MINIREVIEW

The Germ-Free Animal Fed Chemically Defined Diet: A Unique Tool (43286D)

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The original low molecular weight, water-soluble, chemically defined diets (CD diets) for germ-free (GF) rodents were developed because of the difficulty of hand rearing cesarian-derived GF rats, mice, gerbils, and hamsters from birth on artificial formulas containing enough cow's milk protein to ensure adequate growth (1). In 1957, Greenstein *et al.* (2), reported the development of a water-soluble, chemically defined diet for conventional (CV) rats, i.e., rats having a wide range of microbial associates. Consultation with the authors led to our adoption of soluble formulas in which amino acids are substituted for the apparently difficult-to-digest sterilized milk protein. These diets, based on glucose, amino acids, vitamins, and minerals, could be sterilized by filtration without the chemical changes caused by other modes of sterilization. They needed only a supplement of defined fats and fat-soluble vitamins, which could be filter-sterilized and fed separately.

Our studies were thus started because of practical necessity, especially for the independent derivation of GF mouse strains (which might prevent the vertical transmission of virus apparently indigenous to most strains of mice). It was also clear, however, that hand-feeding defined diets could serve to refine our knowledge of nutritional requirements at every stage of the life cycle, and could give further insight into the role played by maternal milk in physiological and immunological maturation.

After considerable adjustment of the original Greenstein formula to meet both infant and GF conditions, it proved possible to hand rear GF rats on CD diet from the first day of life, but only if they had nursed GF rat mothers for at least a few hours after birth (3). GF mice required days of maternal suckling before they could be successfully hand reared on CD diet. For infant rodents, the predigested character of the diet was found to cause more problems than it solved. Its low molecular weight components forced an unpromising choice between high osmolarity and high dilution of the mixture (3).

The hand-feeding studies, had, however, contributed to a rapid adaptation of the Greenstein formula to GF rodents, since infant animals respond quickly to dietary inadequacies. Meanwhile, developments in the biomedical sciences made it increasingly clear that colony-reared (mother-suckled) GF rodents weaned to CD diets could contribute much-needed definition to studies in nutrition, metabolism, physiology, and, especially, immunology. While GF animals fed natural ingredient (NI) diets had lower than CV levels of IgG and IgA globulins (4), IgG had never reached the low levels anticipated early on. The possibility that the GF-CD mouse could make basic contributions to immunology became evident after it was found that ultrafiltration of the CD diet to remove components with a mol wt above 10,000 virtually eliminated antigenic stimulation. Despite the animals' early ingestion of high molecular weight maternal milk, their immune systems showed very little activation (5). This opened the way for both basic and applied immunology based on a truly primary immune response.

Therefore, research with GF-CD diets concentrated on development of chemically defined, water-soluble, antigen-free diets that could sustain GF rodents through repeated litters and generations. This would prove the adequacy of the diet to produce a healthy, well-defined experimental animal never exposed to anything undefined except its mother's milk. Because of the high cost of the diet, most of this development was carried out

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with mice. A few experiments with GF-CD rats indicated that they would respond similarly to mice.

The Germ-Free State

In 1885, Pasteur (6) suggested sterilizing the shell of a fertile egg and placing it in a sterilized incubator with sterilized food and water to hatch, although he admitted to the preconceived belief that life under such conditions would be impossible. But by 1895, the rearing of guinea pigs germ-free for several weeks proved that GF life was indeed possible (7). However, those first GF mammals already showed an abnormal enlargement of the cecum, the major site of microbial fermentation in rodents.

The GF animal provides the ultimate control for testing the effect of microbial associates, particularly their positive contributions, or long-term degenerative effects. In the 1930s, Glimstedt (8) demonstrated a significantly reduced lymphoid system in the GF guinea pig. More surprising was the discovery that the GF state affected morphology, not only of the normally microbe-associated gut, but also of the cardiovascular system (9).

Obviously, GF and CV rats attain different states of homeostasis. These effects have been most extensively studied in the Lobund Wistar rat fed NI diets. Young and young adult GF rats grow at the same rate as their CV counterparts, but do so with less food intake (10), a lower cardiac output and O_2 use (9), and relatively smaller hearts and lungs (10). But in rats cecectomized at an early age, cardiac output and resting O_2 consumption are near normal (11). It has been established that beta-blocking substances originating in the enlarged GF cecum interfere with catecholamine action (12). This activity would block at least part of the norepinephrine-dependent, energy-dissipating thermogenesis of the brown adipose tissue and thereby reduce thermogenesis and related O_2 uptake. It has been established that the core temperature of the GF rat is approximately 0.3°C lower than that of its CV counterpart (13, 14).

As the GF rat grows, there is a decrease in cecal size as a percentage of body weight, from more than 10% at 1 month of age to 3–4% at 6 months. This would reduce beta-blocker activity, and make utilization of dietary energy less effective and more comparable to that of its CV counterpart (15). In the meantime, however, the early lower energy and oxygen demands have resulted in smaller hearts and lungs, a situation that perpetuates itself during the life span of the animal (16), and may be related to its somewhat smaller adult body weight (17).

Substantial differences also exist in gut physiology, such as a lower renewal rate of the intestinal mucosa (18), higher concentrations of pancreatic enzymes and bile acids (19, 20), and different absorption characteristics of various materials (21). Regional blood flow to the liver and the activity of a number of rate-controlling hepatic enzymes suggest a reduced metabolic activity of that organ (10). However, none of these anomalies appears to affect the major functioning of the GF rat and mouse. Reproduction is normal (22) and life span is somewhat extended (16, 23). The endocrine picture, as far as studied, does not show substantial deviations from the CV pattern. Serum thyroid hormone levels tend to be somewhat higher than CV, in apparent divergence from the lower metabolic activity of the GF rat and mouse mentioned earlier, pointing again to the different homeostatic conditions in these animals (10, 24). Serum lipid levels of GF rats were found to be somewhat lower than CV (25). Notwithstanding these differences, function and metabolism of the GF rat and mouse appear sufficiently close to those of the CV rat and mouse to allow translation of most of the experimental results into the "conventional" world.

Development of the Chemically Defined Diet

Germ-free status itself dictated the early changes from the formulation of Greenstein *et al.* (2). Hemorrhages in the first GF-CD rats confirmed what had been found earlier in GF rats fed semi-synthetic diets: that GF rats utilize menadione (vitamin K_3) very poorly as compared with CV rats (26). Menadione had to be replaced by a natural K factor, phylloquinone (vitamin K_1). The synthetic emulsifier, Tween 80, used by Greenstein *et al.* (27) to emulsify the lipids into their one-solution diet caused more severe diarrhea in our GF animals than had occurred in their CV mice. Popliteal lymph nodes hypertrophied, and IgG levels rose above CV levels (28). Others later reported the antigenicity (29) and hepatotoxicity (30) of Tween 80. Thus, the lipids had to be filtered and fed separately from the water solution of the nutrients (31).

Greenstein *et al.* (2) also used the synthetic compound cysteine ethyl ester in place of the poorly soluble cysteine. In our study, this ethyl ester changed the odor, taste, and color of the CD diet and was therefore deleted, since animals can convert methionine to cysteine. Later, Levenson *et al.* (32) showed that cysteine ethyl ester in a Greenstein-type diet caused pancreatic acinar cell atrophy to a small extent in GF rats and to a much greater extent in CV rats. In CV rats, it also caused hemolytic anemia (32). The above indicates the important role of the animal's versatile microflora in determining its response to unnatural compounds.

In the same time frame of this development, the trace minerals selenium, chromium, vanadium, fluorine, tin, and nickel were reported in the literature to be essential. These were added to the diet in the recommended amounts. When linolenate was reported to be also an essential fatty acid, our purified lipid mix modeled on corn oil was replaced by one modeled on

soy oil (Table I; 31). A syndrome of sudden death, with some soft tissue calcification indicated a higher need for Mg than is usually recommended for CV animals (28). This may be connected to calcifications seen in GF C3H mice fed semi-purified diets (33) and to mineral imbalances found in GF guinea pigs and rabbits (34, 35).

Because mother mice needed nesting material for the successful nursing of their young, filter paper strips had to be provided, albeit reluctantly, by our group and by Hashimoto *et al.* (36). We found that GF-CD mice consumed this material to the extent that it comprised 8% of their ingested solids. This addition to the diet reduced cecal enlargement and the incidence of cecal volvulus and trichobezoars (37).

The levels of total and individual amino acids were modified according to literature reports of more effective patterns. The best and final pattern, however, was arrived at by comparing the plasma amino acid pattern of GF-CD diet mice with that of CV mice fed NI diet L-485 (for diet L-485, see Ref. 38). Changes in the dietary amino acids were made until the patterns matched (37). The resulting formulation supported reproduction of CFW mice into the fifth generation, indicating that it contained all nutrients essential for the survival of the species. It thereby met Schultze's (39) 1952 stipulation for proving nutritional adequacy: a defined diet fed to a germ-free animal.

When heart size and resting oxygen consumption were determined in GF-CD mice, values were approximately 30% over those found in GF-NI mice, and even 10% over those in CV-NI mice (40). This contrasted with the situation in both GF rats and GF mice fed NI diets, which showed substantially lower oxygen consumption than their CV counterparts (9, 41). This suggests that GF mice fed CD diet require additional energy to effectively utilize the diet's major dietary components. Other investigators feeding rats amino acid-containing diets have obtained similar results (42, 43).

These CD diets appeared to have little antigenicity for our CFW mouse strain. Immunoelectrophoresis of serum of 70-day-old GF-CD mice showed a virtual absence of IgG antibody. Their white blood cell counts, for both granulocytes and mononuclear cells, were 20–50% of those in GF mice fed NI diet L-485. These GF-NI values were only slightly lower than those of CV CFW mice of the same age maintained on diet L-485. The latter diet contains no casein, which had been found a major source of non-viable, but potentially antigenic microbial forms (44). However, after 1 year on CD diet, GF CFW mice showed appreciable amounts of IgG (44, 45). It should be noted, however, that our GF CFW strain, though having been maintained as a closed colony for 15 years, was not an inbred strain.

Accordingly, later studies were carried out with our inbred C3H/HeCr strain. In addition, sterilization procedures were changed to include prefiltration through a Diaflo membrane, which eliminated all material over 10 kDa. This procedure, together with the elimination of possible animal-to-animal antigenicity, now produced IgA and IgG levels undetectable by immunoelectrophoresis, even in 14-month-old GF-CD C3H mice. It decreased the already low white blood cell counts

Table I. Chemically Defined Diet L-489E14SE per 100 g of Water-Soluble Solids in 300 ml of Ultrapure H_2O[a]

L-Leucine	1.90 g	Ca glycerophosphate	5.22 g
L-Phenylalanine	0.74 g	$CaCl_2 \cdot 2H_2O$	0.185 g
L-Isoleucine	1.08 g	Mg glycerophosphate	1.43 g
L-Methionine	1.06 g	K acetate	1.85 g
L-Tryptophan	0.37 g	NaCl	86.00 mg
L-Valine	1.23 g	$Mn(acetate) \cdot 4H_2O$	55.40 mg
Glycine	0.30 g	Ferrous gluconate	50.00 mg
L-Proline	1.48 g	$ZnSO_4 \cdot H_2O$	40.60 mg
L-Serine	1.33 g	$Cu(acetate)_2 \cdot H_2O$	3.70 mg
L-Asparagine	1.03 g	$Cr(acetate)_3 \cdot H_2O$	2.50 mg
L-Arginine·HCl	0.81 g	NaF	2.10 mg
L-Threonine	0.74 g	KI	0.68 mg
L-Lysine·HCl	1.77 g	$NiCl_2 \cdot 3H_2O$	0.37 mg
L-Histidine·HCl·H_2O	0.74 g	$SnSO_4 \cdot 2H_2O$	0.37 mg
L-Alanine	0.59 g	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.37 mg
Na L-Glutamate	3.40 g	Na_3VO_4	0.22 mg
Ethyl L-Tyrosinate·HCl	0.62 g	$Co(acetate)_2 \cdot 4H_2O$	0.11 mg
α-D-Dextrose	71.40 g	Na_2SeO_3	0.096 mg

[a] Vitamin B mix (0.09 g) containing (in milligrams): thiamine·HCl (1.23), pyridoxine·HCl (1.54), biotin (0.25), folic acid (0.37), vitamin B_{12} (pure; 1.44), riboflavin (1.85), niacinamide (9.2), i-inositol (61.6), Ca pantothenate (12.3), choline·HCl (310). Amounts of lipid nutrients in one measured daily adult dose of 0.25 ml: 0.22 g of purified soy triglycerides: 4.3 μg (7.8 IU) of retinyl palmitate; 0.0192 μg (0.77 IU) of cholecalciferol; 2.2 mg of 2-ambo-α-tocopherol; 4.4 mg of 2-ambo-α-tocopheryl acetate; and 48.0 μg of phylloquinone. The fatty acid content is 12% palmitate, 2% stearate, 24% oleate, 54% linoleate, and 8% linolenate.

even further. Only IgM values remained comparable to those of solid diet-fed GF and CV C3H mice (44).

Hashimoto *et al.* (36) fed a similar CD diet formulation to GF ICR mice and used antisera specific for the different Ig isotypes to quantitate their levels in 5- to 6-week-old animals. IgM levels were only slightly decreased in the absence of antigenic exposure. IgA was not detected in either the serum or intestinal wall of any GF mice, whatever their diet; it was detected only in mice with a viable microflora. The serum IgG level of GF-CD mice was one tenth that of GF-NI mice and one hundredth that of CV-NI mice. The authors concluded that IgG levels were sensitive to both dietary and microbial antigens.

However, after immunization using sheep red blood cells, GF-CD C3H mice at the Lobund Laboratory showed an immune potential as least as good as that of GF-NI or CV-NI mice. This was expressed in their postinoculation levels of anti-SRBC plaque-forming cells, serum IgG, and hemagglutinin (44). Following *in vitro* challenge with phytohemagglutinin and concanavalin A, the spleen cell response of GF-CD mice was at least equal to, and possibly slightly above, that of GF and CV mice fed NI diet (46). In the nonchallenged state, GF-CD spleens showed an amount of IgM-containing cells equal to that in the CV-NI spleen, but showed only 20% as many IgG-containing cells, and no IgA-containing cells.

At this point in time, we concluded that the CD diet L-489E14SE was at least qualitatively adequate for GF C3H mice. Body weights and appearances were satisfactory, and the diet supported reproduction into the fifth generation, although reproduction dropped sharply after the first two litters in any generation (37). Enough animals could now be produced to meet the needs of more sophisticated immunological studies. To this end, we entered into collaboration with the Department of Cell Biology, Immunology, and Genetics of Erasmus University, Rotterdam, the Netherlands.

Immunological Evaluation of GF-CD C3H Mice

Study of the "spontaneously" occurring ("background") Ig synthesis in spleen and bone marrow revealed roughly equal numbers of IgM-secreting cells in GF-CD and CV-NI mice. The number of IgG-secreting cells in the spleen was again comparable in both groups, but was found to be lower in the bone marrow of the GF-CD mice. IgA-secreting cells were drastically decreased. The antibody-specificity repertoire of the background IgM-secreting cells, when tested against five differently haptenized sheep red blood cells, was found to be comparable between the two animal groups, suggesting that the generation of the IgM antibody repertoire was independent of external antigenic stimulation (47).

In a follow-up study, the isotype expression and the specificity repertoire of lipopolysaccharide-reactive B cells were studied. Lipopolysaccharide-activated cultures of spleen and bone marrow cells of GF-CD C3H mice produced similar, or possibly slightly higher, numbers of IgM and IgG_1 producing clones than the cells of their CV counterparts fed NI diet L-485. Again, the specificity repertoire of the stimulated cells appeared not to be influenced by prior antigenic exposure of the intact animals (48). The study also suggested that the capability of IgM-secreting cells to switch to secretion of IgG_1 was not affected by prior antigen exposure (49).

Present Methodology: The Production of GF-CD BALB/c Mice

The continued emphasis on immunological studies then led us to change to BALB/c mice (BALB/cAnN) obtained from the University of Wisconsin because of their special value for immunological studies and for the production of monoclonal antibodies (50). Surprisingly, these mice, maintained on the latest CD formula (L-489E14SE) with ash-free filter-paper bedding, reproduced through nine generations, and through eight litters in one generation, far better than the C3H mice. After an initially somewhat slower growth, their body weights increased more rapidly, so that at 8 weeks of age, body weights compared well with GF and CV solid diet-fed controls. No overt signs of nutritional deficiency were seen in female mice sacrificed for experiment at 18 months of age (31). Determination of "background" Ig-secreting cells and the IgM antibody repertoire confirmed the earlier results obtained with C3H mice (51). Therefore, with CD diet L-489WS14SE (Table I), the major development phase of the CD diets came to an end.

The mice are housed in vinyl Trexler-type isolators initially sterilized with a spray of 2% peracetic acid. The polycarbonate shoe box-type mouse cages (Fig. 1) have lids locally modified to hold the inverted diet and water bottles. The cages' plastic bottoms have been replaced with stainless steel wire mesh false bottoms above removable drip pans.

The water-soluble portion of the diet used since 1981 is shown in Table I. The important practical details about the order and temperature of addition can be found in Reference 31. This portion of the diet is ultrafiltered through an Amicon PM10 membrane in an Amicon TC3E thin-channel filter holder. The solution may be kept at 5°C for 6 weeks, and at room temperature for 1 to 2 weeks. Although the ultrafiltered diet is already free of bacteria, molds, viruses, and molecules larger than 10 kDa, it is passed into the isolator through a sterile nylon filter membrane of 0.2-μm pore size because that is the safest route through the isolator barrier. The diet is fed *ad libitum* in inverted brown bottles having 0.16-cm holes drilled in their lids (Fig. 1). Ultrafiltered water is also available. Whatman

Figure 1. The cage used for germ-free mice fed chemically defined, water-soluble diet, showing the dish for oil, and the collars for water and diet bottles.

(No. 41) ashless filter paper provides ingestible fiber and also serves as bedding and nesting material. It is autoclaved for 25 min at 121°C or irradiated at 4.5 mrad.

Purified soy-derived triglycerides provide the essential fatty acids plus readily available calories. To this end, soy oil has been converted to methylesters, which are vacuum-distilled over a specific range and converted back to triglycerides. Purified fat-soluble vitamins A, D, E, and K are added (Table I). The mixture is not ultrafiltered, based on the assumption that vacuum distillation has achieved the same result. It is then passed into the isolator, as described above for the water-soluble portion of the diet, and dispensed to the mice as single, measured daily doses in stainless steel planchets welded to the stainless steel cage dividers (Fig. 1).

The fact that nine generations of BALB/c mice have been maintained on this diet proves that quantitative, as well as qualitative, nutrient requirements of the BALB/c strain appear to have been met. Life span was limited in male mice by physiological effects of the GF state (cecal enlargement and volvulus) and a special physiological effect of the CD diet (fur balls forming in the cecum and moving out to block the rectum). These hazards are not considered evidence of nutritional deficiency, but mechanical problems connected with the GF state. Since thus far most males have been used for experimental purposes at a relatively young age, no reliable data on their potential life span are available.

Certain composition changes might still produce minor improvements. To be considered here is the lack of queuine in the diet. This tRNA base is normally obtained via the diet or from intestinal microflora production, since it cannot be synthesized by higher mammals. It is inserted posttranscriptionally as queuosine in the "wobble position" of four distinct tRNA, replacing a guanine molecule (52), supposedly to make translation more effective. Although under normal conditions the absence of the resulting queuosine in these transfer RNA does not seem to affect the GF-CD mice, conditions during pregnancy, lactation, and the early postweaning period may be such that optimal efficiency of the protein-synthesizing apparatus becomes a necessity. Nevertheless, the BALB/c colony maintained on this CD diet has provided a healthy, nutritionally defined animal model exposed only to an absolute minimum of exogenous antigenic stimulation.

Recent Immunological Studies

Most recent studies using CD mice have been in the field of immunology. More extensive study of the background antigen-specific spleen and bone marrow cells of the GF-CD BALB/c mice confirmed results obtained earlier with the C3H strain. In GF-CD mice, IgM-secreting cells outnumbered IgG- and IgA-producing cells in both organs. In the comparable CV mice, IgM-secreting cells were dominant in the spleen, but not in the bone marrow. As was found in the case of the C3H mice, these IgM-producing cells seemed to occur in a somewhat higher concentration in the GF-CD mice than in the CV-NI mice. Comparison of the frequency distribution of Ig-secreting cells specific for DNP27-BSA and the anti-idiotype monoclonal antibodies Ac38 and Ac146 in the spleen and bone marrow of GF-CD and CV mice suggested a shift in the distribution of the IgG- and IgA-producing cells specific for these antigens, when compared with the antigen-specific distribution of the IgM-secreting cells. This and other experiments indicate that, while the IgM antibody repertoire is relatively independent of exogenous antigenic stimulation, the shift from IgM to IgG and IgA production appears to be influenced by antigenic exposure (53). This suggests two compartments of background Ig-secreting cells: a stable, endogenously regulated compartment consisting mainly of IgM-secreting cells; and another compartment consisting mainly of IgG- and IgA-secreting cells, whose numbers and specificity repertoire are affected by exogenous antigenic stimulation.

Serum Ig levels of the GF-CD mice correlate well with the numbers of Ig-secreting cells of the various isotypes in the spleen of these mice, whereas in CV mice there exists a clear discrepancy. In serum of CV-NI mice, IgG is the dominant isotype, whereas in the spleen, IgM-secreting cells are clearly in the majority. This difference cannot be explained by the difference

in half-life of the isotypes, and suggests that serum Ig in GF-CD mice reflects the production of the spleen, whereas in CV-NI mice, organs such as bone marrow and lymph nodes contribute considerably to the serum Ig levels, especially for IgG and IgA (54).

The development of the compartment of Ig-secreting cells was studied in GF-CD and CV-NI mice from birth to young adult age. The results suggest that the ontogenetic appearance of Ig-secreting cells in the spleen and the specificity repertoire of the IgM-secreting cells are independent of exogenous antigenic stimulation. However, after birth, the rate of development of the Ig-secreting cell compartment was enhanced by environmental antigenic stimulation (55).

The available repertoire was then investigated by the determination of the usage of V_H gene families in a collection of hybridomas of lipopolysaccharide-reactive B cells derived from the spleen of adult GF-CD BALB/c mice and a hybridoma collection from neonatal splenic B cells of CV origin. Both collections were screened against a large panel of exogenous and endogenous antigens. The results suggest that the available repertoire of adult GF-CD mice resembles that of neonatal CV-NI mice (56, 57).

The serum of the GF-CD mice was also tested for the occurrence of several, presumably "naturally" occurring, antibodies against carbohydrate antigens. Most naturally occurring antibodies against carbohydrate antigens of bacterial origin found in CV-NI mice appear to be caused by exogenous stimulation (54).

Vos (58) confirmed the very low numbers of IgG- and IgA-secreting cells in GF-CD BALB/c mice and then studied the splenic T cell repertoires of these same mice. He found their T cell levels and repertoires to be similar to those of CV-NI mice, and showed that GF-CD T cells could be induced to lymphokine secretion. Thus, there was no defect in GF-CD T or B cells. Their normalcy points to an autonomous activity of the immune system in which the IgG- and IgA-secreting cells appear to be the actual host-defense arm of the system, responding to exogenous challenge.

Cockfield *et al.* (59) studied the extent to which expression of the major histocompatibility complex (MHC) antigen was affected by exogenous microbial or other sources of endotoxin, using the kidney as a representative nonlymphoid tissue (59). They had already established that an intact T cell compartment was not necessary for normal levels of expression, since these occurred in athymic nude mice and CB-17 SCID mice. Working with organs of GF-CD and CV-NI BALB/c mice obtained from the Lobund Laboratory, they found MHC expression in these groups comparable for both Class I and Class II. However, when GF-CD mice were introduced into a CV colony, induction of both Class I and Class II MHC expression occurred. They concluded that, although exposure to bacterial flora or other sources of endotoxin was not required for normal MHC expression, a change in flora may up-regulate expression, probably by inducing the secretion of cytokinins from non-T cells.

Applications: Production and Use of Monoclonal Antibodies

The immune system's responsiveness in GF-CD mice, coupled with its relatively unstimulated state (53), suggested to Gargan *et al.* (50) the use of GF-CD BALB/c mice to generate specific monoclonal antibodies. An antigen injected into such mice gives rise to a much less heterogeneous pool of antibody-producing lymphocytes than it would generate in CV-NI mice. This property permits enhanced immune response to the less immunogenic regions of an antigen. Taking advantage of this, they injected intact cross-linked fibrin into GF-CD mice and obtained an antibody that recognizes an epitopic region unique to the intact fibrin polymeric structure. The antibody does not cross-react with fibrinogen or with any degradation products of either fibrin or fibrinogen. This high specificity, plus its high affinity, makes it uniquely suited for localizing at the site of a fibrin clot or for efficient and safe delivery of coupled clot-dissolving enzymes. By enhancing the generation of antibodies against the rarer immunogenic sites of antigens, the use of GF-CD mice may help in determining the structure of antigens (60).

In another approach, GF-CD BALB/c mice, removed from the isolator to a laminar flow hood to facilitate injection of fibrinogen, proved useful for obtaining an antibody to circulating fibrinogen, even though the mice gradually acquired a microflora in the hood. This antibody does not cross-react with any degradation products of either fibrinogen or fibrin (61). It provides a basis for a latex agglutination assay for plasma fibrinogen, the level of which is proving to be an independent predictor for susceptibility to clot formation in the heart or brain (62).

Conclusion

With the development of CD diet L-489E14SE, it is possible to maintain production colonies of BALB/c mice under GF conditions with totally defined nutritional intake after weaning. These mice are exposed to a minimum of antigenic stimulation and thus provide a unique model in which all exogenous factors may be controlled.

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