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Serum Proteins and Lymphoid Tissues in Germ-Free Mice Fed a Chemically Defined, Water Soluble, Low Molecular Weight Diet

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Summary. Germ-free CFW mice reared on practical type steam sterilized solid diets showed lower than conventional levels of all serum immunoglobulins. Reared on water soluble, low molecular weight, chemically defined diets which had been sterilized by filtration, young adult germ-free mice demonstrated almost total absence of immune globulins. However, in older animals reared on this diet, a gradual increase with age, notably of IgG, became apparent.

Total white blood cell count and differential counts were only slightly and not significantly lower in germ-free mice fed solid diet than in their conventional controls. Germ-free mice fed the water soluble diet, on the other hand, showed substantially lower white cell counts than the animals reared on solid diet.

It is concluded that steam sterilized solid diet constitutes a major source of uncontrolled exogenous stimulation of the lymphoid tissues.

INTRODUCTION

In order to conduct well defined experiments on the origin and production of γ-globulins and specific antibodies, all uncontrolled exogenous antigenic and/or synergistic stimulation should be avoided. Data obtained with germ-free animals demonstrate that, in the absence of a viable microflora, diet may be a major source of antigenic stimulation (Wostmann, 1961; Michael, Whitley and Landy, 1962; Sell, 1964; Wostmann, Pleasants and Bealmear, 1968). Most sterilized solid diets not only contain macromolecular substances of uncertain antigenicity, like casein (Sell, 1964), but often include ingredients which contain large numbers of non-viable microbial forms (Wostmann *et al.*, 1968). Steam sterilization will tend, via Schiff's base formation and other condensation reactions, to increase size and antigenicity of the macro-molecular substances.

Chemically defined water soluble, low molecular weight diets of potentially low antigenicity for rats and mice were first described by Greenstein, Birnbaum, Winitz and Otey (1957). These formulations offer the advantage that bacteria can be removed by Millipore filtration without altering the chemical composition of the diet. At the Lobund Laboratory these diets have been adjusted to the needs of germ-free rats and mice (Pleasants, Reddy and Wostmann, 1968). Five successive generations of germ-free CFW mice were produced

before the small colony became accidentally contaminated, indicating the qualitative nutritional adequacy of the diet. Germ-free mice of the above colony have now been investigated in terms of immunological parameters and compared to germ-free and conventional mice fed natural type solid diet.

MATERIALS AND METHODS

The mice originated from the closely bred Lobund germ-free mouse colony of CFW (Carworth Farm Webster) origin, and from the genetically closely related conventional colony. One group of germ-free mice had been maintained in Trexler-type plastic isolators on the water soluble, low molecular weight and chemically defined diet L 479 E9 (H_2O-diet) described by Pleasants *et al.* (1968). Animals in this group were first, second, third and fourth generation animals. First generation animals were taken from the Lobund germ-free colony at 14 days of age and provided only H_2O-diet. Their mothers were placed with them 1 hour out of every 3 hours until the young were 21 days old. Thus the young mice could adjust gradually from mother's milk to the liquid diet without having access to the solid diet consumed by the mother. Thereafter the animals were housed singly except during mating. Housing consisted of stainless steel wire cages with sloping sides to facilitate access of young mice to overhead diet bottles (Wostmann *et al.*, 1967). Bedding was available only during late gestation and lactation when filter paper nesting material was provided. The other group of germ-free mice was derived directly from the germ-free production colony and reared on practical type solid diet L 485 (Kellogg and Wostmann, 1967). They were housed five to a glass jar on wood shavings, in plastic isolators. Conventional mice reared on diet L 485 were housed similarly but kept in the open animal room. Food and water were available at all times.

Blood samples were drawn from the ophthalmic plexus. Total leucocyte and differential counts were made according to routine procedures (Bealmear and Wilson, 1967). Cellulose acetate electrophoresis was performed on 3× 15 cm strips using a sodium barbital buffer, pH 8·6 (ionic strength 0·06). The strips were stained with amido black and read and integrated with a Chromoscan reflectance densitometer (Joyce, Loebl and Co. Inc., Burlington, Massachusetts). Total protein concentration was determined with a serum protein refractometer. Immunoelectrophoresis was performed on 2·5× 7·5 cm glass slides. Sodium barbital buffer, pH 8·2 (ionic strength 0·1) was used, with Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois) made up to 0·75 per cent in the buffer as a supporting medium. Tissues were fixed in 10 per cent neutral buffered formalin, dehydrated in dioxane, embedded in paraffin, sectioned at 6 μ on a rotary microtome, and stained with haematoxylin and eosin. Examination was by light microscopy at 100×, 450× and 1000× magnification.

RESULTS

At 65–70 days of age the serum of young adult germ-free mice reared on solid diet contained normal amounts of the electrophoretically discernible protein fractions, with the exception of γ-globulin which occurs at a much lower concentration (Table 1). Germ-free mice reared on H_2O-diet showed a similar pattern with normal levels of total protein and albumin, but γ-globulins were mostly below detectable range. Body weights of the mice fed H_2O-diet were approximately 20 per cent lower than those of the mice fed solid diet

TABLE 1

SERUM PROTEINS AND LEUCOCYTES OF 70-DAY-OLD GERM-FREE CFW MICE REARED ON WATER-SOLUBLE AND PRACTICAL TYPE SOLID DIET

Diet		Status	Serum proteins					WBC × 10^3/mm^3		
			Albumin	α*	β	γ	Total	Total	Agran.‡	Gran.§
L 479 E9 (H_2O soluble)	Series I	GF (4 ♂)	2·60 ±0·11	2·57 ±0·15	0·71 ±0·05	0·02 ±0·02	5·9±0·3	4·6±0·7	3·1±0·4	1·5±0·4
	Series II	GF (4 ♂)	2·97 ±0·13	2·32 ±0·08	0·76 ±0·01	0·08 ±0·03	6·2±0·1	2·2±0·2	1·8±0·2	0·4±0·1
L 485		GF (10 ♂)	3·10 ±0·10	1·67 ±0·05	0·90 ±0·06	0·25 ±0·04	6·0±0·1	8·4±0·7	6·0±0·5	2·4±0·5
		Conv (20 ♂)†	2·93 ±0·11	1·55 ±0·14	0·98 ±0·10	0·58 ±0·09	6·0±0·1	9·9±0·1	7·5±0·2	2·4±0·2

* Range between albumin peak and β-globulin fraction marked by transferrin.
† Data on serum protein fractions from six animals only.
‡ Agranulocytes include all mononuclear cells without cytoplasmic granules (e.g. lymphocytes and monocytes).
§ Granulocytes include polymorphonuclear leucocytes or neutrophils, eosinophils and basophils.

(22 *versus* 27 g). The slight retardation in growth is presumably indicative of the failure to ingest sufficient calories directly after weaning when the transition to the low molecular weight formula must be made while caloric need per unit body weight is at its maximum.

Immunoelectrophoresis and further immunological quantitation of the serum of germ-free mice reared on solid diet has generally indicated that all globulins usually regarded as

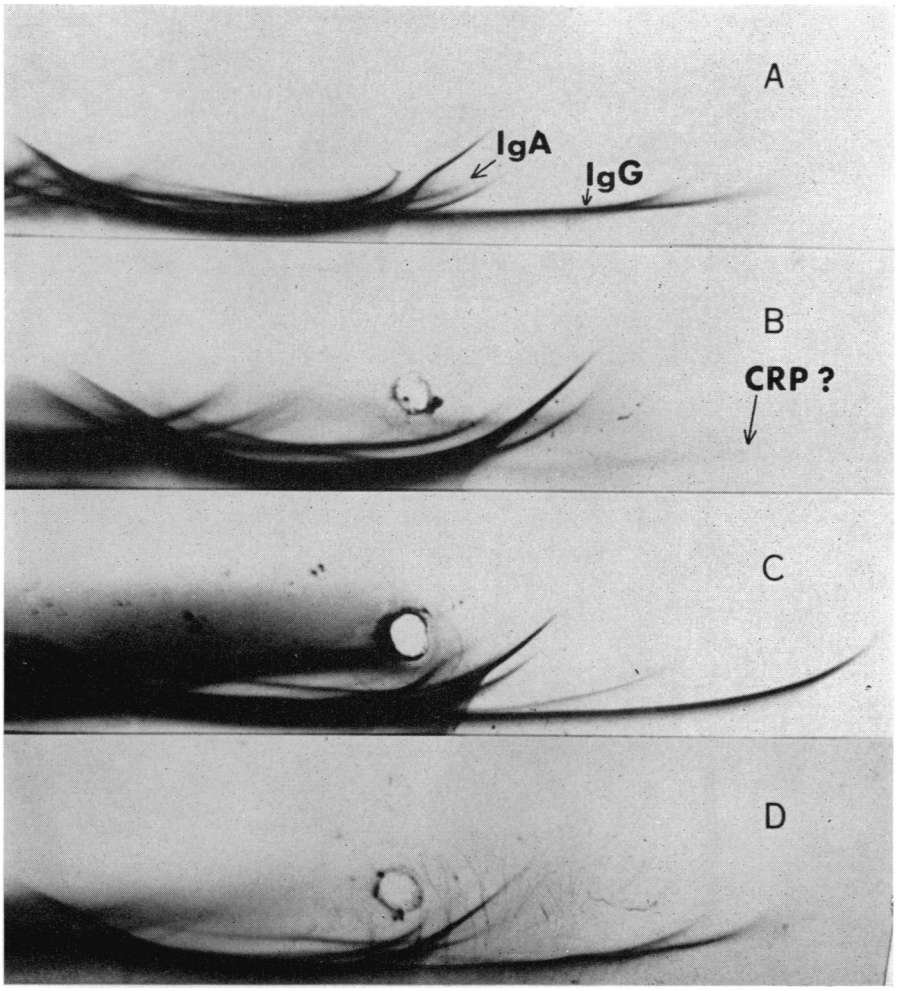

FIG. 1. Cathodal range of mouse serum immunoelectrophoresis patterns. (a) Germ-free, 70-day-old, solid diet; (b) germ-free, 70-day-old, water soluble diet; (c) conventional, 70-day-old, solid diet; (d) germ-free, 330-day-old, water soluble diet. For details see text.

immune globulins are present, although in reduced amounts (Arnason, Salomon and Grabar, 1964; Fahey and Sell, 1965). Fig. 1 showed representative immunoelectrophoresis patterns of germ-free and conventional mice reared on solid diet L 485 and of germ-free mice reared on H_2O-diet L 479 E9. The pattern obtained with serum of 65-day-old germ-free mouse No. 4 reared on solid diet appears to confirm the earlier observations. The IgG fraction shows clearly, and IgA is also indicated. A typical IgM line is usually absent from

the pattern. Serum from mouse No. 242 of comparable age reared on H_2O-diet, however, lacks a sharply delineated IgG fraction although the presence of some IgA is suggested. In the absence of a demonstrable IgG line, two faint arcs become visible. The faster moving one may well be a vestige of IgG_2, while the slower globulin fraction appears in the position where Hurlemann, Thorbecke and Hochwald (1966) indicate C-reactive protein (CRP). Conventional mouse serum demonstrates the usual spectrum of immune globulins.

DISCUSSION

The present findings demonstrate that antigenicity of dietary origin can be a major source of uncontrolled exogenous stimulation. This had already been suggested by the effect of bovine milk proteins in the diets for germ-free rabbits and guinea-pigs on serum γ-globulin levels (Wostmann, 1961; Sell, 1964), and appears to be further confirmed by the WBC counts in Table 1. Here the character of the diet rather than the microbial status appears to be the determining factor. The rather insignificant difference in total and differential count between germ-free and conventional CFW mice reared on solid, practical type diet confirms earlier observations (Bealmear and Wilson, 1967; Boggs, Chervenick, Marsh, Pilgrim, Cartwright and Wintrobe, 1967). A substantial reduction was achieved, however, by rearing the animals on a chemically defined low molecular weight diet which was sterilized by filtration instead of by heat or radiation.

The character of the diet did not appear to affect the histological appearance of the lymph nodes and spleen. Lymph nodes were characteristic for the germ-free state (Pollard, 1966), with dense nodules predominating and an occasional inactive centre visible in the nodule. Some plasma cells were present in the inactive centres but few, if any, mitotic figures were in evidence. The intermediate and medullary zones were sparsely populated with plasma cells and reticulum cells. There were large areas of white pulp in all the spleens examined, but no active centres, a few plasma cells, and few mitotic figures. Suprisingly the livers of germ-free mice reared on H_2O-diet demonstrated a more than two-fold increase in Kupffer cells when compared to animals fed the solid diet.

Eventually even in the case of germ-free mice reared on these relatively antigen free diets a major build up of γ-globulins occurs. One 8-month-old animal demonstrated a well defined IgG_1 line, and at 11 months of age all mice tested showed the full qualitative spectrum of mouse immunoglobulins with the possible exception of IgM (Fig. 1).

The reduction of dietary antigenicity achieved in the H_2O-diets appears to result in minimal exogenous stimulation as depicted by the low WBC values, and by the very low concentrations of IgG in young adult mice. However, a gradual build up of immune globulins with age still occurs. Residual dietary antigenicity caused by chemical impurities in the diet would constitute one possible source of stimulation. Steps had already been taken to reduce the antigenicity of the earlier water-soluble diets. In particular Tween 80 had been removed from the diet after it was found to cause diarrhoea and enlargement of certain lymph nodes (Wostmann *et al.*, 1967). The amino acids of the diet received additional charcoal purification, and were all United States National Research Council grade. Since the completion of the above studies, we have introduced the PM-10 Diaflo Ultrafiltration System (Amicon Corp., Lexington, Massachusetts) which claims a 10,000 molecular weight cut off. Residual antigenic stimulation could also result from animal to animal contact during mating periods, or from local tissue degeneration caused by constant exposure to the toxic factors occurring in the cecum of the germ-free rodent (Gordon,

1967). Efforts are underway to reduce further the antigenicity of diet and environment, and to elucidate the origin and character of the γ-globulins which gradually appear in the serum of older germ-free mice fed chemically defined, low molecular weight water soluble diet.

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