Nutritional outcome and immunocompetence in mice fed on a diet containing raw field beans (*Vicia faba*, var. minor) as the source of protein

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Feeding growing mice on diets containing raw field beans (*Vicia faba* var. minor) as the only source of protein brought about an impairment in growth, muscle mass and liver weight. No changes in food consumption were observed, but the food intake:weight gain ratio was increased in those animals. Plasma protein, triacylglycerols and cholesterol values were not affected by the dietary treatment although serum glucose and zinc levels fell after legume intake as well as the number of circulating erythrocytes. The relative enlargement of thymus and spleen in the legume-fed mice was apparently accompanied by a reduction in the cell number and an increase in cell size, while the protein synthesis capacity followed differentiated patterns in both tissues when assessed through protein, DNA and RNA determinations. The haemagglutination titres and the number of rosette-forming cells were lower in those animals fed on the field bean diet as well as the splenic lymphocyte responses to phytohaemagglutinin, Concanavalin A or lipopolysaccharide mitogens used to evaluate the functional status of T and B lymphocytes. The present study describes, apparently for the first time in mice, the involvement of field bean intake in some immunological disturbances affecting both humoral- and cell-mediated aspects of the immune response.

Field bean (*Vicia faba* L.): Immunocompetence: Mice: Growth: Nutritional status

The interaction between nutrition and immune function is bidirectional (Chandra, 1988). Thus, nutritional status influences host immunocompetence, while infections and immune impairment bring about detrimental effects of nutrient utilization (Gershwin *et al.* 1985; Stinnet, 1987).

The nutritional value of field beans (*Vicia faba*) has been traditionally attributed to their high protein content, although these seeds are also good sources of carbohydrate, minerals and B-vitamins (Hebblethwaite, 1983). However, the presence of certain anti-nutritional factors (ANF) and the imbalance in sulphur amino acids (Eggum, 1986; Gupta, 1987) have been associated with some undesirable biochemical and physiological alterations (Eggum, 1986; Huisman *et al.* 1990*a*) including impairment of growth (Martínez & Larralde, 1984; Hasdai *et al.* 1989; Rubio *et al.* 1990), reduced nutrient digestion and absorption (Villanueva *et al.* 1987; Macarulla *et al.* 1989), lower nitrogen balances (Martínez & Larralde, 1984; Keith & Bell, 1988; Huisman *et al.* 1990*b*) and inhibition of some pancreatic activities (Crass *et al.* 1987; Hasdai *et al.* 1989).

In the literature the only information found about the nutritional utilization of legumes

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

	Casein diet (C)	Field bean (*Vicia faba* var. minor) diet (V)
Casein*	208	—
Field bean	—	700
Saccharose	312	100
Wheat starch	312	100
Olive oil	50	50
Cellulose	60	—
Mineral mix†‡	45	40
Vitamin mix§	10	10
Choline chloride	3	3
Energy content (kJ/kg)	15600	15500
Crude protein (nitrogen × 6·25)	20·0	20·0

* Methionine (10 g/kg) was added to casein diet.
† Harper mixture containing (g/kg): NaCl 139·3, KI 0·8, K_2HPO_4 389·1, $MgSO_4.7H_2O$ 57·3, $CaCO_3$ 381·4, $FeSO_4.7H_2O$ 27·0, $MnSO_4.H_2O$ 4·0, $CuSO_4.5H_2O$ 0·5, $CoCl_2.6H_2O$ 0·02.
‡ Supplemented with 124 and 77 mg $ZnSO_4.7H_2O$/kg diet to obtain diets with 30 mg Zn/kg diet.
§ Harper mixture containing (mg/g): retinol 0·6 mg, cholecalciferol 0·95 mg, tocopherol 3·0, menadione 1·5, *p*-aminobenzoic acid 145·0, Ψ-inositol 32·5, nicotinic acid 15·0, calcium pantothenate 5·7, riboflavin 1·5, cyanocobalamin 6·0, biotin 63·0. Sufficient lactose (carrier) was added to make up 1 g.

by mice and the effect on immune competence is concerned with soya bean (Bounous & Kongshavn, 1985), which has a different nutritional and agricultural value from that of other crops cultured in the EC. In the context we have evaluated, apparently for the first time in mice, the impact of feeding a legume (*Vicia faba*) widely harvested in Europe on growth rate, organ weights and nucleic acid content as well as on plasma biochemical values and humoral- and cell-mediated immune indicators, which is of particular interest in long-term feeding schedules where nutritionally mediated immunological disturbances and infectious diseases can develop.

MATERIALS AND METHODS
Animals and diets
Recently weaned male Swiss albino mice (4 weeks old) obtained from Letica S.A. (Barcelona, Spain) and weighing about 19–22 g were randomly assigned to the dietary groups of eight animals each. The diets, shown in Table 1, contained casein (C) or raw *Vicia faba* seeds (V) as the source of protein. Individual body-weights and food intake were recorded daily for 28 d. The animals were housed in polypropylene cages with wire-meshed bottoms. Room temperature was maintained between 20 and 22°, with controlled humidity and a 12 h day–night cycle. Feed and water were provided *ad lib.* in specially designed feeders to minimize feed wastage.

Antigen and immunization pattern
Sheep erythrocytes (SRBC) were used as the antigen in these studies. Fresh blood was collected from a healthy sheep in sterile Alsever's solution. Before use, this was washed three times in sodium chloride solution (8·5 g/l).

All mice of the experimental groups were inoculated intraperitoneally 4 d before the end of the experiment with 0·2 ml SRBC suspension (100 g/l; approximately 5×10^8 SRBC) in

sodium phosphate-buffered saline (9 g NaCl/l, pH 7·2), since the production of specific antibodies to SRBC is optimally exacerbated after this period of administration (Hudson & Hay, 1989).

Haemagglutination assay

Blood was collected by cardiac puncture under diethyl ether anaesthesia at the end of experiment. Serum was separated by centrifugation (1000 g, 10 min), and stored at $-20°$ until analysed.

Serum haemagglutinating-antibody titres were determined by the microtitre method of Sever (1962) as described by Radix et al. (1983). Serum was heated at 56° for 30 min to inactivate complement and prevent lysis in agglutinin titrations. Agglutination was observed after incubation of SRBC with double dilutions of serum at 37° for 1 h. Serum titres are expressed as logarithms to the base 2 of maximum reciprocal serum dilutions causing agglutination.

Serum and blood measurements

Serum zinc levels were measured by atomic absorption spectrophotometry at 214 nm, previously diluted 4-fold with twice distilled water as described elsewhere (Park et al. 1986). Serum protein levels were determined according to the Lowry et al. (1951) method, by using bovine serum albumin (Sigma Chemical Co.) as a standard. Serum glucose, cholesterol and triacylglycerols were determined by enzymic methods (Bergmeyer, 1974).

Circulating blood cells were counted in a haemocytometer. Packed cell volume values were determined using capillary tubes by centrifugation (1000 g for 10 min) and erythrocyte mean corpuscular volumes (MCV) were calculated using the formula:

$$\text{MCV } (\mu\text{m}^3) = \frac{\text{packed cell volume } (\%) \times 10}{\text{erythrocyte (millions/mm}^3)}.$$

Spleen manipulation

Using aseptic techniques, spleens were removed, weighed and placed in cold NaCl solution (8·5 g/l). Then, spleens were repeatedly punctured and slightly pressed to extract lymphocytes. Lymphocytes were isolated by centrifugation on Ficoll–Hypaque density gradients (Böyum, 1968) and washed three times with saline solution. Erythrocytes were sometimes lysed by instantaneous osmotic shock with distilled water. Lymphocytes were finally suspended in culture medium (RPMI-1640 supplemented to 100 ml fetal calf serum/l, 2 mM-L-glutamine, 100 units penicillin/ml, and 100 μg streptomycin/ml). The cells were counted in a haemocytometer, viability determined by the trypan blue exclusion method, and adjusted to contain 1×10^6 and 10×10^6 viable spleen lymphocytes for mitogen stimulation and rosette-forming cell (RFC) assays respectively.

Test for RFC

This determination was performed according to the method of McConnell et al. (1969) with minor modifications (McFarlane & Hamid, 1973). Equal volumes (0·2 ml) of lymphocyte suspension (2×10^6 viable lymphocytes) and SRBC (10 g/l) were mixed in an ordinary test-tube and then gently centrifuged at 400 g for 12 min at room temperature. The cell deposit was resuspended with the aid of a fine Pasteur pipette and 25 μl were examined for RFC using both high- and low-power objectives. Usually duplicate counts were done.

Lymphocyte proliferation assays

Microassay plates were set up with triplicate wells for each mitogen concentration. Each well contained 1×10^5 viable spleen lymphocytes in 0·1 ml culture medium.

Mitogens were added to each well and the covered plates incubated in a high humidity and a carbon dioxide atmosphere (50 ml/l) at 37° for 2 d. Then the samples were pulsed with 1 μCi [methyl-^{3}H]thymidine and harvested 24 h later. The amount of [methyl-^{3}H]thymidine incorporated was determined by liquid-scintillation counting. Raw values (counts/min; cpm) obtained in such assays were expressed as a stimulation index (SI), which is:

$$SI = \frac{\text{[methyl-}^3\text{H]thymidine uptake in stimulated culture (cpm)}}{\text{[methyl-}^3\text{H]thymidine uptake in unstimulated culture (cpm)}}.$$

The mitogen concentrations were 5 μg/ml culture for *Escherichia coli* lipopolysaccharide S (LPS) and concanavalin A (Con A), and 10 μg/ml culture for phytohaemagglutinin (PHA).

DNA and RNA determinations

Muscle and liver protein contents were determined according to the method of Lowry *et al* (1951), and the analyses of DNA and RNA by the technique of Munro & Fleck (1966) as described by Eisemann *et al.* (1989).

Statistical analysis

The results are presented as the arithmetic means with their standard errors for each control and experimental group. Differences among the mean of groups were evaluated using the Student's two-tailed *t* test and *P* values $\leqslant 0.05$ were considered to be significant.

RESULTS

Growth performance and food intake measurements

A marked growth rate reduction was found in those mice given diet V as compared with diet C. Food consumption was similar in both dietary groups although higher values for the food efficiency ratio (food intake:weight gain) were observed in the legume-fed mice. Moreover, muscle gastrocnemius and liver weights were smaller in animals fed on diet V, while an enlargement was observed in thymus and spleen on a relative basis (Table 2).

Lymphoid tissue nucleic acid contents

In the thymus and spleen, legume intake induced marked losses of DNA, while protein:DNA ratio was elevated, suggesting a possible reduction in cell number associated with an increase in cell size. The RNA:protein ratio remained unchanged in the spleen in both dietary treatments, but was significantly reduced in the thymus, which contrasts with the protein content measurements (Table 3).

Serum and blood measurements

Plasma levels of protein, triaclyglycerols and cholesterol were similar in both dietary treatments as well as leucocyte counts and packed cell volume values. By contrast, plasma Zn and erythrocyte counts were lower after field bean intake, while erythrocyte MCV was elevated (Tables 4 and 5).

Immunological response assessment

Feeding mice on a diet containing legume as the source of protein reduced haem-agglutination titres and SRF cell numbers without changes in cell viability (Table 6).

The humoral immune response to LPS mitogen was depressed in the group given diet V

Table 2. *Initial and final body-weights and organ weights feed conversion ratio and daily food intakes of mice given diets containing raw field beans* (Vicia faba *var. minor; V*) *or casein* (C)*

(Mean values with their standard errors)

Dietary treatment...	C		V		Statistical significance of difference:
	Mean	SE	Mean	SE	P <
Body-wt					
Initial (g)	21·4	0·2	21·2	0·2	NS
Final (g)	35·3	1·1	28·4	0·8	0·001
Organ wt					
Liver (g)	1·90	0·09	1·41	0·06	0·001
Gastrocnemius muscles (mg)	312	11	261	7	0·001
Kidneys (mg)	599	26	376	3	0·001
Spleen (mg)	128	7	147	8	NS
Thymus (mg)	44	5	54	6	NS
Daily food intake (g/d)	4·6	0·1	4·3	0·1	NS
Body weight gain/protein intake (g/g)	0·54	0·04	0·30	0·04	0·001
Feed conversion ratio (g intake /g gain)	9·5	0·7	20·6	3·1	0·001

NS, not significant.
* For details of diets and dietary regimen, see Table 1 and p. 494.

Table 3. *Protein and DNA concentration, protein:DNA, RNA:protein of spleen and thymus of mice given diets containing raw field beans* (Vicia faba *var. minor; V*) *or casein* (C)*

(Mean values with their standard errors)

Dietary treatment...	C		V		Statistical significance of difference:
	Mean	SE	Mean	SE	P <
Spleen					
Protein (mg/g)	163·1	7·5	134·5	10·4	0·05
DNA (mg/g)	17·52	1·13	12·57	1·17	0·05
Protein:DNA (mg/mg)	7·55	1·84	10·97	1·13	0·05
RNA:protein (μg/mg)	20·09	3·22	24·68	2·49	NS
Thymus					
Protein (mg/g)	169·1	4·2	167·5	5·2	NS
DNA (mg/g)	55·46	1·54	36·63	2·40	0·001
Protein:DNA (mg/mg)	3·06	0·09	4·65	0·20	0·001
RNA:protein (μg/mg)	42·79	0·90	24·3	1·19	0·001

NS, not significant.
* For details of diets and dietary regimen, see Table 1 and p. 494.

Table 4. *Serum protein, glucose, cholesterol, triacylglycerols and zinc of mice given diets containing raw field beans* (Vicia faba *var. minor; V*) *or casein* (C)*

(Mean values with their standard errors)

Dietary treatment...	C		V		Statistical significance of difference:
	Mean	SE	Mean	SE	P <
Protein (mg/l)	48·2	3·5	51·0	1·1	NS
Glucose (mg/l)	2610	160	2200	100	0·05
Cholesterol (mg/l)	1450	70	1510	120	NS
Triacylglycerols (mg/l)	1110	110	1420	130	NS
Zinc (μg/l)	1890	70	1250	50	0·001

NS, not significant.
* For details of diets and dietary regimen, see Table 1 and p. 494.

Table 5. *Leucocyte and erythrocyte counts, packed cell volume* (PCV) *and mean corpuscular volume* (MCV) *of mice given diets containing raw field beans* (Vicia faba *var. minor; V*) *or casein* (C)*

(Mean values with their standard errors)

Dietary treatment...	C		V		Statistical significance of difference:
	Mean	SE	Mean	SE	P <
Leucocyte count (/mm^3)	8463	907	8281	422	NS
Erythrocyte count (10^6/mm^3)	9·08	0·11	8·05	0·13	0·001
PCV (%)	46·9	1·1	46·0	0·9	NS
MCV (μm^3)	51·7	0·9	57·3	1·8	0·01

NS, not significant.
* For details of diets and dietary regimen, see Table 1 and p. 494.

Table 6. *Viability of lymphocytes, haemagglutination titres and spleen rosette-forming cells* (RFC) *of mice given diets containing raw field beans* (Vicia faba *var. minor; V*) *or casein* (C)*

(Mean values with their standard errors)

Dietary treatment...	C		V		Statistical significance of difference:
	Mean	SE	Mean	SE	P <
Viability (%)	97·2	0·4	98·3	0·6	NS
Haemagglutination titres	5·6	0·3	3·3	0·4	0·001
Spleen RFC (%)	2·6	0·3	1·3	0·2	0·001

NS, not significant.
* For details of diets and dietary regimen, see Table 1 and p. 494.

Table 7. *Stimulation index (SI) from lipopolysaccharide (5 µg/ml; LPS), phytohaemagglutinin (10 µg/ml; PHA) and concanavalin A (5 µg/ml; Con A) mitogens of spleen lymphocytes of mice given diets containing raw field beans* (Vicia faba *var. minor; V) or casein (C)**

(Mean values with their standard errors)

Dietary treatment...	C		V		Statistical significance of difference:
	Mean	SE	Mean	SE	P <
SI (cpm/cpm):					
LPS	4·08	0·60	2·18	0·23	0·05
PHA	4·64	0·56	2·67	0·33	0·01
Con A	14·70	2·27	6·70	1·27	0·001

cpm, counts/min.
* For details of diets and dietary regimen, see Table 1 and p. 494.

as well as the transformation of helper T cells by Con A or the proliferation of most T cells using PHA mitogen (Table 7), which were estimated as indicators on cell-mediated immunological responsiveness.

DISCUSSION

Legume intake affects growth performance in different species of experimental or productive animals. Thus, situations of impairment of growth have been observed in rats (Martínez & Larralde, 1984), chickens (Rubio *et al.* 1990), pigs (Bond, 1981; King *et al.* 1983; Huisman *et al.* 1990a) and ruminant livestock (Bond, 1981; Williams *et al.* 1984).

The inclusion of field beans and other legumes in the diet reduces digestibility (Crass *et al.* 1987; Villanueva *et al.* 1987) and absorption of nutrients (Macarulla *et al.* 1989), accompanied by a reduced N retention (Martínez *et al.* 1987) which has been attributed to the occurrence of some naturally occurring toxicants (Liener, 1989) or the imbalance in S amino acids (Prakash & Migra, 1988). In an early experiment it was found that heat treatment and amino acid supplementation of field-bean diets only improved growth performance slightly (Bello *et al.* 1972).

The possible involvement of several ANF such as polyphenols, protease inhibitors, lectins, etc., in the process of growth retardation has been reported (Eggum, 1986; Gupta, 1987; Oliveira *et al.* 1988; Hasdai *et al.* 1989), and has been attributed to a decrease in whole-body protein synthesis rather than changes in protein breakdown (Martínez *et al.* 1987). In this context, it has also been reported repeatedly that the inclusion of raw legumes as the source of protein brings about a number of biochemical and physiological alterations (Gupta, 1987; Larralde & Martínez, 1989), such as impairment of exocrine pancreatic function (Hasdai *et al.* 1989) and intestinal digestibility (Villanueva *et al.* 1987) as well as changes in gut integrity (Kilshaw & Slade, 1982; Ridout *et al.* 1988) and motility (Goodlad & Mathers, 1990), which can lead to adverse effects on nutrient utilization and immune responsiveness of the mucosal-associated lymphoid tissue and secretory Ig A (Barratt *et al.* 1979; Sissons *et al.* 1989). On the other hand, the impairment of mucosal integrity may induce malabsorption or macromolecular uptake of antigens, or both, since it has been suggested that diarrhoea in soya-bean-fed calves is caused by an immediate type of hypersensitivity (Sissons *et al.* 1989); however, a specific sensitivity to some proteins cannot be discarded.

The stunting of growth in mice fed on a legume diet was not associated with changes in food consumption. Hence, a concomitant increase in food intake:weight gain ratio was observed in the legume-fed animals, similar to that previously reported in rats (Martínez & Larralde, 1984) and chickens (Rubio *et al.* 1990).

Feeding animals on field beans as the only source of protein resulted in lower liver and gastrocnemius muscle weights compared with controls. Conversely, a relative hypertrophy was found in thymus and spleen of the legume-fed mice, which could be explained, at least in part, by an immune reaction to antigenic proteins occurring in the seeds, as previously suggested (Bell *et al.* 1978; Sissons *et al.* 1989). Furthermore enlargement of pancreas and intestine induced by feeding legume diets has been reported in rats and guinea-pigs (Oliveira *et al.* 1988; Hasdai *et al.* 1989).

Biochemical assessment of plasma lipids showed no differences between groups, which is supported by previous findings with normal rats (Martínez *et al.* 1986), although a hypo-lipidaemic role has been attributed to legumes in hypercholesterolaemic rats (Mengheri *et al.* 1985) and normo-cholesterolaemic men (Shutler *et al.* 1989; Mendis & Kum-aransunderam, 1990).

The levels of glucose were lower in diet V-fed mice compared with the controls, a finding similar to that previously obtained by feeding rats with kidney beans or soya beans which was also accompanied by low levels of serum insulin (Grant *et al.* 1987). Thus, a possible beneficial effect of diets based on leguminous seeds in the management of diabetes has been identified, and this has been attributed to the storage carbohydrates (Hockaday, 1982).

On the other hand, a number of reports have established that the presence of legume protein in animal diets increases mineral requirements (Harland, 1989; García-López *et al.* 1990), which is in good agreement with the reduced values for plasma Zn and erythrocyte counts obtained, which were associated with an increase in erythrocyte cell size. These biochemical observations would indicate reduced bioavailability of Fe and Zn in legume diets with clear implications in growth, metabolism and immunity (Martínez *et al.* 1985; Harland, 1989; Suttle & Jones, 1989; Chandra, 1991).

Tissue DNA content, protein:DNA or RNA:protein ratios have often been estimated as indirect and stationary indicators of cell number, cell size and protein synthesis capacity respectively in nutritional and metabolic studies, despite some limitations (Waterlow *et al.* 1978). Our results indicate that cellularity is affected in the lymphoid tissue of legume-fed animals. Thus, the number of cells is reduced in both the thymus and spleen, which was associated with an apparent cell hypertrophy. However, the protein synthesis capacity, as assessed by RNA:protein ratio, showed a differential pattern in both lymphoid tissues, since no changes were found in the spleen, although a marked reduction was observed for this variable in thymus.

The humoral immune response to SRBC antigens can be measured in haemagglutination assays, which provide information about the Ig M response (Verma *et al.* 1988). Our results show that haemagglutination titres are markedly reduced in the legume-fed mice, a finding similar to that previously reported in situations of Zn deficiency or protein restriction (Hansen *et al.* 1982). Also, γ-globulin concentration has been reported to be decreased in rats fed on a diet containing field beans as the source of protein (Martínez *et al.* 1986).

Binding of RFC, which are members of the B and T lymphocyte series, is due to antibody or antibody-like receptors present on the cell surface (Garvey *et al.* 1981). Legume intake reduced by half the number of RFC, which suggests that both humoral- and cell-mediated immune responses could be affected.

The selective stimulation and blast transformation of T and B cells by mitogenic agents has proved to be an adequate tool for the study and recognition of antigenic activation and characterization of different lymphocyte subpopulations (Gershwin *et al.* 1985). Lectins

such as PHA and Con A extracted from legumes specifically stimulate most T cell series and T helper cells respectively, while B cell transformation is specifically achieved by mitogenic agent LPS.

The lectin content of field beans is generally considered to be low (Grant *et al.* 1983). However, the possible impact of these haemagglutinins on immunocompetence and nutritional outcome is a matter of controversy and should be taken into consideration when examining these results (Ferguson, 1985).

The influence of legume protein and lectins on the immune system is a rather special case of reactivity because it may depend on both antigenic determinants of the protein (Barratt *et al.* 1979) and the immunobiochemical reactivity of lectins (Pusztai *et al.* 1986) and could explain, at least in part, the relative deficiency in nutritional performance of animals fed on those legume diets (Smith & Sissons, 1975), as well as some disturbances of the immune response (Ferguson, 1985).

The SI values obtained from tritiated thymidine incorporation into lymphocyte DNA were always lower in the legume-fed mice, indicating that T and B cell populations are functionally affected by field bean intake. Additionally, it could be suggested that the reduction in helper T lymphocyte response would influence the intrinsic capacity of the B lymphocytes to respond to an immunogenic stimulus.

The role played by individual nutrients in the observed changes in immune responsiveness could not be established by our study. However, it has been suggested that legume protein (Bounous & Kongshavn, 1985), the lower mineral bioavailability in the diets (Forbes, 1984; Dowd *et al.* 1986) and lectins occurring in these seeds (Pusztai *et al.* 1986) would be involved in those processes, without discarding other ANF.

A different hypothesis concerning the mechanisms by which legume diets act on immune responsiveness emphasizes metabolic and hormonal changes resulting from bean consumption (Grant *et al.* 1987) or Zn deficiency (Giugliano & Millward, 1987) as well as protein- or lectin-dependent changes in gut-associated microflora (Bounous *et al.* 1983; Pusztai *et al.* 1986).

It can be concluded that feeding mice on diets containing field beans as the source of protein brings about an impairment in growth rate, muscle mass and liver weight, while an enlargement of the thymus and spleen was observed. Furthermore, legume intake caused a number of immunological disturbances affecting both humoral- and cell-mediated immune responses, which have not apparently been evaluated previously with mice as the experimental model and field beans as the source of protein. These findings have clear implications, such as infections and nutritionally-mediated immunological disturbances, in animal nutrition as well as in clinical nutrition, in situations of long-term feeding of this legume.

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