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Immune components of the intestinal mucosae of ageing and protein deficient mice

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Summary. The studies were designed to investigate the effect of ageing and low protein (4%) diet on antibody-dependent cell-mediated cytotoxicity (ADCC) and IgA concentration in the intestine. Both ADCC activity and IgA concentration were shown to reach mature levels at 17 weeks old. The effect of ageing was greater on ADCC activity which showed a drastic drop in activity at 75 weeks old than IgA which had only a slight decline in concentration at this age. The low protein diet has a greater suppressive effect on IgA concentration than on ADCC activity. As the duration of feeding low protein to the young mice increased, greater decline in IgA concentration in the intestine was observed. There was, however, no significant difference from the normal mice in ADCC activity and IgA concentration of aged and adult mice given short term (8 weeks) low protein diet.

INTRODUCTION

The frequent exposure of gastro-intestinal (GI) tract to chemicals and micro-organisms in the food has made the GI tract an important barrier to pathogenic invasion of the body (Watson & McMurray, 1979). In the GI tract the host immune defences need to provide adequate and constant surveillance. One of these defences is the production of secretory immunoglobulin A (sIgA) by plasma cells near the mucosal surface (Brandtzaeg 1974). The sIgA secreted into the intestine binds bacteria and viruses which reduces their attachment to the mucosal cells and subsequent growth. In addition to sIgA production by mucosal lymphocytes, there are other mucosal lymphoid cells in the intestine with potential roles in host defence. The nature of these mucosal lymphoid cells and their roles in mucosal immunity of the undernourished are still very much unknown. Recently techniques have been developed to isolate these intestinal mucosal lymphoid cells (IMLC) from human (McDermott, Franklin, Jenkins, Kodner, Nash & Weinrieb, 1980), rabbit (Rudzik & Bienenstock, 1974) and guinea-pig (Battandier, Bundy, O'Neill, Bienenstock & Nelson, 1978). IMLC were found to be capable of carrying out cell-mediated immune mechanisms (Battandier *et al.*, 1978; MacDermott *et al.*, 1980). However, the larger body sizes in rabbit and guinea-pig and the difficulty in obtaining human intestines make them less applicable in certain experimental studies. Therefore, we have developed a technique to isolate mucosal lymphocytes from mouse intestine. In this study, we applied this technique to characterize the effect of ageing on aspects of mucosal immunity in the intestine as measured by antibody-dependent cell-mediated cytotoxicity (ADCC) and IgA level in the intestinal

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secretion. Since incidence and severity of intestinal diarrhoea is increased by undernutrition, we also examined the effect of low protein diet (4%) on mouse intestinal ADCC and IgA production in young and aged mice.

MATERIALS AND METHODS

Animals

Inbred BALB/c mice were bred in the animal facilities in the Department of Foods and Nutrition at Purdue University using breeding stock purchased from Jackson Laboratory, Bar Harbor, Maine. This mouse strain has a mean lifespan of 25 months. Mice with ages ranging from 3 weeks to 75 weeks old were used in the study.

Experimental protocol. After weaning at 21 days of age, mice were quartered eight per cage and fed isocaloric diets of 20% protein (control) and 4% protein (low protein) and water *ad libitum* (Table 1). Mice were killed 1–25 weeks after commencing the diet. Results for each point of time, described as the total number of weeks on diet, represent data obtained from eight to twelve individual mice. Ageing female mice 47 and 64 weeks old which had been reared on commercial lab chow were then fed the isocaloric 20% protein and 4% protein diets for 8 weeks.

Glass bead column

A glass bead column was used to aid separation of mucous and intestinal tissue debris from the intestinal lymphocytes suspension. The column consists of a 10 ml plastic syringe filled to the 2 ml mark with 3 mm diameter siliconized glass beads (SGB, Potters Industries, Inc., Hasbrouck Heights, N.J.) and packed to the 6 ml mark with 1 mm SGB. Finally, the column was filled with additional 3 mm diameter SGB to the 10 ml mark and washed twice with 10 ml of phosphate-buffered saline (PBS) before being sterilized and stored at room temperature until needed.

Table 1. AIN semipurified mouse diets*

Composition	High protein (%)	Low protein (%)
Casein high nitrogen	20·0	4·0
DL-Methionine	0·3	0·3
Cornstarch	15·0	15·0
Sucrose	50·0	66·0
Fibre-celufil	5·0	5·0
Corn oil	5·0	5·0
AIN mineral mix	3·5	3·5
AIN vitamin mix	1·0	1·0
Choline bitartrate	0·2	0·2

*U.S. Biochemical Corporation, Cleveland, Ohio. Diets Nos. 10662 (20% protein) and 17804-P (4% protein).

Isolation of intestinal mucosal lymphoid cells

The procedures for the isolation of intestinal mucosa lymphocytes were a modification of those described previously (Battandier *et al.*, 1978 and Rudzik & Bienestock, 1974). Mice were killed by cervical dislocation and intestine from each mouse was carefully removed and trimmed free of Peyer's patches. A segment of intestine severed at the stomach–duodenum junction and at the ileum-ascending colon junction was used for isolation of intestinal mucosal lymphocytes. The intestinal content was washed out using 1 ml of cold PBS (pH 7·2) and then centrifuged at 500 ***g*** using a Damon/IEC HN-511 centrifuge. Supernatant was collected for determination of IgA. Measurement of IgA was for the 1 ml wash which included the fluid in the intestine. The total recovered volume was routinely 0·8 ml in supernatant and 0·2–0·3 ml in solid sediment. The intestine was washed again with 10 ml of PBS (pH 7·2) containing 100 u./ml penicillin and 100 μg/ml streptomycin (PN-Strep) (Grand Island Biological Co. Grand Island, N.Y.). It was then cut into several fragments of approximately 7 cm long in a Petri dish containing 10 ml of cold RPMI-1640 medium supplemented with 5% foetal calf serum (FCS) and PN-Strep. Fragments of intestine were carefully cut open longitudinally to expose the inside and with a 10 ml syringe plunger, the mucosa cells were gently rubbed off until the intestine appeared transparent. The cell suspension was then pipetted into a disposable plastic tube (17 × 100 cm) (Falcon, Oxnard, Calif.) and vortexed for 15 sec before being allowed to sit for 30 min to let heavier tissue debris settle. The supernatant was carefully pipetted out into the glass bead column and the filtrate was centrifuged once at 220 ***g*** for 5 min and then at 500 ***g*** for 10 min. Finally the cells were resuspended in 4 ml of RPMI medium containing PN-Strep and 10 μg/ml of gentamycin (Schering Corp., Kenilworth, N.J.). The viability of the lymphocytes was determined by trypan blue exclusion and cells were adjusted to a concentration of $2{\cdot}4 \times 10^5$ lymphocytes/ml.

Enumeration of T and B lymphocytes

The number of theta and Ig positive lymphocytes isolated from mouse intestinal mucosa were determined by direct and indirect immunofluorescent staining (Moller, 1961). The B lymphocytes were stained directly using fluorescein-labelled rabbit anti-mouse Ig, whereas T lymphocytes were first bound by rabbit anti-mouse theta antibody and then by fluorescein-labelled Goat anti-rabbit Ig antibody (Cappel Laboratories, Cochranville, Pa). A total of 300 cells were counted from each of the duplicate samples.

Cytolytic assay

Antibody-dependent cell-mediated cytotoxicity assay (ADCC) using gut lymphocytes was performed according to a procedure described earlier for spleen lymphocytes (Haffer, Freeman & Watson, 1979). Briefly, using a 3:1 effector to target cells ratio, $8{\cdot}0 \times 10^4$ antibody-coated rabbit anti-SRBC (Cappel Laboratories, Cochranville, Pa) ^{51}Cr-labelled sheep red blood cells (SRBC) were added to $2{\cdot}4 \times 10^5$ intestinal mucosa lymphocytes in triplicate and incubated for 18 hr at 37° in 5% CO_2–95% air. The medium used in this assay was RPMI-1640 supplemented with 10% foetal calf serum, penicillin, streptomycin, and gentamicin. One millilitre of the supernatant was harvested from each tube and amount of chromium release was measured in an automatic gamma spectrometer. The percentage lysis was computed using the following formula

$$\% \text{ lysis} = \frac{\text{Test c.p.m.} - \text{spontaneous c.p.m.}}{\text{Total c.p.m.} - \text{spontaneous c.p.m.}} \times 100.$$

Intestinal secretory IgA

The concentration of IgA in supernatants collected from intestinal washing were measured using goat anti-mouse IgA (α chain specific) (Cappel Laboratories, Cochranville, Pa) in radial immunodiffusion assay (Mancini, Carbonara & Heremans, 1965). Duplicate wells were set up for each test sample and the diameter of the precipitation ring was measured using a Precision Viewer (Hyland Div. Travenol Lab. Inc., Costa Mesa, Calif.). Known concentrations of mouse IgA (Litton Bionetrics, Frederick, Md) were used as control for quantifiable measurement of the concentration of intestinal IgA.

Statistical analysis

The mean, standard error and paired *t* tests programmed on a Texas Instrument Model 58 calculator were used in analysis of the data.

RESULTS

Enumeration of T and B lymphocytes

Segment of intestine severed at the stomach–duodenum junction and at the ilem-acending colon junction was used for isolation of intestinal mucosal lymphocytes. Three-week-old mice have smaller intestinal weights and this was reflected in the total number of cells isolated from each intestine (Table 2). The ratio of T and B lymphocytes in the intestine of 3-week-old mice was approximately 1:1, whereas 8-, 18- and 25-week-old mice each had a T to B cells ratio of approximately 2:1.

Table 2. Percentage of T and B cells in intestinal lymphocytes isolated from mice of various ages

Age* (weeks)	Total cells, intestine ($\times 10^5$)	Intestinal Lymphocytes		Intestinal weight (g)
		B (%)	T (%)	
3	10·5±1·5†	46·5±1·5	43·0±3·0	0·66±0·25
8	14·0±1·0	29·8±1·5	60·7±2·0	0·84±0·04
18	18·5±1·0	35·8±1·0	54·0±2·2	1·35±0·08
25	20·0±2·3	37·0±1·6	61·7±1·8	1·38±0·12

*BALB/c mice reared on normal lab chow.
†Mean ±SE.

Effect of ageing on intestinal ADCC

The intestinal lymphocyte ADCC activity was measured in mice that were fed isocaloric normal protein (20%) diet beginning at 3 weeks of age (Fig. 1). The percentage of lysis by 3-week-old mice intestinal lymphocytes on antibody-coated SRBC was 7·71%. As the animals became mature, intestinal ADCC activity increased. By 17 weeks, the ADCC activity of intestinal lymphocytes reached its peak with 37·21% lysis. This level of activity remained relatively unchanged through 50 weeks of age among the animals assayed. However, by 75 weeks of age, intestinal ADCC activity had dropped drastically although its level of 15·66% lysis still was twice that of the immature 3-week-old mice.

Effect of ageing on intestinal IgA

Quantitative measurement of intestinal IgA was performed on mice ranging from 7 to 75 weeks of age (Fig. 1). Immature 6-week-old mice have an intestinal IgA

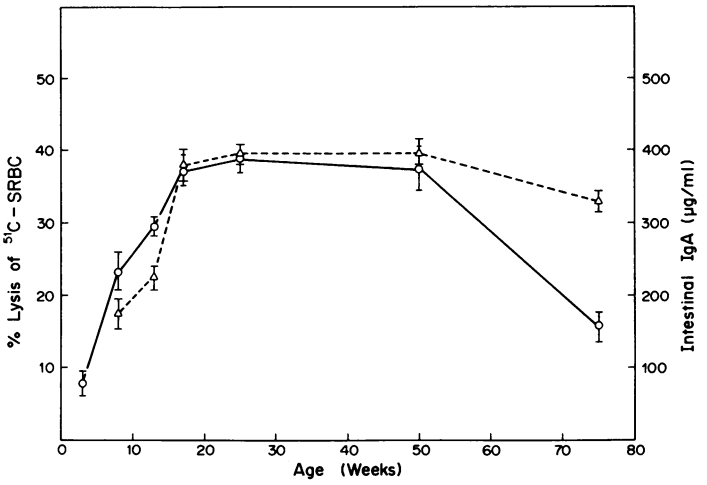

Figure 1. The effect of ageing on intestinal ADCC activity (o—o) and IgA concentration (▵—▵).

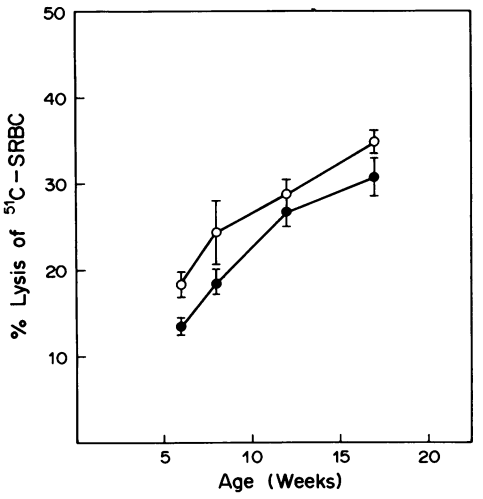

Figure 2. Effect of protein malnutrition on intestinal ADCC activity of immunologically immature BALB/c mice. (o—o) 20% protein diet, (●—●) 4% protein diet.

concentration of 174·00 μg/ml. Concentration of IgA increased as animals became mature. By 17 weeks of age, IgA in the intestine had reached a concentration of 380·8 μg/ml. This level of IgA remained fairly constant in mice from 17 weeks to 50 weeks old. Thereafter, there was a significant drop in IgA concentration from the mature level to 331·00 μg/ml in the 75-week-old mice ($P < 0.05$).

Effect of low protein diet on intestinal ADCC and IgA

Mice at 3 weeks old were placed on isocaloric normal (protein, 20%) or restricted (protein, 4%) diet. Subsequently, intestinal ADCC activity (Fig. 2) and IgA level (Fig. 3) were measured at 6, 8, 12 and 17 weeks of age. The ADCC activity in the normal protein diet group increased gradually from 18·36% lysis to 34·75% lysis by 17 weeks. The low protein diet group

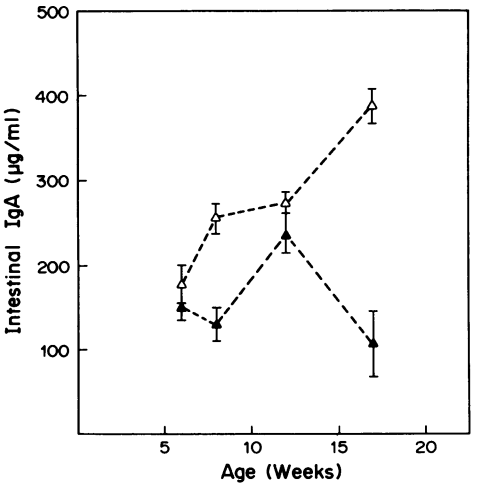

Figure 3. Effect of protein malnutrition on intestinal IgA concentration of immunologically immature BALB/c mice. (▵--▵) 20% protein diet, (▲--▲) 4% protein diet.

also showed gradual increase from 13·58% lysis to 30·73% lysis at 17 weeks. However, there were significant differences in the intestinal ADCC activities between the normal and low protein diet mice at 6, 8 and 17 weeks ($P < 0.05$). The difference between the two groups was greatest at 6 weeks ($P < 0.005$) whereas no difference was observed among the 2 groups at 12 weeks of age.

The levels of intestinal IgA in mice fed the control diet increased from 177·86 μg/ml at 6 weeks old to 387·0 μg/ml at 17 weeks. However, the 4% protein diet group had a slightly lower IgA concentration than controls at 6 weeks of age (Fig. 3). At 8 weeks old, IgA concentration had decreased to 130 μg/ml. This was followed by an increase to 237·0 μg/ml at 12 weeks but by 17 weeks the intestinal IgA level decreased again to 106 μg/ml. No difference in the level of IgA between the two groups at 6 weeks but significant differences were observed at 8 ($P < 0.05$), 12 ($P < 0.05$) and 17 weeks ($P < 0.005$).

The results of the low protein diet on ADCC and IgA level of 55- and 70-week-old mice are presented in Table 3. Mice were given low protein diet 8 weeks before death for ADCC and IgA assays. No significant difference in ADCC and IgA level was found between the normal controls and the low protein diet mice at 55 or 75 weeks old.

Table 3. Effect of low protein diet on intestinal IgA secretion and ADCC activity of intestinal lymphocytes in aged mice

Age* (weeks)	ADCC (% lysis ^{51}Cr-SRBC)		IgA (μg/ml)	
	Control	Low protein	Control	Low protein
55	40·0±5·4†	39·5±2·4	384±16	368±40
70	20·5±4·8	25·5±3·6	313±21	335±29

*Female mice grown for 47 and 62 weeks respectively, then fed normal (20%) or low (4%) protein diet for 8 weeks.
†Mean ±SE.

DISCUSSION

In this study, we have demonstrated an age-associated decline in CMI in the intestine using an *in vitro* ^{51}Cr release assay (ADCC). The results from this assay show a 60% decline from the maximum adult value by the time the animals were 75 weeks old. The decline in intestinal ADCC activity was not associated with T–B cells ratio. However, changes in T-cell functions measured by PHA responsiveness correlated with spleen cells' ADCC activity and both have been shown to decline with age in various strains of mice (Menon, Jaroslow & Koesterer, 1974; Becker, Roubinian, Feldman, Blackman, Klajman & Talal, 1979; Haffer *et al.*, 1979). Previous studies in our laboratory using BALB/cJ spleen cells showed an 11% decline in ADCC activity between mice 45 weeks old and 125 weeks old (Haffer *et al.*, 1979). Thus there appears to be different rates of decline in intestinal ADCC activity and spleen ADCC activity due to ageing with a greater decline in the intestinal ADCC than splenic ADCC. The incidence of cancer of the gastrointestinal tract has been shown to occur more frequently in older people (Neurdenberg, 1955; Segi, 1955; Haenszel, 1958; Geboes & Bossaert, 1977). Although genetic defects (Dorn & Cutler, 1956; Strudwick, Ewing & White, 1964) and dietary factors (Dungal, 1955; Quisenberry, 1955; Segi, 1955) have been considered as some of the possible causes, the decline in intestinal ADCC activity with age as shown in this study could be a factor in the increased incidence or severity of gastrointestinal neoplasia.

Intestinal IgA concentration plateaued and declined at the same time as that of intestinal ADCC activity. The effect of ageing on intestinal IgA response was less severe than the intestinal ADCC activity as there was only a 17% decline in IgA concentration. The statistically significant ($P < 0.05$) drop in intestinal IgA level in 75-week-old mice indicates the beginning of the decline in host gut defence capacities due to ageing. The intestinal IgA levels and the ADCC activity of the moderately protein malnourished young animals were less than age-matched controls by 6 weeks on the diet. However, the ageing animals on the low protein diets for 8 weeks had similar ADCC and intestinal IgA levels to age-matched controls. This suggests that their immune responses are more resistant to suppression or growth retardation than young animals which is in agreement with previous studies of other aspects of cellular immune function (Watson & Haffer, 1980, Watson & McMurray, 1979).

Severe protein malnutrition has been known to affect humoral (Jose, Stutman & Good, 1973; Reinhardt & Steward, 1979) and cellular immunity (Chandra, 1972; Edelman, Suskind, Olson & Sirisinha, 1973; Watson & McMurray, 1979) of the protein-deprived host. However, certain parameters of CMI such as resistance to certain tumours have been shown to be enhanced following mild/moderate

protein restriction in the diet (Ross & Bras, 1971, 1973; Bell & Hazell, 1975; Haffer *et al.*, 1979). In our study, we have demonstrated a lower level of intestinal ADCC activity in the protein deprived mice. Although the intestinal ADCC activity showed continuous increase with age, it was consistently lower than that of the normal control mice. Studies using 4% protein diets have shown retarded development of CMI responses which eventually (15–20 weeks) reached the levels of aged-matched controls (Haffer *et al.*, 1979; Watson & Safranski, 1981). Our results using intestinal lymphocytes to measure ADCC agree with those of Haffer *et al.* (1979). They found a suppression of ADCC mediated by splenic lymphocytes which did not return to control values after prolonged exposure of the mice to low protein diet whereas PHA mitogenesis and mixed lymphocyte functions did show a diet-caused delayed maturation. Nevertheless, our results also support those of Jose *et al.* (1973) who found a decrease in ADCC activity against mastocytoma in animals fed low protein diets at weaning.

The effect of moderate protein deprivation was greater on intestinal IgA where the level of IgA was increasingly suppressed with the increase duration of protein restriction in the diet. McMurray, Rey, Casazza & Watson (1977) in their study on severely malnourished Colombian children found great reduction in sIgA concentration in the tears of these children. Guinea-pigs fed 8% protein diet also showed significantly lower sIgA concentration in tears and vaginal secretions (Watson, Horton & Clinton, 1977). However, the suppression of IgA production in protein malnutrition is not permanent since renourishment of the subjects can restore the IgA level back to normal (Watson & McMurray, 1979). Nevertheless, the decrease in IgA concentration in the intestine following protein restricted diet is likely to increase the susceptibility of the host to infection.

Clearly ageing and protein malnutrition exert their effect differently on the immune responses of the gut. While ageing has greater effect on the intestinal ADCC activity, it has less effect on the level of IgA in the intestine. Conversely, low protein diets were shown to have greater effect on intestinal IgA concentration but lesser effect on ADCC. Furthermore ADCC in the gut was shown for the first time to be susceptible to nutritional stress. Previously this had been shown to occur only using spleen cells in the older mice and only after long-term dietary restriction (Watson & Haffer, 1980). This study also indicates that a short-term protein restriction may alter cellular and secretory immune functions in the intestinal tract of immunologically-immature but not mature mice. The mechanisms of suppression are still under investigation, but may be caused by generation of suppressor cell types similar to those which occur in the normal ageing of BALB/c mice. This study, using a secretory immune function, also confirms the work summarized by Weindruch, Kristie, Cheney & Walford (1979) and Watson & Safranski (1980) who showed that long-term, low protein diets result in early suppression of some aspects of cellular immunity. Additional studies are underway to define further the effect of ageing and diets on gut immunity and how dietary intake can influence maturation of the immune responses in the young and ageing host.

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