of splenocytes isolated ($\times 10^6$)	154	12	25	129	15	26	109	11	24	76	8	24	NS	P<0·0002	NS
No. of MLN cells isolated ($\times 10^6$)	23	2	25	21	2	26	7	1	25	8	3	23	NS	P<0·0001	NS
No. of Peyer's patch cells isolated ($\times 10^6$)	6·3	0·4	12	5·8	0·7	11	5·4	0·7	13	5·1	0·8	10	NS	NS	NS

MLN, mesenteric lymph node.
*Data were analysed by two-way ANOVA. P-values represent significance of main effects diet and strain. Where an interaction occurs between diet and strain across a row (diet × strain), significant differences are identified by least square means and are denoted by different superscripts (P<0·05).
For details of diets and procedures, see p. 646.

7) per twelve villi) compared to control diet-fed BBdp rats (42 (SEM 5) per twelve villi) or either BBdr group.

Phenotype distribution in the spleen, mesenteric lymph nodes and Peyer's patches

BBdp rats were T-cell lymphopenic as defined by significantly lower proportions of T-cells (W3/13 +, CD5 + W313 + cells) in all tissues examined (P<0·0001; Table 3). This lymphopenia affected both the CD4 + (P<0·0001) (Peyer's patches P=0·04) and CD8 + (P=0·0191) T-cell subsets in all tissues examined (Table 3). Diet, however, did not significantly affect the phenotype distribution of immune cells from the spleen or MLN of BBdp or BBdr rats. Feeding FF to BBdr rats resulted in a significantly higher proportion of CD8 + T-cells in the Peyer's patches compared to feeding the control diet (P=0·003; Table 3). CD8 + T-cells were virtually absent in Peyer's patches from BBdp rats (Table 3). Feeding FF to BBdp rats resulted in a higher proportion of B-cells in the Peyer's patches compared to BBdp rats fed the control diet or either BBdr group (Table 3). The proportion of CD3 + lymphocytes in BBdr rats was 46 (SEM 2)% (n 14), 43 (SEM 2)% (n 14) for the control and FF diets, respectively. CD3 was not measured in BBdp rats.

Response by splenocytes, Peyer's patch cells and mesenteric lymph node cells to mitogens

The incorporation of [^{3}H]thymidine uptake by splenocytes (2237 (SEM 131) decays per min, n 63) and MLN (589 (SEM 104) decays per min, n 52) cultured in the absence of a mitogen did not differ among groups (means are presented as combined from all groups). Splenocytes and MLN-derived lymphocytes from BBdr rats had a higher stimulation index to both ConA and PMA + ionomycin than did cells from BBdp rats (P<0·001, Fig. 2(a) and (b), respectively). For BBdp rats, diet did not significantly affect the mitogen response of splenocytes and MLN cells (Fig. 2). Splenocytes and MLN cells from BBdr rats fed FF had a significantly higher stimulation index (37 (SEM 6), n 13, and 337 (SEM 73), n 10, respectively) after incubation with PMA + ionomycin than did splenocytes (P=0·001) and MLN cells (P=0·038) from BBdr rats fed the control diet (19 (SEM 2), n 16, and 196 (SEM 28), n 8, respectively) (P=0·001; Fig. 2). Incorporation of [^{3}H]thymidine by cells from Peyer's patches was not measured.

Unstimulated splenocytes and MLN did not produce IL-2, IFN-γ or TNF-α. When stimulated with both ConA and PMA + ionomycin, splenocytes from BBdr rats produced significantly more IL-2 than splenocytes from BBdp rats (P<0·0001; Table 4). For BBdr animals, the concentrations of the other T_h1 (IFN-γ, TNF-α) cytokines in supernatants from both splenocytes and MLN cells did not differ between diets (Table 4). There were no significant differences between rat strains or between diet groups in the production of IFN-γ following PMA + ionomycin stimulation by splenocytes. MLN cells from BBdr rats produced significantly more ConA-stimulated IFN-γ than BBdp rats (P=0·004); however, TNF-α production by MLN cells did not differ by strain or diet (Table 4).

T_h2 cytokine concentrations in supernatants from both BBdr and BBdp stimulated splenocytes were significantly different between diet treatments. The production of IL-10 by unstimulated splenocytes (352 (SEM 24) pg/ml, n 58) did not differ among

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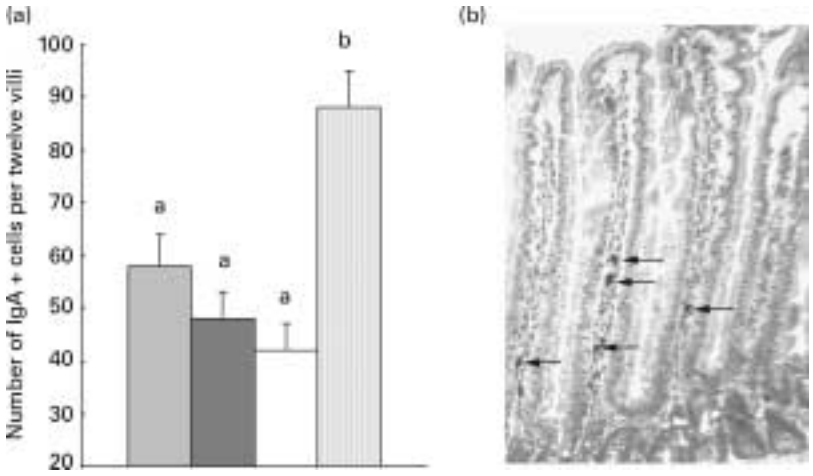

Fig. 1. The effect of diet (control v. fermentable fibre (FF)) and strain (diabetes-prone BioBreeding rat (BBdp) v. diabetes-resistant BioBreeding rat (BBdr)) on the number of IgA+ cells in the jejunum of BBdp and BBdr rats (▒, BBdr control; ■, BBdr FF; □, BBdp control; ▥, BBdp FF). (a), Values represent group means with their standard errors of the means depicted by vertical bars (n 12). [a,b] Mean values with unlike superscript letters were sigificantly different ($P < 0.05$). Positively stained cells were counted in four villi per section (three sections per sample) at four equally spaced predetermined positions in the cross-section. Data were analysed by a two-way ANOVA (diet × strain) and least square means where interactions between main effects occurred. (b), Representative immunostained jejunal section from a BBdp rat fed control diet (magnification 250 ×). ← indicates lamina propria cells that are positive fo IgA. For details of diets and procedures, see p. 646.

strain or diet groups. IL-4 was not detected in supernatants from unstimulated cell cultures. ConA-stimulated IL-10 production was significantly higher in FF-fed BBdp and BBdr rats ($P=0{\cdot}009$; Table 4) compared with those fed the control diet. Additionally, BBdr splenocytes produced significantly more IL-10 than BBdp splenocytes ($P=0{\cdot}03$; Table 4). Significant effects of strain and diet were observed with PMA + ionomycin-stimulated IL-4 production, with BBdr cells producing larger amounts of IL-4 than BBdp cells ($P=0{\cdot}02$; Table 4), and both strains produced significantly less IL-4 when fed the FF diet ($P=0{\cdot}02$; Table 4). Stimulation of immune cells from the spleen, MLN and Peyer's patches with ConA did not result in any significant differences in the production of the T_h3 cytokine, TGF-β (Table 4). TGF-β was not detected in supernatants from unstimulated cell cultures.

Discussion

In the present study, we studied the response of the immune system of BBdr and BBdp rats to a dietary component known to affect the intestinal milieu by altering immunological (Field et al. 1999b; Yamada et al. 2003), morphological (Andoh et al. 1999; Kleessen et al. 2003) and microbial (Kleessen et al. 2001; Buddington et al. 2002) parameters in the gut. Dietary FF is present in infant weaning foods, more notably wheat-based cereals, and is present in the weaning diets of rats. As the BBdr rats responded to the addition of FF in the diet in an expected manner, BBdp rats showed a failure to respond appropriately to what might be considered a 'normal' dietary challenge at weaning. In diabetes-resistant rats, feeding a FF compared to cellulose resulted in an increased small intestinal length, higher proliferative (stimulation) index from both splenocytes and MLN, and an increased proportion of CD8 + T-cells in the Peyer's patches. We did not observe an effect of diet on the number of IgA-bearing cells in the jejunum from BBdr rats. However, feeding FF to BBdp rats did not affect the same parameters. In fact, BBdp rats had both a higher proportion of B-cells in the Peyer's patches and a higher number of IgA + cells in the jejunum when fed a diet containing FF, a response not observed in BBdr rats.

Weaning is a crucial period, immunologically, for a young animal (Cummins et al. 1989, 1991; Thompson et al. 1996). The introduction of adult foods, beginning at weaning, exposes the intestinal immune cells to more foreign protein antigens compared to dam's milk (Menezes et al. 2003), and affects the bacterial flora in the gut (Edwards & Parrett, 2002), thereby challenging the relatively 'naïve' immune system of the suckling animal. These changes drive the development of the immune system (Ouwehand et al. 2002). Accordingly, intestinal and peripheral immune activation, such as an increase in soluble and intestinal IL-2 receptor expression, and increased intestinal inflammatory cytokine mRNA, have been reported in human subjects (Cummins et al. 1994), rodents (Thompson et al. 1996; Masjedi et al. 1999) and piglets (Pie et al. 2004), respectively, at the time of weaning.

Evidence of immune 'activation' was observed in BBdr rats fed FF after splenocytes and MLN cells were stimulated with PMA + ionomycin, but was not observed in BBdp rats. In BBdr rats, the mechanism by which feeding FF might cause increases in this response by lymphocytes to PMA + ionomycin is not known. PMA + ionomycin activate lymphocytes by bypassing membrane receptors. Differences observed between diet groups after PMA + ionomycin but not ConA stimulation (a mitogen that targets a lectin receptor on, or near, the T-cell receptor) suggests that the mechanism of increased [^{3}H]thymidine uptake by lymphocytes after FF feeding may be independent of the T-cell receptor. PMA + ionomycin-stimulated IL-2 production by BBdr splenocytes did not differ between diets, providing further support for a T-cell receptor-independent effect. The effect of FF on the response to PMA + ionomycin may be mediated via other lymphocyte subsets, as IL-2 is produced exclusively by T-cells (Waldmann, 1993).

In both the BBdr and BBdp rat strains, feeding the FF diet increased the mitogen-stimulated production of T_h2 cytokines in splenocytes compared with the control diet. Feeding FF to both BBdr and BBdp rats increased IL-10 production in response to ConA and decreased IL-4 production in response to PMA + ionomycin. The effect of feeding FF on the ability of cells from BBdr animals to respond to mitogens has not been well characterised. A study

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Table 3. The effects of diet (fermentable fibre (FF) or control) and strain (diabetes-resistant BioBreeding (BBdr) or diabetes-prone BioBreeding (BBdp)) of rats on phenotype distribution in the spleen, mesenteric lymph node (MLN) and Peyer's patches*

		BBdr						BBdp								
		Control			FF			Control			FF			Significance†		
Tissue	Phenotype	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Diet	Strain	Diet × strain
Splenocytes	Total T-cells (CD5 +)	29	2	16	31	3	13	22	5	7	23	5	9	NS	NS	NS
	Pan T-cells (W3/13 +)	30	3	11	32	3	13	19	3	7	19	2	10	NS	P=0·0002	NS
	CD5 + /W3/13 +	22	3	13	29	2	10	11	2	7	14	3	10	NS	P<0·0001	NS
	CD8 + (cytotoxic/suppressor) T-cells	14	1	15	16	2	15	9	1	11	12	1	9	NS	P=0·0191	NS
	CD4 + (helper) T-cells	22	1	14	21	2	15	11	2	11	13	1	11	NS	P<0·0001	NS
	Ig + B-cells	22	2	13	20	1	14	37	3	11	43	3	10	NS	P<0·0001	NS
	Monocytes/macrophages	8	1	13	7	1	14	13	2	11	12	1	9	NS	P=0·0002	NS
	Natural killer cells	9	1	14	7	1	14	8	2	7	13	2	9	NS	NS	NS
MLN cells	Total T-cells (CD5 +)	69	2	12	73	2	13	24	2	18	25	1	14	NS	P<0·0001	NS
	Pan T-cells (W3/13 +)	72	3	11	74	3	14	26	2	12	27	2	11	NS	P<0·0001	NS
	CD5 + /W3/13 +	66	1	11	62	3	15	20	1	12	20	1	14	NS	P<0·0001	NS
	CD8 + (cytotoxic/suppressor) T-cells	20	2	14	20	1	17	6	1	14	4	1	12	NS	P<0·0001	NS
	CD4 + (helper) T-cells	54	2	13	56	2	12	23	2	16	20	2	16	NS	P<0·0001	NS
	Ig + B-cells	15	1	12	15	1	15	41	3	16	45	3	16	NS	P<0·0001	NS
	Monocytes/macrophages	3	0·6	9	5	0·6	15	9	2	6	9	2	11	NS	P<0·0001	NS
Peyer's patch cells	Total T-cells (CD5 +)	22	2	7	24	1	12	10	1	9	9	1	11	NS	P=0·02	NS
	Pan T-cells (W3/13 +)	20	2	6	23	2	9	9	2	7	10	2	8	NS	P<0·0001	NS
	CD5 + /W3/13 +	16	2	7	18	1	10	5	0	7	4	0	8	NS	P<0·0001	NS
	CD8 + (cytotoxic/suppressor) T-cells	4[a]	1	10	10[b]	1	9	0[c]	0	9	1[c]	0	9	P=0·003	P<0·0001	P=0·011
	CD4 + (helper) T-cells	18	2	10	15	1	10	7	1	9	7	1	10	NS	P=0·04	NS
	Ig + B-cells	55[a]	2	9	54[a]	2	12	54[a]	2	9	63[b]	2	10	NS	NS	P=0·01

*Cells were obtained from BBdr or BBdp rats fed one of two similar diets differing only in the composition of fibre. Values were determined by immunofluorescence and are a proportion (%) of total gated cells.

†P-values represent significance of main effects diet and strain analysed by a two-way ANOVA. Where an interaction occurs between diet and strain (diet×strain) across a row, significant differences are identified by least square means and are denoted by different superscripts (P<0·05).

For details of diets and procedures, see p. 646.

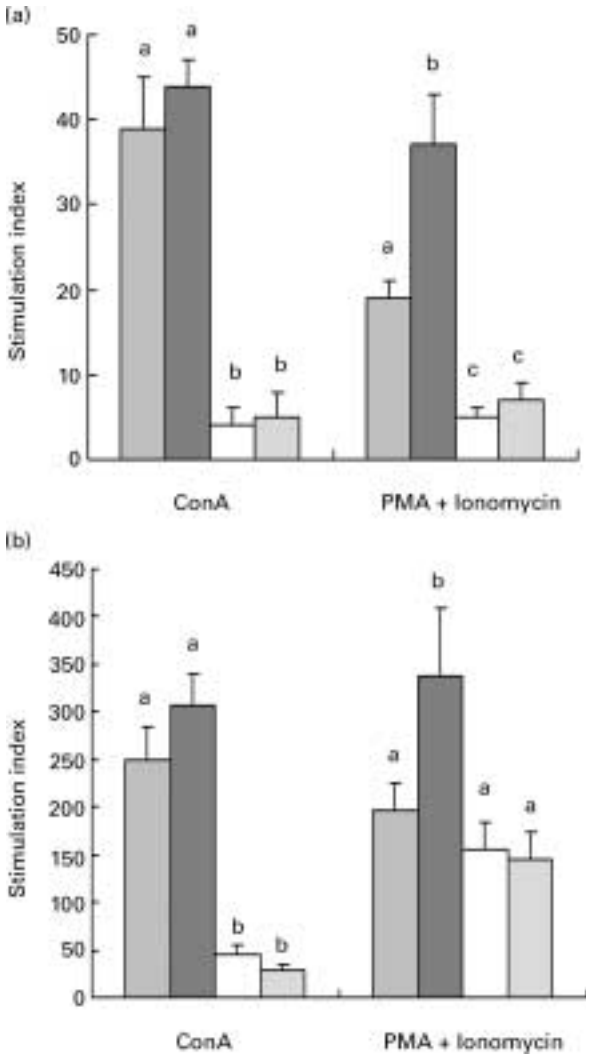

Fig. 2. The effect of diet (control v. fermentable fibre (FF)) and strain (diabetes-prone BioBreeding rat (BBdp) v. diabetes-resistant BioBreeding rat (BBdr)) on the uptake of [^{3}H]thymidine by spleen (a) and mesenteric lymph node (b) lymphocytes (▒, BBdr control; ■, BBdr FF; □, BBdp control; ▥, BBdp FF). Values represent group means with their standard errors of the means depicted by vertical bars. ^{a,b} Mean values within a mitogen with unlike superscript letters were significantly different ($P<0{\cdot}05$). Stimulation index was calculated by dividing the decays per min of stimulated cells by the decays per min of unstimulated cells. Data were analysed by a two-way ANOVA (diet × strain) and least square means where interactions between main effects occurred. ConA, concanavalin A; PMA, phorbol myristate acetate. For details of diets and procedures, see p. 646.

by (Roller et al. 2004) found that ConA-stimulated Peyer's patch cells from rats fed inulin/fructo-oligosaccharides produced more IL-10 than cellulose-fed rats, and another study where fructo-oligosaccharides were fed to mice revealed that IL-10 production by Peyer's patch CD4 + T-cells was increased (Hosono et al. 2003). Others have speculated that diets high in FF may have an anti-inflammatory effect (Rodriguez-Cabezas et al. 2003), perhaps through the production of SCFA by intestinal microflora (Andoh et al. 1999), and our findings support this. While feeding FF increased production of IL-10 after ConA stimulation in lymphocytes from BBdp rats, the amount of IL-10 produced was still significantly less than that observed from lymphocytes from BBdr rats.

Table 4. The effects of diet (fermentable fibre (FF) or control) and strain (diabetes-resistant BioBreeding (BBdr) or diabetes-prone BioBreeding (BBdp)) of rats on mitogen-stimulated cytokine production by splenocytes, mesenteric lymph node (MLN) cells and Peyer's patch cells*

			BBdr						BBdp						Significance†	
			Control (pg/ml)			FF (pg/ml)			Control (pg/ml)			FF (pg/ml)				
Tissue	Mitogen	Cytokine	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Diet	Strain
Splenocytes	ConA	IL-2	2420	324	8	2395	295	11	393	64	8	487	74	10	NS	P<0·0001
		IL-10	652	29	10	904	107	10	471	43	10	738	123	12	P=0·009	P=0·03
		TGF-β	128	20	12	147	32	11	161	23	10	128	14	13	NS	NS
	PMA + ionomycin	IL-2	4871	191	6	4029	342	6	1782	260	9	1628	275	10	NS	P<0·0001
		IFN-γ	591	95	8	520	187	8	890	192	8	545	101	12	NS	NS
		IL-10	690	207	9	798	99	13	476	51	9	419	51	11	NS	NS
		IL-4	35	6	10	21	4	7	21	6	10	10	2	9	P=0·02	P=0·02
MLN cells	ConA	IFN-γ	1782	242	10	1620	287	12	987	299	9	790	185	9	NS	P=0·004
		TNF-α	469	67	14	436	58	16	320	34	7	345	85	9	NS	NS
		TGF-β	408	113	8	629	115	6	810	168	8	836	38	9	NS	NS
Peyer's patch cells	ConA	TGF-β	147	28	13	162	10	8	226	46	7	151	44	10	NS	NS

ConA, concanavalin A; IFN-γ, interferon-γ; PMA, phorbol myristate acetate; TGF-β, transforming growth factor-β.
*Cells were obtained from BBdr or BBdp rats fed one of two similar diets differing only in the composition of fibre. All assays were performed in duplicate and only samples with a CV of less than 15 % between duplicates were included in the statistical analyses.
†P-values represent significance of main effects diet and strain analysed by a two-way ANOVA ($P<0{\cdot}05$ is considered significant). No diet × strain interactions occurred between groups.
For details of diets and procedures, see p. 646.

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Germ-free mice have reduced numbers of IgA-producing cells in the intestine compared to conventionally housed mice (Crabbe et al. 1968), suggesting that the number of IgA-bearing cells in the lamina propria is antigen-dependent. Thus, an increased number of IgA + cells in the lamina propria of BBdp rats after FF feeding may be indicative of an increased exposure to antigen by cells in the Peyer's patches and lamina propria. Inulin is a well-established prebiotic fibre, which has been shown to increase the amount of lactobacilli and bifidobacteria in the intestine (Roland et al. 1995; Buddington et al. 2002). The changes in bacterial colonisation of the small intestine, as well as the large intestine, after feeding a diet containing inulin could potentially have stimulated B-cell activation in the Peyer's patches and subsequent migration to the lamina propria. B-cells can be exposed to antigen and activated in the Peyer's patches before they migrate to the lamina propria to secrete polymeric IgA (Kagnoff, 1993).

Several possible mechanisms could contribute to an increased antigen exposure in the Peyer's patches of BBdp rats. For example, a higher intestinal permeability has been reported in BBdp compared with BBdr rats prior to the age of disease onset, independent of diet (Meddings et al. 1999). Furthermore, increased intestinal inflammation, as evidenced by mucosal myeloperoxidase activity, has been observed in BBdp rats before the age of disease onset possibly contributing to a greater intestinal permeability (Hardin et al. 2002). Feeding a diabetogenic diet to BBdp rats further increases myeloperoxidase activity in the small intestine (Courtois et al. 2004b), thereby providing a mechanism by which intestinal B-cells might be exposed to antigen. Interestingly, the proportion of Ox12 + cells (B-cells) was significantly higher in the Peyer's patches from BBdp rats fed the FF diet, consistent with the higher number of IgA + cells in the lamina propria.

The observation that feeding FF to BBdr rats did not affect the number of IgA+ cells in the lamina propria is somewhat unusual. Other investigators have reported that feeding FF such as gum arabic, glucomannin, celfur, lactulose or curdlan, increased the number of IgA-bearing cells in the caecum of non-diabetic animals (Kudoh et al. 1998, 1999). We measured IgA expression in the jejunum, where the concentration of bacterial antigens is known to be lower than in the caecum (Hao & Lee, 2004), and this may explain the lack of change with diet in the number of IgA-expressing cells in BBdr animals. It has been reported that feeding inulin/fructo-oligosaccharides increased the concentration of sIgA in the ileum and caecum in rats (Roller et al. 2004) and 24 h faecal samples from mice (Hosono et al. 2003). In addition, Peyer's patch cells from mice fed fructo-oligosaccharides stimulated with a mitogen from gram-positive bacteria produced more IgA than controls (Hosono et al. 2003). We did not measure the amount of IgA produced and although we did not see a change in the number of IgA-producing cells in the jejunum of control rats, this does not exclude the possibility that FF might affect the production of sIgA.

It has been demonstrated that feeding a diet containing FF can increase the proportion of CD8 + cells in the Peyer's patches (Field et al. 1999b), intra-epithelial lymphocytes in the small intestine (Field et al.1999b; Nagai et al. 2000; Ishizuka & Tanaka, 2002), in the caecal mucosa (Ishizuka & Tanaka, 2002) and in the colorectum (Nagai et al. 2000). In the present study, we confirmed that feeding a diet containing FF results in a higher proportion of CD8 + cells in small intestinal Peyer's patches of BBdr animals compared with feeding a diet containing only cellulose as the fibre source. It might be predicted, based on movement between tissues, that phenotype changes observed in the Peyer's patches would be reflected in some way in the MLN. This was not the case in the present study and likely due to the very small proportion of CD8 + cells in Peyer's patches ($<10\,\%$ of total cells) compared to the much larger population of CD8 + cells in the MLN of these rats that comprised cells also migrating from the peripheral blood and intraepithelial regions of the gut.

As inulin is an established prebiotic, and in vitro and in vivo studies using probiotics lactobacilli and bifidobacteria have been shown to have effects on immune function (reviewed by Schley & Field, 2002), it is very likely that the effects on immune parameters measured in this study are in part due to a change in the intestinal microflora. Mechanisms by which a change in the microflora might affect immune function in healthy animals is through contact with live bacteria or bacterial components with immune cells in the gut, through bacterial metabolic by-products such as SCFA, and an alteration in the production of mucins (reviewed by Schley & Field, 2002). The potential differences in microbial colonisation between strains were not addressed in this study. The consequences of such differences in colonisation remain to be elucidated.

The gastrointestinal tract has been implicated in the pathogenesis of diabetes in both human subjects and animal models (Vaarala, 2002). In the present study, we have shown that BBdp rats have an abnormal response to a normal dietary ingredient when introduced at weaning. BBdr rats show evidence of immune activation after being fed a diet containing FF, while BBdp rats show evidence of immune exposure without appropriate activation. It is possible that improper antigen handling and processing at a time when immune development is occurring may affect the later outcome of diabetes in these diabetes-prone rats if the appropriate environmental trigger is present.

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