The oral immunogenicity of BioProtein, a bacterial single-cell protein, is affected by its particulate nature

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The bacterial single-cell protein BioProtein (BP; Norferm Danmark, Odense, Denmark), produced by fermentation of natural gas with methanotrophic bacteria, is a potential protein source for man and animals. For human consumption, removal of the nucleic acid is necessary. Preliminary studies have shown that ingested BP induces a specific immune response. The objective of the present study was to characterize the type of response, its development over time and product-related causative factors. Mice were fed with diets containing 60 g nucleic acid-reduced BP/kg, 240 g nucleic acid-reduced BP/kg, 240 g untreated BP (basic BP)/kg or 240 g casein/kg (control). In another study, mice were fed 240 g basic BP/kg, whole cell-free BP-culture homogenate or control diet. The immune response was monitored using an ELISA for BP-specific immunoglobulin in blood and BP-specific immunoglobulin A in blood and saliva. Ingested BP induced a steady specific mucosal and systemic immune response, characterized by a dose-dependent production of immunoglobulin and immunoglobulin A in blood and immunoglobulin A in saliva. Basic BP and nucleic acid-reduced BP induced identical responses. However, feeding mice BP-culture homogenate induced immunoglobulin A in saliva but there was no systemic response. The antibodies from BP-fed mice cross-reacted with BP-culture homogenate revealing the presence of the same antigenic components in the two products despite the different oral immunogenicity. Thus, ingestion of BP induces a persistent mucosal and systemic immune response of which the systemic response can be avoided by ingesting a BP preparation free of whole cells. This indicates the importance of the non-particulate constitution of single-cell protein products intended for human or animal consumption.

Single-cell protein: BioProtein: Oral immunogenicity: Particulate food antigen

Single-cell protein (SCP) is considered as a potential non-conventional protein source for human food and animal feed to help food-supply problems worldwide in an inexpensive manner. SCP is protein extracted from microbial biomass derived from fermentation of algae, fungi or bacteria, typically by using waste materials as substrate. BioProtein (BP) is a SCP developed by the company Norferm Danmark (Odense, Denmark) and was approved by European Union for use in animal and fish feed in 1995. BP is spray-dried biomass of a mixed methanotrophic bacterial culture grown on natural gas.

Compared with algae and fungi, bacteria have the advantages of rapid growth rate and high protein content of good quality (Kuhad *et al.* 1997; Anupama & Ravindra, 2000), but disadvantage of a high content of nucleic acids, up to 160 g/kg dry weight. Due to the health complications caused by accumulation of uric acid in the body, this has hampered the use of bacteria as a source of SCP on a commercial basis for human consumption (Anupama & Ravindra, 2000). Consequently, for the purpose of investigating the potential of BP for human consumption, Norferm Danmark has, in addition to the basic BP product, developed a nucleic acid-reduced BP product using a heat-shock method (Larsen & Joergensen, 1996).

Even though micro-organisms have played a role in the human diet since ancient times, their utilization has been restricted to micro-organisms naturally occurring in the environment used as fermenting agents, and thus only forming a minor part of the final product. In the case of SCP such as BP, the product consists solely of a microbial material that, although never reported to be pathogenic (Whittenbury & Krieg, 1984), is of an origin with no history of dietary use. Therefore, besides proper evaluation of nutritive value, there is a requirement to evaluate the safety aspects of SCP products thoroughly. Accordingly, a sub-chronic toxicity study of rats was recently completed by Mølck *et al.* (2002) to address safety aspects of nucleic acid-reduced BP: the study showed that feeding nucleic acid-reduced BP to rats for 13 weeks activated the immune system, seen as changes in cells from the gut-associated immune system and induction of a systemic antibody response towards the nucleic acid-reduced BP.

The context in which a specific antigen is exposed to the gut mucosa has been shown to have a profound influence

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on the immune response generated towards the antigen, and may in some instances endow the antigen with signals that stimulate a productive response. Unlike a pure soluble antigen, a replicating or particulate antigen, as well as the presence of immune stimulating adjuvant components, are factors of great importance in rendering an otherwise tolerogenic antigen immunogenic (O'Hagan *et al.* 1989; Strobel & Mowat 1998). SCP such BP differs from common food protein sources of animal or vegetable origin in some important aspects in relation to the factors mentioned earlier. Consisting solely of microbial material, BP has indeed a particulate constitution and contains microbial adjuvant components such as endotoxins that are potentially capable of stimulating the immune response.

In view of the critical importance of the nature of a food protein for the resulting immune response upon ingestion, the objective of the present study was to characterize the immune response known to develop toward ingested nucleic acid-reduced BP and to identify product-related causative factors. For this purpose, mice-feeding studies using various doses, feeding times and different preparations of BP including nucleic acid-reduced BP, basic BP and a whole-cell-free preparation, were carried out. The results showed that ingestion of BP as a whole-cell-free preparation avoided the persistent systemic antibody response otherwise generated towards basic BP, indicating that the physical preparation of SCP products, such as BP intended for human or animal consumption, is indeed an important factor beyond the components *per se* for the immune response induced upon ingestion.

Materials and methods

Test materials

The BP products were provided by Norferm Danmark. BP is a mixed culture product consisting of approximately 90 % *Methylococcus capsulatus* (Bath), 6–8 % *Alcaligenes acidovorans*, 1 % *Bacillus brevis* DB4 and 1 % *Bacillus firmus* DB5. Basic BP is a spray-dried granulated culture material containing approximately (g/kg): protein (Kjeldahl analysis) 660, fat 90, ash 70, crude fibre 10, N-free extract (mainly carbohydrate) 110, water 60. Nucleic acid-reduced BP is BP culture material further processed to reduce nucleic acids by a heat-shock method that activates endogenous nucleases. For optimal stimulation of the nucleases, Fe is added in this step. Basic BP contains 100–110 g RNA–DNA/kg whereas nucleic acid-reduced BP contains only 20–30 g/kg.

A BP-culture homogenate free of whole cells was prepared especially for experimental use by homogenizing the bacterial culture suspension (Rannie homogenizer, 100 MPa; APV, Albertslund, Denmark) and subsequently ultracentrifuging at 14 000 g for 20 min at 4°C (Varifuge 20RS with a HCT15·1500 continuous flow rotor, 1·5 l; Heraeus Instr., Hanan, Germany) to remove remaining whole cells. The resultant supernatant fraction was stored at −20°C until use. The protein content in the BP-culture homogenate was determined to be 350 mg/ml by amino acid analysis based on ion-exchange chromatography of a HCl hydrolysate

using *o*-phthalaldehyde post-column derivatization for detection, as described by Barkholt & Jensen (1989).

For immunization and use in assays for BP-specific antibody and cell reactions, an extract of BP was prepared by shaking 500 mg finely ground BP with 25 ml carbonate buffer (0·05 M, pH 9·6) for 30 min, centrifuging for 10 min at 3000 g and collecting the supernatant fraction. The pellet was then resuspended in another 25 ml carbonate buffer and incubated overnight at 4°C while stirring. After another centrifugation, the resultant supernatant fraction was pooled with the supernatant fraction obtained from the first extraction step. The extract was divided into portions to avoid freeze–thaw cycles and stored at −20°C until use. The protein content in the final BP extract was determined by amino acid analysis as described earlier.

Animals and housing

Female BALB/c A mice, aged 8–10 weeks, were purchased from M&B (Ry, Denmark) and acclimatized for 1 week before use in experiments. Mice were held four to six per cage at 20–22°C with a 12 h light–dark cycle in a negative-pressure ventilated area. They were housed in protected environment cages (Techniplast, Buguggiate, Italy) with filter covers that create a barrier at cage level. Water and food were provided *ad libitum*. Weight changes were recorded weekly. All animal studies were approved by the Danish Animal Experiments Inspectorate and the rules for care and use of animals formulated in the *Council of Europe Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* were followed.

Experimental design of feeding studies

In a long-term feeding study, mice were randomly allocated into five groups each with five mice. For 8 weeks, four of the groups were fed well-defined diets containing either 0 (control), 60 or 240 g nucleic acid-reduced BP/kg or 240 g basic BP/kg (Table 1). The fifth group were fed 240 g nucleic acid-reduced BP/kg for the first 2 weeks only and then fed the control diet for the remaining 6 weeks. The concentrations of macronutrients, vitamins and minerals were adjusted according to the amount of added BP.

In another study, mice were randomly allocated into three groups each with six mice. Two of these groups were fed the control diet and 240 g basic BP/kg respectively for 4 weeks. The third group was fed for 4 weeks with the control diet but with the addition of BP-culture homogenate to the drinking water (175 mg protein/ml); this corresponded to a BP-derived protein intake equal to that of the group fed 240 g basic BP/kg (approximately 0·8 g protein/mouse per d), based on a daily feed and water consumption of 4–5 g and 4–5 ml respectively. The amount of water consumed was recorded.

From all mice blood was collected weekly from the retro-orbital plexus and diluted in PBS–Triton X-100 (0·01 M-PBS with 1·0 ml Triton X-100/litre, pH 7·4) and stored at −20°C until analysis. Saliva samples were obtained from all groups with the exception of the group

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

Component	Feed		
	0 g/kg	60 g/kg	240 g/kg
BioProtein (approximately 750 protein/kg)*	0	60	240
Caseinate (approximately 890 protein/kg)	180	130	0
Sucrose	34	34	32
Yellow dextrin	34	34	32
Maize starch	306	304	288
Potato starch	306	304	288
Mineral mixture	28	28	28
Vitamin B mixture	12	12	12
Soyabean oil with fat-soluble vitamins	50	45	30
Cellulose	50	50	50

* Norferm Danmark, Odense, Denmark.

fed only 60 g basic BP/kg by holding a pre-weighed wad of cotton in the mouth of the mouse for 2 min and then reweighing the cotton wad to calculate the amount obtained. The net weight of captured saliva was approximately 15 mg. The saliva was diluted 32-fold by adding PBS–Triton X-100 to the tube containing the cotton wad. After gentle shaking for 5 min, the samples were stored at $-20°C$ until analysis.

Antibody titre determination by ELISA

BP-specific antibodies in blood and saliva were determined by ELISA. Microtitre plate wells (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl BP extract prepared as described earlier at a concentration of 35 µg protein/ml carbonate buffer. After washing four times in wash buffer (PBS–Triton X-100 diluted 1:10), 100 µl serially diluted sample was added to each well and the plate was incubated for 1 h at 37°C. After washing again, the plate was incubated for 1 h further with 100 µl secondary antibody per well. For total immunoglobulin (Ig) determination, a horseradish (*Amoracia rusticana*) peroxidase-conjugated rabbit anti-mouse Ig antibody (Dako, Glostrup, Denmark) was used. For determination of BP-specific IgM, IgG_1, IgG_{2a} and IgA, rabbit anti-mouse antibody specific to the respective antibody isotypes (Zymed, San Francisco, CA, USA) were used and an additional step, involving 1 h incubation with horseradish peroxidase-conjugated swine anti-rabbit Ig antibody (Dako) was carried out. The plate was developed by adding 100 µl substrate solution containing 3,3',5,5'-tetramethylbenzidin per well and incubated for 10 min. The reaction was stopped by adding 100 µl 2 M-phosphoric acid per well and the absorbance at 450 nm with 630 nm as reference was measured. A positive control consisting of pooled blood collected at day 21 from mice immunized on days 0 and 14 with BP extract in Freund's incomplete adjuvant was included in all ELISA runs. All samples were run in duplicate. Results were determined as titres, defined as the sample dilution giving an absorbance of 0.2.

In vitro *spleen cell proliferation assay*

Single cell suspensions were prepared aseptically from each mouse spleen by mechanical means and centrifuged for 10 min at 300 g. For removal of erythrocytes, the cells were treated with NH_4Cl (8·3 g/l, 5 min on ice) and then washed twice in Dulbecco's modified Eagle's medium (BioWhittaker Europe, Verviers, Belgium) supplemented with 100 µg penicillin and 100 IU streptomycin/ml. Finally, cells were resuspended in serum-free medium (X-vivo 10; BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM-L-glutamine, 100 µg penicillin/ml and 100 IU streptomycin/ml and cultured as 3×10^5 cells/225 µl per well in quadruplicate in a ninety-six-well flat-bottomed plate (Nunc) with 50 µg BP extract/ml prepared as described earlier or PBS as control. After incubation for 3 d at 37°C in 5 % CO_2, cells were pulsed for another 20 h with [^{3}H]thymidine (37·00 kBq/ml; Amersham International, Amersham, Bucks., UK). After harvesting onto glass-fibre filter-mats using a twelve-well cell harvester (Automash 2000; Dynex, Denkendorf, Germany), the cell proliferation was determined by measuring [^{3}H]thymidine incorporation by liquid scintillation counting (Tri-Carp; Packard, Meriden, NJ, USA).

SDS–PAGE and immunoblotting

BP-culture homogenate (4 mg protein/ml) dissolved 1:2 and boiled for 5 min in Tris–SDS sample buffer (0·1 M-Tris-base, SDS (80 g/l), glycerol (240 ml/l), Coomassie (0·25 g/l), pH 6·8) containing 40 mM-dithiothreitol was subjected to SDS–PAGE (125 V for 1 h) using a 10–20 % gradient tricine gel (Novex; Invitrogen, Groningen, The Netherlands) (Schagger & vonJagow 1987). SeeBlue (Invitrogen) was used as standard marker.

The SDS–PAGE gel was blotted onto a nitrocellulose membrane (0·45 µm, 100 × 80 mm) for 100 min at 64 mA using 3-(cyclohexylamino)propanesulfonic acid buffer (0·1 mol/l methanol (100 ml/l), pH 11·0). The membrane was blocked for unspecific binding by incubating for 15 min in TBS–T buffer (50 mM-Tris-base, 0·3 M-NaCl, Tween 20 (10 g/l), pH 10·3). Then the membrane was cut appropriately into strips, which were incubated overnight at room temperature with blood (diluted 1:160 in TBS–T buffer) from mice at day 0 (control for background response) and day 28 of feeding with 240 g BP/kg. The strips were washed three times for 10 min with TBS–T buffer and then incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-mouse Ig (Dako). The strips were washed

and developed in the dark with Nitroblue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as colour substrate. The strip with the standard marker was blocked for 15 min with blocking buffer (Dako), rinsed for 5 min in incubation buffer (Dako) and then incubated overnight at room temperature with citrate buffer (50 mM, pH 3·0). Then the strip was stained for approximately 30 min with freshly prepared colloidal Au sols solution (Moeremans *et al.* 1985).

Statistic analysis

Data was analysed using Statistical Analysis Systems statistical software package (version 6.12; SAS Institute, Cary, NC, USA). For the feeding studies involving data collection over time from the same animals, two-way ANOVA with repeated measures on one factor was performed using the 'proc mixed' procedure to determine the effect of group, day and the interaction between group and day. If significant, the analyses were followed by a difference of least squares means test to determine effects of groups at individual times. For analysis of cell proliferation data, a one-tailed unpaired t test with Welch's correction was used, as equal variance could not be assumed in the control and the dosed group. $P \leq 0.05$ was considered significant. To improve visualization and simplicity of graphs, results are presented as mean values subtracted from the mean of the control to correct for background variations, with the standard errors of the difference set as error bars.

Results

8-Week feeding study

In order to examine in more detail the immune response found in previous studies (Mølck *et al.* 2002) to be induced towards ingested BP, an 8-week BP-feeding study in mice was undertaken. Blood and saliva samples (collected weekly) were subjected to ELISA analysis to monitor the course of the systemic and mucosal humoral immune response respectively. No significant difference in weight gain was observed between the control and the BP-fed groups.

Feeding mice a diet containing 240 g nucleic acid-reduced BP/kg induced a statistically significant specific systemic antibody response within 1 week of feeding, measured as total BP-specific Ig in blood (Fig. 1(a)). The response increased rapidly during the first 2 weeks and then after an intermediate reduction, reached a steady-state level at about week 5, lasting the remainder of the experimental period. Further analysis of blood samples revealed a modest but significant peak level of BP-specific IgM (titre 1·2 (SE 0·2) greater than control value, $P \leq 0.023$) after 1–2 weeks that afterwards rapidly declined (results not shown), while the BP-specific IgG (measured as IgG$_1$ and IgG$_{2a}$) abruptly increased in this period and continued increasing until reaching a steady state level (Fig. 3).

Feeding mice only 60 g nucleic acid-reduced BP/kg also induced a significant but much less profound BP-specific Ig response in blood; this did not reach a statistically

significant level until day 14, as compared with day 7 for the group fed 240 g nucleic acid-reduced BP/kg (Fig. 1(a)). Likewise, the response remained significantly below the level of the group fed 240 g nucleic acid-reduced BP/kg ($P \leq 0.0001$) from day 7 onwards.

A significant response of BP-specific IgA was found in both blood and saliva for nucleic acid-reduced BP-fed mice (Fig. 1(b and c)). Looking at the mice fed 240 g
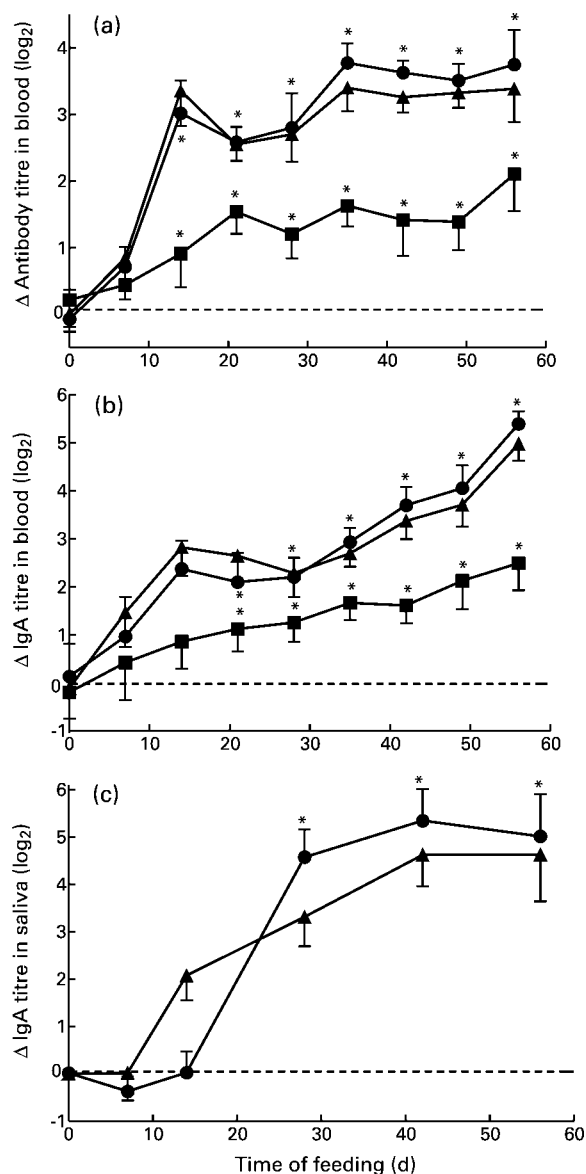
Fig. 1. BioProtein (BP; Norferm Danmark, Odense, Denmark)-specific immunoglobulin (Ig) in blood (a) IgA in blood (b) and IgA in saliva (c) monitored by ELISA for mice fed for 56 d with 60 g nucleic acid-reduced BP/kg (■), 240 g nucleic acid-reduced BP (●) or 240 g basic BP/kg (▲) (n 5 per group). For details of diets and procedures, see Table 1 and pp. 170–171. Results are presented as mean values with the mean value of a control group subtracted. For all measurements of the control group, mean values were 10·5 (SEM 0·2) for Ig in blood, 6·2 (SEM 0·2) for IgA in blood and 6·1 (SEM 0·3) for IgA in saliva, representing the average unspecific background. Standard errors are shown by vertical bars. Mean values were significantly different from those of the control group (two-way ANOVA with repeated measures on one factor): *$P \leq 0.05$.

nucleic acid-reduced BP/kg, the IgA response in blood increased rapidly until day 14 where it stabilized until day 28 and then further increased during the remaining time of assessment. The BP-specific IgA in saliva, however, emerged not until some time after day 14, when it increased swiftly and then stabilized at about day 28. Thus, it appears that the increase in anti-BP IgA response in saliva coincided with the transient stabilization of the blood anti-BP IgA and furthermore, that at the time of saliva anti-BP response stabilization, the anti-BP IgA in blood started to increase again.

To address whether the nucleic acid reduction processing of BP modifies the effect of BP on the immune system, a comparison of the immune response of mice fed nucleic acid-reduced BP v. basic BP was performed. No statistically significant difference appeared between the two BP products for either of the tested antibodies including BP-specific total immunoglobulin and IgA (Fig. 1) and IgG_1 and IgG_{2a} tested in blood only at day 0 and 56 (results not shown).

To assess the immune response towards BP after ingestion of BP has ceased, a group of mice was fed 240 g nucleic acid-reduced BP/kg for 2 weeks and then fed the control diet for the remaining 6 weeks of the experimental period. For the first 4 weeks, the total BP-specific serum antibody response did not differ statistically from that of the group fed 240 g nucleic acid-reduced BP/kg continuously (Fig. 2(a)). However, beginning at day 28, i.e. 1 week after feeding nucleic acid-reduced BP was discontinued, the serum antibody response declined steadily as opposed to the group continuously fed nucleic acid-reduced BP, showing that the continued exposure sustained the antibody production. Interestingly, when examining the IgG subclass antibodies IgG_1 and IgG_{2a} (Fig. 3), the IgG_1 response declined considerably subsequent to discontinuing nucleic acid-reduced BP feeding, whereas the IgG_{2a} was completely unaffected and did not differ statistically at any time from the response of the group fed nucleic acid-reduced BP continuously. After ceasing nucleic acid-reduced BP feeding, the BP-specific IgA response in blood declined to an insignificant level by day 28, while the BP-specific IgA in saliva remained at a substantial level (Fig. 2(b)) showing that the BP-specific IgA production is induced solely at mucosal sites with no manifest systemic IgA response.

BioProtein-specific cellular response

BP-specific cell activity was measured after feeding BP as the spleen cell proliferative response towards in vitro BP extract stimulation for mice fed 240 g nucleic acid-reduced BP/kg for 14 d compared with the control group (Fig. 4). The result showed a statistically significant response (P=0·0012), indicating that T cells have been activated.

Feeding study with BioProtein-culture homogenate

For the purpose of investigating whether the observed immunogenic potency of BP is influenced by the particulate nature of the product, a study was completed based on feeding mice for 4 weeks with an amount of whole

cell-free homogenate of BP culture, equal, regarding protein intake, to a group of mice fed 240 g basic BP/kg. A non-BP-fed control group was also included. The intake of BP-derived protein either from BP-culture
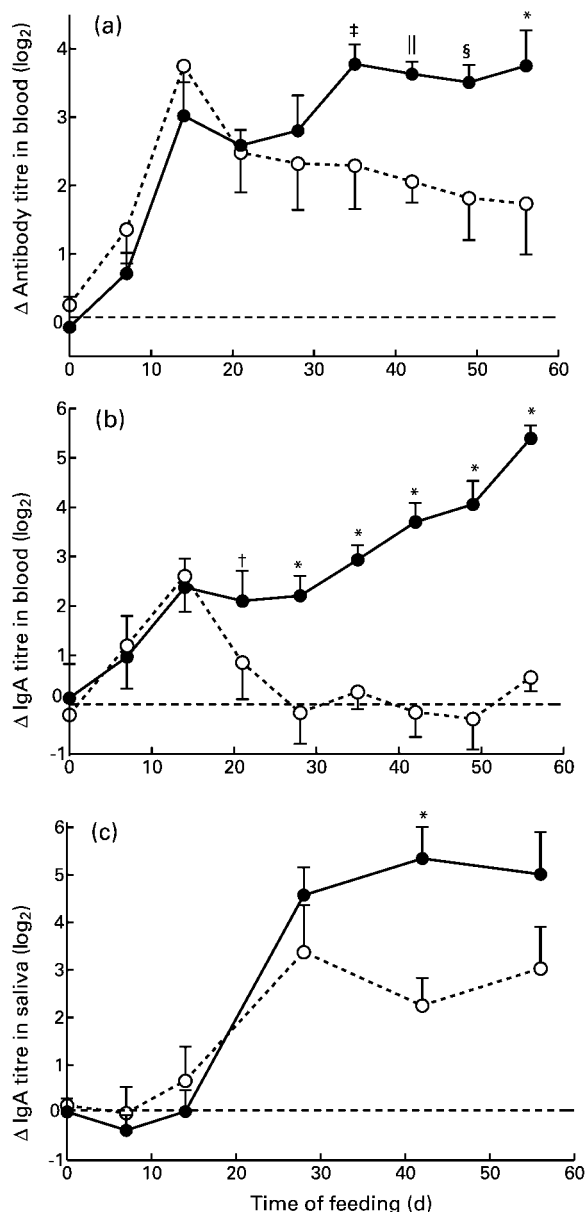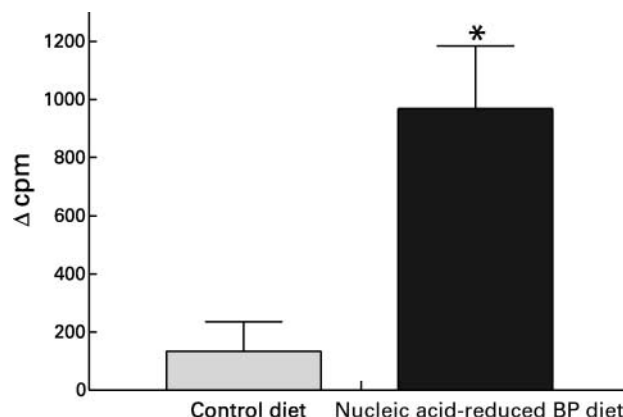

Fig. 2. BioProtein (BP; Norferm Danmark, Odense, Denmark)-specific immunoglobulin (Ig) in blood (a) IgA in blood (b) and IgA in saliva (c) monitored by ELISA for mice fed 240 g nucleic acid-reduced BP/kg on days 1–14 and then control diet on days 15–56 (O) or 240 g nucleic acid-reduced BP/kg on days 1–56 (●) (n 5 per group). For details of diets and procedures, see Table 1 and pp. 170–171. Results are presented as mean values with the mean value of a control group subtracted. For all measurements of the control group, mean values were 10·5 (SEM 0·2) for Ig in blood, 6·2 (SEM 0·2) for IgA in blood and 6·1 (SEM 0·3) for IgA in saliva, representing the average unspecific background. Standard errors are shown by vertical bars. Mean values were significantly different between the groups shown (two-way ANOVA with repeated measures on one factor): *P≤0·0001, †P=0·022, ‡P=0·0029, §P=0·0007, ‖P=0·0004. Other differences were not statistically significant (P≥0·05).
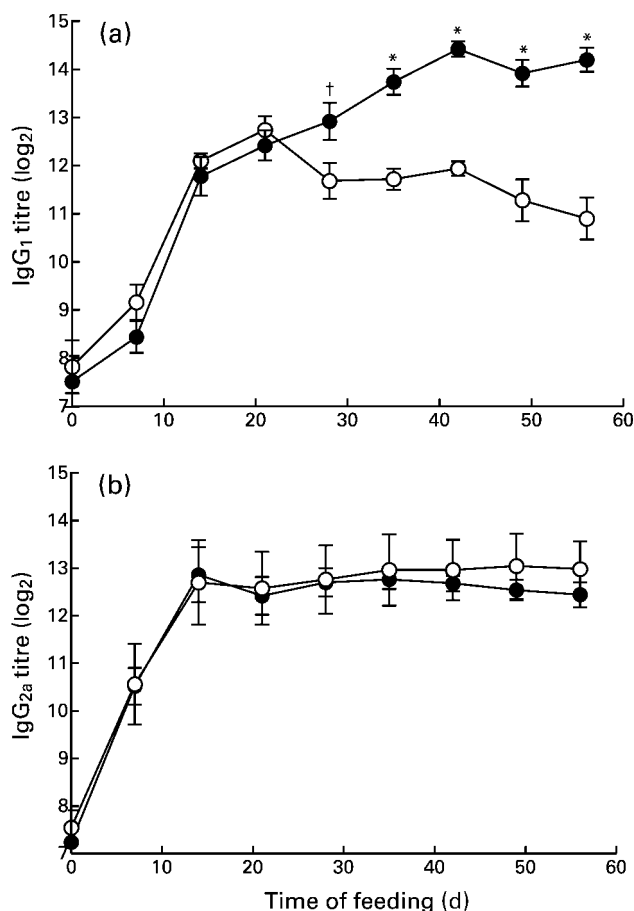
Fig. 3. BioProtein (BP; Norferm Danmark, Odense, Denmark)-specific immunoglobulin (Ig) G_1 (a) and IgG_{2a} (b) monitored by ELISA in blood from mice fed 240 g nucleic acid-reduced BP/kg on days 1–14 and then control diet on days 15–56 (○) or 240 g nucleic acid-reduced BP on days 1–56 (●) (*n* 5 per group). For details of diets and procedures, see Table 1 and pp. 170–171 Results are presented as mean values with their standard errors shown by vertical bars. Background values of the control-fed animals were not measured. Mean values were significantly different between the groups shown (two-way ANOVA with repeated measures on one factor): *$P \leq 0.0001$, †$P = 0.0138$. Other differences were not statistically significant ($P \geq 0.05$).


Fig. 4. Proliferation of spleen cells from mice fed 240 g nucleic acid-reduced BioProtein (BP; Norferm Danmark, Odense, Denmark)/kg for 14 d. Spleen cells were stimulated *in vitro* with either nothing (background) or BP extract (50 μg protein/ml) for 3 d before pulsing for 20 h with [^{3}H]thymidine. For details of diets and procedures, see Table 1 and pp. 170–171. Results representing delta counts per min (Δcpm) are shown as mean values with their standard errors shown by vertical bars (*n* 4 per group). Mean values were significantly different from those of the control group (one-tailed unpaired *t* test with Welch's correction, as equal variance could not be assumed in the control and the test group): *$P = 0.0012$.

homogenate supplied through the drinking water or via the diet did not vary significantly between the two BP-fed groups at any time during the feeding period.

Interestingly, the BP-culture homogenate-fed mice did not develop a significant BP-specific systemic serum antibody response, as did the mice fed basic BP (Fig. 5(a)). However, though not statistically significant ($P = 0.21$), a modest serum BP-specific IgA response seemed to appear at day 28 (Fig. 5(b)). Surprisingly, the mice fed the BP-culture homogenate developed a BP-specific IgA response in saliva of a magnitude not statistically different from that induced in mice fed basic BP (Fig. 5(c)), clearly showing that the mucosal immunogenicity of BP-culture homogenate is retained in the absence of systemic immunogenicity. In examining whether the immunogenic compounds were removed during the processing of BP-culture homogenate explaining the absence of systemic immunogenicity of BP-culture homogenate, the blood

samples were further analysed for BP-culture homogenate-specific antibodies with ELISA. The mice fed BP-culture homogenate still failed to have specific antibodies whereas the BP-fed mice showed a significant response (Fig. 6(a)), demonstrating that the very same antigens are present in BP as in BP-culture homogenate. This was further substantiated by the appearance of bands in immunoblots of BP-culture homogenate and serum from mice fed basic BP (Fig. 6(b)).

Discussion

The gut-associated lymphoid tissue is capable of discriminating between harmless antigens coming from the diet or resident microflora and antigens derived from pathogens or toxic agents. The outcome of exposure of foreign antigens to mucosal tissue ranges from induction of non-responsiveness (tolerance), induction of systemic priming to induction of a local mucosal IgA response (Strobel & Mowat, 1998). Although the regulating mechanisms are far from clear, it is believed that tolerance is the default response to pure soluble antigens, whereas productive immunity is induced only when the antigen is endowed with particular immune activating signals (Strober & Coffman, 1997). Under healthy conditions, however, human subjects (Husby *et al.* 1985; Barnes *et al.* 1988; Ahmed *et al.* 1997; Rumbo *et al.* 1998), as well as rodents (Coombs *et al.* 1983; Wold *et al.* 1989; Kim *et al.* 1993), may develop a weak systemic and mucosal antibody response to long-term dietary antigen exposure. Such antibody responses vary a great deal among individuals and typically occur only transiently in young individuals and tend to decline with age, probably due to development of tolerance (Husby *et al.* 1985; Dannaeus 1993; Ahmed *et al.* 1997).

The present study showed that mice fed for 8 weeks with 240 g nucleic acid-reduced BP/kg consistently
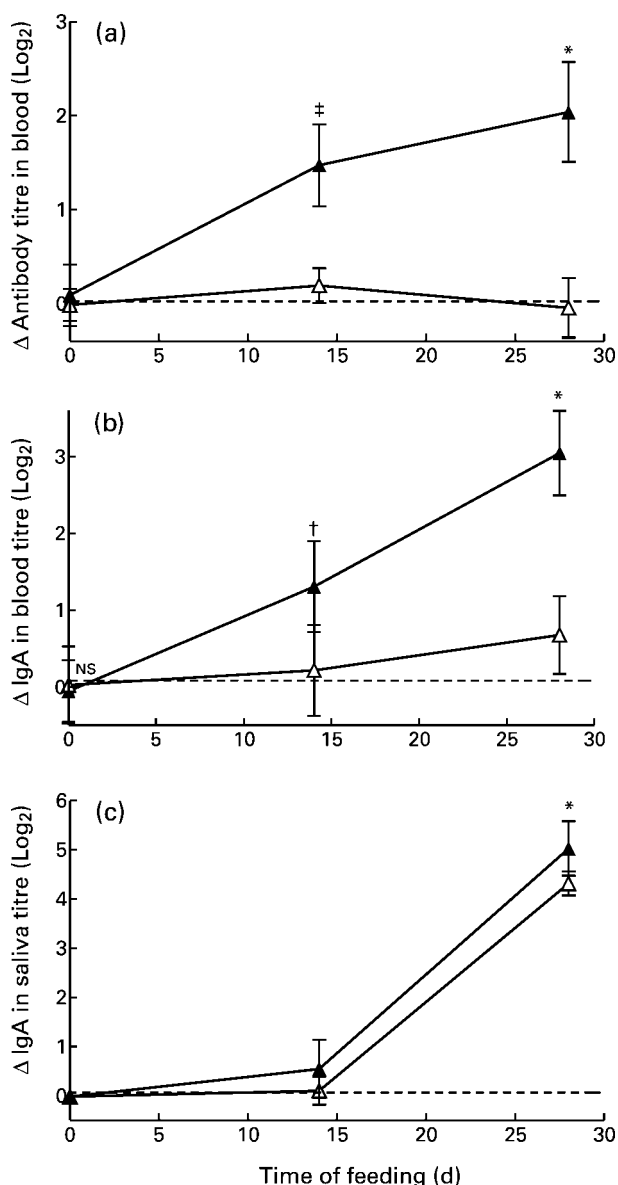
Fig. 5. BioProtein (BP; Norferm Danmark, Odense, Denmark)-specific immunoglobulin (Ig) in blood (a) IgA in blood (b) and IgA in saliva (c) monitored by ELISA for mice fed for 28 d with 240 basic BP/kg (▲) or BP-culture homogenate (△) with equal BP-derived protein intake (n 5 per group). For details of diets and procedures, see Table 1 and pp. 170–171. Results are presented as mean values with the mean value of a control-fed group subtracted. Standard errors are shown by vertical bars. For all measurements of the control group, mean values were 9·2 (SEM 0·3) for Ig in blood, 5·8 (SEM 0·4) for IgA in blood and 5·1 (SEM 0·1) for IgA in saliva, representing the average unspecific background. Mean values were significantly different from those of the control group (two-way ANOVA with repeated measures on one factor): *$P \leq 0.0001$, †$P = 0.029$, ‡$P = 0.0004$. Other differences were not statistically significant ($P \geq 0.05$), including between BP-fed groups in (c).

developed a clear and persistent BP-specific immune response characterized by both a systemic (Ig in blood) and a mucosal (IgA in saliva) response (Fig. 1). The immune response was moreover found to be T-cell dependent, as specific splenocyte proliferation took place upon *in vitro* stimulation with BP. Quantitative assessment of the

response magnitude could not be made on the basis of the present study due to the relative form of the results obtained by ELISA. However, the response patterns were very clear and remarkably consistent among the mice, with no animals not responding to ingested BP, indicating an enhanced oral immunogenicity of BP compared with ordinary food antigens. The course of the systemic antibody response resembled that of a typical antibody response generated against immunogens administered parenterally, featuring an initial rapid IgM production succeeded by a much stronger IgG response (Goding, 1996). The serum IgA level decreased readily while saliva IgA persisted after discontinuing BP feeding, which indicates that the IgA response detected in serum originated solely from mucosally triggered B cells occurring transiently in blood, since it is known that IgA^+ B cells triggered in the gut mucosa recirculate through lymph and blood before homing to mucosal effector sites (Macpherson *et al.* 2001). This could also explain the temporary discontinuance of the increase in serum IgA at the time where the saliva IgA response started to develop (about day 14) as IgA^+ B cells may begin homing to mucosal sites by this time.

The processing of SCP to reduce the nucleic acid content is one factor conceivably influencing the constitution of SCP: it has in fact been suspected to be involved in increased oral immunogenicity of SCP products, as clinical tolerance trials in human subjects have indicated a higher potential of the nucleic acid-reduced SCP products to induce allergic reactions than the unaltered products (Scrimshaw & Murray, 1995). In the present study, mice ingesting 240 g basic BP/kg developed a specific immune response not statistically significantly different at any time from that developed against nucleic acid-reduced BP, suggesting that the nucleic acid-reduction processing of BP had no effect on its oral immunogenicity. Potential difference in the capacities of basic BP and nucleic acid-reduced BP to induce BP-specific IgE was not specifically addressed in the present study. However, when considering the production of the IgG subclasses IgG_1 and IgG_{2a} (measured on day 56, results not shown), no statistical difference occurred between basic BP and nucleic acid-reduced BP. IgG_1 and IgE production is supported by T-helper (Th) 2 cells functioning in humoral immunity against exogenous antigens including allergy, whereas IgG_{2a} production is supported by Th1 functioning in cell-mediated immunity (Fujihashi *et al.* 1997). Thus, eliciting identical IgG_1 and IgG_{2a} responses suggests that no difference exists in the oral immunogenicity of nucleic acid-reduced BP *v.* basic BP with respect to the type of response (Th1 and 2) evoked by the two products.

Prolonged exposure of BP to the gut-associated lymphoid tissue owing to heavy accumulation in macrophages in the mesenteric lymph nodes, as observed in the study by Mølck *et al.* (2002), may represent another factor potentially involved in the strong antibody response induced towards ingested BP. Moreover, prolonged exposure may differentially affect the response pattern favouring a Th1 cell response, as the IgG_{2a} response is selectively sustained in contrast to the abating IgG_1 response subsequent to termination of BP feeding. The half-life of circulating IgG_1
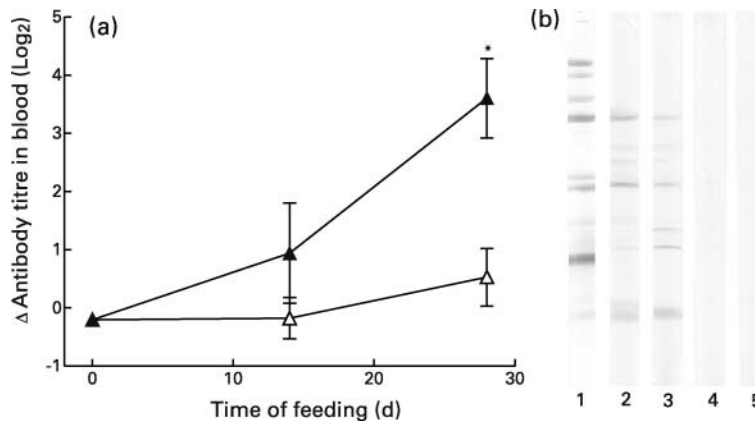
Fig. 6. (a) BioProtein (BP; Norferm Danmark, Odense, Denmark)-culture homogenate-specific immunoglobulin in blood from mice fed for 28 d with 240 g basic BP/kg (▲) or BP-culture homogenate (△) (n 5 per group). For details of diets and procedures, see Table 1 and pp. 170–171. Values are means with the mean value of a control-fed group subtracted. Standard errors are shown by vertical bars. For all measurements of the control group, mean value was 7·9 (SEM 0·3), representing the average unspecific background. Mean values were significantly different from those of the control group (two-way ANOVA with repeated measures on one factor): *$P \leq 0.0001$. Other differences were not statistically significant ($P \geq 0.05$). (b) Immunoblots of BP-culture homogenate with blood from mice fed 240 g basic BP/kg for 28 d (lanes 2–3) or 0 d (control; lanes 4–5). Lane 1 is Au-stained blot of SeeBlue (Invitrogen, Groningen, The Netherlands) standard.

and IgG_{2a} is the same (Kerr & Loomes 1994), suggesting that the factors supporting the Th2 response may be more efficiently cleared from immune exposure than the Th1-driving factors.

The particulate nature of an antigen is yet another factor that greatly enhances oral immunogenicity (O'Hagan *et al.* 1989; Ermak & Giannasca 1998). Interestingly, the studies employing basic BP in comparison with BP-culture homogenate clearly revealed that the whole-cell, and thus highly particulate, appearance of BP induced both a local response and a systemic immune response, whilst the BP-culture homogenate, representing identical antigens as in basic BP but in a non whole-cell context, induced only a mucosal response. This demonstrates that factors apart from the antigens *per se* such as the particulate nature are responsible for the systemic immunogenicity of BP.

The lymphoid aggregates termed Peyer's patches form important inductive sites of the gut-associated lymphoid tissue for initiation of a mucosal IgA response (Brandtzaeg, 1998). Peyer's patches are specialized for sampling particulate materials (macromolecules, viruses and bacteria), whereas soluble antigens are more poorly sampled. Particulate antigens sampled by Peyer's patches have been found to be selectively translocated to the mesenteric lymph nodes and further to systemic lymphoid tissue such as the spleen according to their size (Jenkins *et al.* 1994; Nakase *et al.* 2001). Particles of approximately 4 μm are efficiently transported to the spleen, whereas both smaller and larger tend to be retained in the Peyer's patches. Moreover, the dissemination of the particulate antigen in the body seems to be a key factor in regulating the induction of systemic and mucosal immune responses (Tabata *et al.* 1996; Matsunaga *et al.* 2000). Tabata *et al.* (1996) found that when particulate antigen is translocated to the spleen, a systemic IgG immune response will take place, whereas when retained in Peyer's patches only a mucosal IgA response will be initiated. These facts could explain the distinct responses induced towards basic BP and

BP-culture homogenate. As basic BP consists of bacteria and bacterial fragments having a particle size range optimal for being partly systemically translocated and partly retained in Peyer's patches, both a systemic and a mucosal immune response is efficiently induced. Though being free of whole bacterial cells hindering a systemic response, BP-culture homogenate still contains cell fragments that might be efficiently retained in Peyer's patches and thus a mucosal IgA response is generated. These findings are further supported by the findings in the study by Mølck *et al.* (2002) showing that nucleic acid-reduced BP appeared to be absorbed from the intestine and transported to the mesenteric lymph nodes, and furthermore seemed likely to undergo a more general dissemination as the liver was also affected. In accordance with this, studies by Dahlgren *et al.* (1991) clearly show that only when genetically incorporated into gram-negative bacteria, ovalbumin become orally immunogenic, whereas soluble ovalbumin co-administered with the very same bacteria induces no response. Likewise, studies by Wold *et al.* (1989) show that bacterial antigens exhibit greater oral immunogenicity than dietary antigens.

Despite the fact that the particulate nature of antigens seems to play a very important role in the oral immunogenicity, no such strong immune response occurs to ingested dairy lactic acid bacteria or to the residing gut flora, suggesting that other factors are also involved. Moreover, IgA specific to common dietary antigens is normally not present in saliva in detectable amounts, as was the case for BP-culture homogenate and for basic BP and nucleic acid-reduced BP. Certain microbial products such as lipopolysaccharide are recognised through pattern recognition receptors on antigen presenting cells and mediate an activating signal (Medzhitov & Janeway 2000). The main organism in the BP culture, *Methylococcus capsulatus* (Bath), contains lipopolysaccharide as an integral part of the cell membrane (Fjellbirkeland *et al.* 1997) and thus, at first sight, represents an obvious candidate for an adjuvant component in BP. However, studies have proved the

complexity of lipopolysaccharide as a mucosal adjuvant as when lipopolysaccharide is co-administered orally with certain antigens it has been found to enhance oral tolerance (Khoury *et al.* 1990). Moreover, lipopolysaccharide is inherently abundant in the mucosal lumen of the intestine (Haman *et al.* 1998). Interestingly, recent studies have shown a diversity of the effect of lipopolysaccharide derived from different bacterial species on their antigen presenting cells stimulation, which could explain a specific property in force for the lipopolysaccharide of *M. capsulatus* (Pulendran *et al.* 2001).

The presence of oral tolerance, as traditionally detected by feeding the antigen and observing for a reduced immune response to a subsequent immunization compared with a non-fed control group, was not specifically addressed in the present study. Studies on this subject are ongoing in our laboratory. Moreover, exhibiting oral immunogenicity, BP holds a potential of being a mucosal adjuvant affecting the immune response towards co-present antigens. Studies on this topic are also ongoing in our laboratory.

In conclusion, BP possesses an oral immunogenicity as both a systemic and mucosal BP-specific antibody response is induced upon ingestion. No differences were found between basic BP and nucleic acid-reduced BP in this matter. The particulate nature of BP played a crucial role for the initiation of the systemic antibody response, which was avoided by processing the BP culture to a product free of whole bacterial cells. A significant mucosal IgA response still occurred.

For future applications of SCP such as BP for animal and human consumption, the present study clearly showed that detailed immunological investigations are needed as an important part of the general safety assessment and to identify and characterize the causative factors in attempt to develop safe products. Failure to recognize the importance of appropriate processing, such as the elimination of a particulate constitution shown in the present study, could lead to unnecessary rejection of SCP products as a dietary protein source.

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