Immunocompetence in relation to a heat-processed diet (Maillard reaction) in weanling rats

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Diets containing unheated casein (CD; control) or a casein-glucose mixture (CGD) previously heated at 140° for 2 h were fed to two groups of young rats for 21 d. Differences in body weight, feed consumption, thymus and spleen growth, protein metabolism and *in vivo* immune response were then determined. For this last experiment, animals were inoculated with sheep erythrocytes (SRBC) on day 15 to provide an immunological challenge. No changes were seen in body weight, feed consumption or feed conversion ratios. Neither were significant differences found in spleen weight, protein content, DNA content, DNase (*EC* 3.1.4.6) activity or lymphocyte count, suggesting that spleen cell growth remained similar in all the animals studied. The CGD induced marked increases in thymus DNA content whilst the protein:DNA ratio became lower. Spleen RNA content was similar in all rats, but thymus RNA content was 29 % lower in the CGD group, although this difference did not reach statistical significance. This fact might be a consequence of the low RNase (*EC* 2.7.7.16) activity and RNase:RNA ratios in the thymus glands of CGD-fed animals. Further, the number of splenic plasma cells secreting anti-SRBC antibodies (direct plaque-forming cells) was significantly decreased in the same group. It might be concluded that both diets are adequate for rat growth and that the differences observed in the thymus of CGD-fed rats may be directed towards preserving tissue function. Nevertheless, the CGD did cause immunological disturbances affecting the humoral immune response.

Heat-processed diet: Maillard reaction: Immunocompetence: Thymus: Spleen

Processed foods are increasingly common in the human diet. Heat treatment is widely used to improve the taste and texture of food in commercial and home processing (grilling, baking, boiling, etc.). However, the heating of food proteins is known to have undesirable consequences. The Maillard browning reaction is one of the most important. This reaction occurs between reducing sugars and amino compounds (Adrian, 1974) and may alter the nutritional properties of food by reducing the availability of essential amino acids (mainly lysine). It may also reduce the digestibility of food and form potentially toxic compounds such as D-serine and D-tyrosine (Friedman *et al.* 1984). This should be taken into account when heat-processed food is consumed and further research is required to define the possible nutritional risks of its consumption (Finot, 1990).

In a study of the metabolism (absorption, excretion, etc.) of Maillard reaction products (MRP), Hurrell (1990) showed different physiological effects according to the Maillard reaction conditions. Matsuda *et al.* (1990) and Öste *et al.* (1990) studied the allergenic

capacity of some proteins subjected to the Maillard reaction but their results were inconclusive.

The available literature concerning the effects of MRP on the nutritive value of foods is controversial and confused, and research is required to determine the effects of these compounds on tissues. Many cells of the immune system are known to rely on metabolic pathways whose mediators employ a range of nutrients as cofactors (Chandra, 1992). Some of these nutrients may act directly on the cells of the immune system, and alter their capacity to recognize foreign stimuli, leading to food allergies (Gershwin *et al.* 1985; Metcalfe, 1985).

Therefore, an investigation was made into protein metabolism in thymus and spleen cells of rats fed on diets containing either unheated casein or a heated protein + sugar mixture as the protein source. The *in vivo* immune response of the experimental animals was also studied. The latter is of particular interest in long-term feeding schedules since nutritionally-mediated immunological disturbances and infectious diseases may develop.

MATERIALS AND METHODS

Animals and diets

Three-week-old male Wistar rats (from the Instituto de Nutrición y Bromatología (CSIC), Madrid, Spain), weighing about 52 g were randomly assigned to two dietary groups of sixteen animals. As a source of protein the diets contained either raw casein (CD) or a mixture of casein + glucose (CGD) previously heated at 140° for 2 h (Table 1). Animals were housed in individual polypropylene cages with wire-mesh bases and maintained at a temperature between 20 and 22° and at 95% humidity. A 12 h day–night cycle was established. All animals had free access to feed and water. Individual body weights and feed intakes were recorded daily for 21 d.

Experimental designs and antigen and immunization patterns

The experimental animals were allowed a 3 d period of adaptation to the diets. After following the diets for 21 d the immune responses of all animals were tested against an immunological challenge provided by sheep erythrocytes (SRBC; BioMérieux, Marcy l'Etoile, France). Before use, the cells were washed three times in NaCl solution (8·5 g/l).

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

	Casein diet (CD)	Casein–glucose diet (CGD)
Casein (unheated)	107·6	–
Methionine	2	2
Casein + glucose (previously heated)	–	153·1
Sucrose	375·8	353
Wheat starch	375·8	353
Olive oil	50	50
Cellulose	50	50
Mineral mix*	37·7	37·7
Vitamin mix*	1·2	1·2
Energy content (kJ/kg)	16120	16120
Protein (N × 6·25)	100	100

* Mineral and vitamin mixes were added to each diet according to the National Academy of Sciences (1978).

Optimal production of anti-SRBC antibodies is seen after 5 d (Hudson & Hay, 1989), therefore, animals were inoculated intraperitoneally on day 16 with $1{\cdot}2 \times 10^9$ SRBC/0·5 ml saline solution per 100 g body weight. At the end of the experimental period, animals were weighed and their spleens and thymus glands removed under diethyl ether anaesthesia.

Manipulation of spleen and thymus and preparation of tissue extracts

Eight spleens belonging to eight animals of each experimental group were used to assess spleen cell counts and the number of direct plaque-forming cells (DPFC). Using aseptic techniques, spleens were removed, weighed and placed in cold balanced salt solution (BSS) (NaCl 136·9 mmol/l, KCl 5·37 mmol/l, $CaCl_2$ 1·26 mmol/l, $MgSO_4.7H_2O$ 0·65 mmol/l, Na_2HPO_4 0·34 mmol/l, $MgCl_2.6H_2O$ 0·49 mmol/l, KH_2PO_4 0·44 mmol/l, glucose 5·55 mmol/l, $NaHCO_3$ 4·17 mmol/l; pH 7·2–7·4). Cell suspensions were prepared by passing spleens through 100-mesh wire screens into BSS. Large clumps of cells were allowed to settle and the remaining suspension was gently agitated using a Pasteur pipette. Cells were counted using an automatic counter (Coulter Counter, Hialeah, FL, USA), and suspensions of 3×10^6 spleen lymphocytes were prepared for the DPFC assay. The spleens of the remaining eight rats of each experimental group were divided into two portions. One half was used to analyse spleen protein content and enzymic activities and the other to determine DNA and RNA contents.

The thymus glands of all sixteen rats were removed and divided into two portions. One portion of each gland was used to determine enzymic activity whilst the other was used to estimate DNA and RNA content.

The samples of spleen and thymus tissue, used to measure protein and enzymic activities respectively, were homogenized (Ultra-Turrax, Staupen i. Breisau, Germany) in cold buffer pH 7·4 (0·15 M-NaCl, 5 mM-$NaHCO_3$) to produce a 200 g/l tissue homogenate. Solids were removed by centrifugation at 500 *g* for 10 min and the supernatant fractions stored at −20° until use. In the remaining samples of spleen and thymus, DNA and RNA contents were determined after extraction with TCA.

Determination of protein, DNA and RNA contents

Spleen and thymus protein contents were determined by the Biuret reaction (Weichselbaum, 1946). RNA content was determined using the method of Munro & Fleck (1966) and DNA content assessed according to the method of Burton (1956).

Enzyme activity measurements

Acid deoxyribonuclease (*EC* 3.1.4.6) and acid ribonuclease (*EC* 2.7.7.16) activities were assayed according to the methods of McDonald (1955) and Kalnitsky *et al.* (1959) with minor modifications (sample incubation periods were 45 min for DNase and 60 min for RNase).

Evaluation of direct plaque-forming cells response

At 5 d after inoculation with SRBC, the DPFC response was assayed in eight rats from each experimental group using the haemolytic plaque assay described by Cunningham & Szenberg (1968). SRBC were used as target cells. The reaction mixture contained a 150 µl spleen-cell suspension in a BSS medium, 40 µl rabbit complement (Behring, Hoechst-

Behring, France) and a 20 μl SRBC suspension in phosphate buffered saline–glucose (PBS–glucose) (NaCl 0·15 mol/l, Na_2HPO_4 8·45 mmol/l, NaH_2PO_4 1·85 mmol/l). Slides were incubated for 1 h at 37° before plaques were counted.

Statistical analysis

Results are presented as arithmetic means with their standard errors. Differences between the group means were evaluated using the pooled *t* test (Statistical Analysis Systems Institute Inc., 1989). Variables showing a high degree of variability e.g. DPFC (Table 7) were checked for the presence of outliers. Where the variabilities of the two treatment groups differed markedly the means were compared using an approximate *t* test for unequal variances (separate *t* test) (BMDP, 1992). Probability values of $P < 0·05$ were considered to be significant.

RESULTS

Growth performance and food intake measurements

Initial and final body weight and weight gain were similar in both dietary groups. No differences were seen between the groups for feed and protein consumption. Feed conversion ratio remained unchanged in both dietary treatments (Table 2).

Cellular growth in lymphoid tissues

No differences were found between the two groups for spleen cell growth (Table 3). Spleen weight and protein and DNA contents as well as tissue lymphocyte count (see Table 7) were similar in both experimental groups. Neither were differences seen in acid DNase catalytic activity (Table 3). In the thymus (Table 4), CGD intake induced a marked increase in DNA content (mg/g thymus) ($P = 0·026$) whilst the protein:DNA ratio diminished ($P = 0·032$). Nevertheless, no significant statistical differences were found between the groups for DNase activity or thymus lymphocyte count.

Table 2. *Initial and final body-weights, daily feed intakes and feed conversion ratio of rats fed on diets containing unheated casein (CD) or heated casein + glucose (CGD) as protein source**

(Mean values with their standard errors for sixteen animals per dietary group)

Dietary treatment...	CD		CGD		Statistical significance of difference†
	Mean	SE	Mean	SE	
Initial body weight (g)	51·25	0·77	51·93	0·94	NS
Final body weight (g)	133·45	9·19	145·00	5·26	NS
Weight gain (g/rat per d)	4·11	0·46	4·66	0·27	NS
Daily feed intake (g)	10·96	0·73	12·19	0·43	NS
Daily protein intake (g)	1·21	0·08	1·33	0·05	NS
Body-weight gain/protein intake (g/g)	3·37	0·21	3·51	0·12	NS
Feed conversion ratio (g intake/g gain)	2·75	0·18	2·64	0·09	NS

* For details of diets, see Table 1.
† Degrees of freedom: CD 15, CGD 15.

Table 3. *Spleen cellular growth of rats given diets containing unheated casein (CD) or heated casein–glucose (CGD) as protein source**

(Mean values with their standard errors for eight animals per dietary group)

Dietary treatment...	CD		CGD		Statistical significance of difference†
	Mean	SE	Mean	SE	
Spleen index‡	2·60	0·19	2·97	0·29	NS
Spleen weight (g)	0·34	0·04	0·39	0·04	NS
Protein (mg/g spleen)	89·84	1·56	86·54	2·45	NS
Protein (mg/spleen)	31·08	3·58	34·30	2·98	NS
DNA (mg/spleen)	5·32	0·75	5·87	0·95	NS
DNA (mg/g spleen)	15·40	0·80	14·44	1·03	NS
Protein/DNA (mg/mg)	5·93	0·36	6·20	0·64	NS
DNase (U/spleen)	5·65	0·65	6·12	0·58	NS
DNase (U/mg protein)	0·18	0·01	0·18	0·02	NS
DNase/DNA (U/mg)	1·10	0·11	1·19	0·26	NS

* For details of diets, see Table 1.
† Degrees of freedom: CD 7, CGD 7.
‡ Spleen weight ($\times 10^3$)/final body weight.

Table 4. *Thymus cellular growth of rats fed on diets containing unheated casein (CD) or heated casein–glucose (CGD) as protein source**

(Mean values with their standard errors for sixteen animals per dietary group)

Dietary treatment...	CD		CGD		Statistical significance of difference†
	Mean	SE	Mean	SE	
Thymus index‡	3·08	0·38	3·20	0·27	NS
Thymus weight (g)	0·43	0·07	0·46	0·04	NS
Protein (mg/thymus)	23·34	4·85	23·30	2·54	NS
Protein (mg/g thymus)	54·03	4·02	50·23	2·46	NS
DNA (mg/thymus)	8·89	1·61	11·97	1·62	NS
DNA (mg/g thymus)	20·81	0·82	25·43	1·56	$P=0·026$
Protein/DNA (mg/g)	2·63	0·24	2·02	0·17	$P=0·032$
DNase (U/thymus)	5·83	1·23	5·40	0·89	NS
DNase (U/mg protein)	0·22	0·01	0·24	0·20	NS
DNase/DNA (U/mg)	0·64	0·07	0·47	0·05	NS
Lymphocytes (cells $\times 10^6$/thymus)	569	64	587	81	NS

* For details of diets, see Table 1.
† Degrees of freedom: CD 15, CGD 15.
‡ Thymus weight ($\times 10^3$)/final body weight.

RNA metabolism in lymphoid tissues

RNA contents and RNA:protein ratio were similar in the spleen and thymus of rats in both dietary treatments (Tables 5 and 6). However, RNase activity (RNase/protein) ($P=0·034$) and RNase/RNA ($P=0·019$) were lower in thymus glands from animals of the CGD group (Table 6).

Table 5. *Spleen RNA metabolism of rats fed on diets containing unheated casein (CD) or heated casein–glucose (CGD) as protein source**

(Mean values with their standard errors for eight animals per dietary group)

Dietary treatment...	CD		CGD		Statistical significance of difference†
	Mean	SE	Mean	SE	
RNA (mg/spleen)	3·73	0·44	4·19	0·47	NS
RNA (mg/g spleen)	10·93	0·47	10·51	0·46	NS
RNA/protein (mg/mg)	0·12	0·008	0·12	0·008	NS
RNase (U/spleen)	0·25	0·03	0·27	0·03	NS
RNase (mU/mg protein)	7·90	0·40	7·85	0·42	NS
RNase/RNA (mU/mg)	65·90	5·60	64·6	2·10	NS

* For details of diets, see Table 1.
† Degrees of freedom: CD 7, CGD 7.

Table 6. *Thymus RNA metabolism of rats fed on diets containing unheated casein (CD) or heated casein–glucose (CGD) as protein source**

(Mean values with their standard errors for sixteen animals per dietary group)

Dietary treatment...	CD		CGD		Statistical significance of difference†
	Mean	SE	Mean	SE	
RNA (mg/thymus)	3·49	0·5	4·90	0·67	NS
RNA (mg/g thymus)	8·12	0·51	10·44	0·70	NS
RNA/protein (mg/mg)	0·17	0·02	0·21	0·01	NS
RNase (U/thymus)	0·11	0·02	0·08	0·01	NS
RNase (mU/mg protein)	4·83	0·42	3·57	0·13	$P=0·034$
RNase/RNA (U/mg)	3·14	0·004	1·71	0·001	$P=0·019$

* For details of diets, see Table 1.
† Degrees of freedom: CD 15, CGD 15.

Assessment of immunological response

The spleen lymphocyte count was similar in both experimental groups (Table 7). The number of splenic plasma cells secreting anti-SRBC antibodies (DPFC) was significantly reduced in CGD-fed rats, both in absolute terms ($P=0·01$) and when expressed per 10^6 spleen cells ($P=0·004$).

DISCUSSION

The dietary intake and body-weight values (Table 2) recorded in this study provide evidence that a casein + glucose diet can be considered nutritionally adequate for rat growth. All animals showed a positive weight gain during the experimental period. These results agree with those reported by Kimiagar *et al.* (1980) in a study of rats fed on a diet containing 100 g MRP/kg for 1 month. Though damage to the lysine component of the heat-treated diet could be expected (this amino acid is the most affected by heat treatment in the presence of reducing sugars (Hurrell *et al.* 1983)) no adverse effects were seen with respect to the variables mentioned earlier. According to Said *et al* (1974), adaptation to

Table 7. *Direct plaque-forming cells (DPFC) response in spleen of rats fed on diets containing unheated casein (CD) or heated casein–glucose (CGD) as protein source**

(Mean values with their standard errors for eight animals per dietary group)

Dietary treatment...	CD		CGD		Statistical significance of difference†
	Mean	SE	Mean	SE	
Lymphocytes‡ (cells $\times 10^6$/spleen)	347	11	384	24	NS
Spleen DPFC ($\times 10^{-3}$)§	582	97	254	18	$P=0{\cdot}01$
DPFC (/10^6cells)§	1647	238	664	32	$P=0{\cdot}004$

* For details of diets, see Table 1.
† Degrees of freedom: CD 7, CGD 7.
‡ Pooled *t* test.
§ Separate *t* test.

lysine-free diets is clear and marked. Rats given no lysine still show apparently adequate intakes and lose weight and tissue much more slowly than animals fed on diets deficient in threonine, isoleucine or total S amino acids. This may explain the normal rat growth observed in the present study.

No significant differences were found between the groups with respect to spleen weight, spleen-somatic index, spleen DNA levels, or spleen DNA catabolism (Table 3) or RNA metabolism (Table 5). In the same way, Kimiagar *et al.* (1980) observed no spleen weight loss in rats fed on MRP for 1 month. Similar results were reported by Martínez *et al.* (1992) in spleens from mice which had been fed on either casein or *Vicia faba* as a protein source. However, Srivastava *et al.* (1975), Muñoz *et al.* (1981) and Martinez *et al.* (1992) found that spleen protein and DNA contents decreased in rats fed on altered quality and/or quantity of protein. Under malnutrition conditions, Kenney *et al* (1968) and Srivastava *et al.* (1975) reported that rat spleen RNA levels were reduced. In such conditions spleen RNA catabolism remains unmodified in order to maintain RNA content as much as possible (Muñoz *et al.* 1981).

Thymus cell atrophy (Table 4) was observed in CGD-fed animals, caused mainly by higher (26 %) DNA content in the thymus. A high variability was found for these data, and as a consequence, the difference between the means was not detected as significant. When DNA content was expressed per weight unit (g) of thymus, a statistically significant difference was found. Low DNA levels in lymphoid organs (Srivastava *et al.* 1975) and thymus lymphocyte counts have been reported under malnutrition conditions (Bell *et al.* 1976). With respect to thymus DNA catabolism, DNase/mg DNA was 36 % lower in the CGD group than in the CD group, although no statistically signficant difference was detected. Muñoz *et al.* (1981) observed no modifications in acid DNase activity of the thymus glands of growing rats fed on a low-protein diet for 20 d. However, after 30 d these authors reported increased DNase levels, leading to organ involution.

Thymus RNA levels were statistically similar in the two groups, in spite of the fact that the absolute and relative thymus RNA contents were 29 and 22 % higher respectively in the CGD group than in the CD group. This may be due to lowered RNase catabolic activity (Table 6). These results are similar to those of Muñoz *et al.* (1981) who reported lower thymus RNA catabolism in growing rats fed on a protein-deficient diet for 3 weeks. Since non-significant differences were seen between the two groups in either protein or RNA levels, no significant difference was found in protein synthesis capacity (RNA:protein

ratio), although the mean of the CGD group was 19 % higher than that of the CD group, possibly as a consequence of the data variability observed in the CD group.

The humoral immune response to SRBC was measured in the DPFC assay, a technique which provides information on the immunoglobulin M (IgM) response (Hudson & Hay, 1989). The results of this investigation show that the DPFC count was markedly reduced in the CGD-fed rats (Table 7) in spite of the spleen lymphocyte count remaining unmodified. Moreover, it seems relevant to point out that the CD group's DPFC responses showed great variation while in the CGD group this was smaller. It might indicate that the heated diet modulates the immune response more tightly. Impairment of immunity has been found by other authors (Stolzner & Dorsey, 1980; Petro & Bhattacharjee, 1981; Woodward & Miller, 1991; Woodward *et al.* 1992) in mice maintained in protein and amino acid-deficient conditions. According to Bounous & Kongshavn (1985), the effects of altered dietary protein type on the humoral immune response are not exerted only on the rate of primary B-lymphocyte production in the bone marrow, but also alter the functional responsiveness of the B lymphocytes themselves, during their activation and differentiation in the peripheral lymphoid tissues. Dysfunction of antigen-presenting cells may also occur (Boswell *et al.* 1980; Conzen & Janeway, 1988). This would modify the complex sequence of events in B lymphocyte activation, clonal expansion and antibody secretion. Moreover, it has been pointed out that glycosylated proteins, formed after long-term exposure to glucose, are recognized and endocytosed by rat sinusoidal liver cells and peritoneal macrophages (Vlassara *et al.* 1985; Takata *et al.* 1988). In view of these findings, it might be suggested that the macrophages of rats fed on the heated protein–glucose mixture would respond to dietary glycosylated proteins. A possible deficiency in amino acids and a possible response by macrophages could contribute to the depressed humoral immune function observed in the present study.

It may be concluded that both diets are adequate for rat growth. The modifications observed in the thymus glands of animals fed on CGD may be directed towards preserving tissue function. Nevertheless, the heat-treated CGD did cause immunological disturbances affecting the humoral immune response, something that has not been evaluated previously when using rats as an experimental model and heated protein + reducing sugar as the dietary source of protein. There is, therefore, a possibility that nutritionally-mediated immunological disturbances, both in animals and man, could occur in long-term consumption of heat-processed foods.

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