Experimental Amyloidosis in Mice: Effect of high and low protein diets

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ABSTRACT Results of past studies concerning the relation of high and low protein diets to the development of sodium caseinate-induced amyloidosis in mice have been somewhat conflicting. A study was therefore made to reanalyze these factors using the more chemically defined diets now available. C3HeB/FeJ male mice were fed the experimental diet when 9 weeks of age; 2 weeks later caseinate injections were initiated and were given 5 times a week for 10 weeks. All animals were weighed weekly and spleen and liver weights were taken at autopsy. The proportion of amyloid deposits in sections of the spleen and liver was determined by the Chalkley method for the quantitative morphologic analysis of tissues. Neither the high casein nor low casein diets prevented the development of amyloidosis. The results suggest that the high casein diet tends to favor the development of amyloid disease. Although the low casein diet tended to inhibit the development of amyloid disease, the results were inconclusive. Morphologically, these effects were observed primarily in the liver. Organ weight data and survival of the injected animals suggest that these effects pertain to the general picture of amyloid disease in these animals.

That diet can affect the development of experimental amyloidosis in mice has been established (1—4). However, the results of the various studies have been somewhat conflicting. Ku and Simon (1) reported that a low protein diet reduced the percentage of mice in which amyloidosis could be induced by caseinate injections, but found no specific effect in animals fed a high protein diet. Jaffe (4) observed that feeding a diet composed entirely of dried beef heart inhibited or delayed the onset of experimental amyloid disease. Grayzel et al. (2) reported that animals fed an “adequate” diet and a diet supplemented with powdered whole liver showed a delayed onset of caseinate induced amyloidosis as compared with animals fed a high protein diet (60% protein).

Unfortunately, these dietary studies cannot be strictly compared since they varied widely in the natural and semipurified constituents of which the various diets were composed. Thus, the question of the relation of nutrition to the development of experimental amyloidosis is unresolved. The present paper reports the effects of diets high and low in a single protein, casein, as compared with a commercial stock diet on the development of caseinate induced amyloidosis in mice.

MATERIALS AND METHODS

Ninety strain C3HeB/FeJ male mice, obtained from the Jackson Laboratory, Bar Harbor, Maine, were used. The animals were assigned to the various dietary groups by means of random numbers.

Diets. The stock or “normal” reference diet was a commercial stock diet,1 having the following composition: wheat germ meal, ground whole wheat, dried skim milk, dehulled 50% soybean meal, corn oil, brewer’s yeast, stabilized vitamin A and D, salt and ferric citrate; protein, minimum 19.0%, fat, minimum 7.5%, fiber, maximum 2.0%, and N.F.E., minimum 52.0%. The high casein and low casein diets were commercial purified diets.3

The high casein diet had the following composition: (in percent) “vitamin-free” casein, 64; sucrose, 22; corn oil, 8; brewer’s yeast USP, 2; salt mixture USP II, 4; and vitamin mixture. The low casein diet had the following composition: (in percent) casein, 8; starch, 78; corn oil, 10; salt mixture USP XIV, 4; and vitamin mix-

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2 Old Guilford Mouse and Rat Breeder Pellets, Emory Morse Company, Guilford, Connecticut.

3 Obtained from Nutritional Biochemicals Corporation, Cleveland.
ture. The content of vitamins per 45.5 kg of diet in the high and low casein diets was: vitamin A conc (as the acetate, 200,000 units/g) 4.5 g; vitamin D conc (as calciferol, 400,000 units/g), 0.25 g; α-tocopherol, 5.0 g; ascorbic acid, 45.0 g; inositol, 5.0 g; choline chloride, 75.0 g; menadione, 2.25 g; p-aminobenzoic acid, 5.0 g; niacin, 4.5 g; riboflavin, 1.0 g; pyridoxine-HCl, 1.0 g; thiamine-HCl, 1.0 g; Ca pantothenate, 3.0 g; biotin, 20 mg; folic acid, 90 mg; and vitamin B₆, 1.35 mg.

Casein. The casein solution was prepared as follows: 10 g of casein were dissolved with slight heating in 200 ml of 0.25% NaOH. This solution was dialyzed in the cold against distilled water for 48 hours to remove excess NaOH. After dialysis the casein solution was reduced to 100 ml final volume by rapid evaporation, which resulted in a 10% solution of casein. The casein solution was stored at zero to 4° and made up fresh weekly.

Different batches of the casein used varied greatly in their solubility, and their solubility in aqueous solutions depended to a great extent upon the pH of the solvent. Several batches of the casein were tested for solubility in NaHCO₃ and NaOH and that which gave the clearest solution was chosen for this and future studies (control no. 9393).

Procedure. Three groups of 30 animals each were used, twenty of which were injected with a casein solution, and ten of which were not injected. At 9 weeks of age, each group was fed one of the diets described: commercial pellet, high casein or low casein. Water and food were given ad libitum. Paired feeding studies were not conducted. The animals were weighed the day that the diets were initiated and every week thereafter.

Two weeks after the diets were initiated, injection of the casein solution was started and continued for 10 weeks. Each animal designated for injection was given 0.3 ml (30 mg) of the 10% casein solution subcutaneously 5 times a week for 10 weeks. Control animals received no injections. The injection sites were rotated daily.

At the termination of the experiment the animals were weighed, killed by cervical dislocation and the spleens and livers removed and weighed on a torsion balance. Samples of liver, spleen and both kidneys were fixed in neutral formalin for 48 hours, washed overnight in running tap water, dehydrated and embedded in paraffin (56 to 58°). Each spleen, immediately after fixation, was cut transversely into quarters and so oriented at embedding that uniform samples of the whole spleen were available for histologic study.

The blocks were sectioned at 5 μ and all sections routinely stained with hematoxylin and eosin. Selected material from each group was also stained with crystal violet (5) at different pH levels, congo red (6), and thioflavin T (7).

The proportion of amyloid in the liver and spleen (hematoxylin and eosin-stained material) was determined by the Chalkley (8) method for the quantitative morphologic analysis of tissues. Briefly, this method is based on the concept that a point moving randomly through a tissue or organ will impinge upon each component of that tissue or organ in proportion to the volume occupied by each component. It was felt that this method would give more objective quantitation of the amyloid in an organ than the rather subjective quantitative methods based on visual estimates of amyloid deposits compared with arbitrary standards.

Body weights, organ weights and the data obtained from the Chalkley method for quantitative morphologic analysis of tissues (amyloid quantitation) were analyzed by one-way analysis of variances; where significant differences were found, Tukey's honest test was used to determine the nature of the significance. Survival data were tested for significance by the chi-square test with Yate's correction. Statistical comparisons of body weights and organ weights were made within the control groups and within the injected

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4 Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation.
3 Hammersten Quality Casein (control no. 9393), Nutritional Biochemicals Corporation.
7 Wollman, A. L. 1967 Studies on the site of action of the anti-androgen ciproterone acetate and on the range of androgenic compounds against which this anti-androgen is effective. Thesis, Appendix, pp. 132-141.
groups of animals, but not between these groups.

RESULTS

Control animals

All control (uninjected) animals remained healthy in appearance throughout the experiment. At autopsy no evidence of infections or other pathologic processes were observed. Histologically, no overt abnormal changes were noted in the spleens, livers or kidneys of these animals. However, there appeared to be fewer megakaryocytes in the spleens of the animals fed low casein than in those fed the commercial pellet and high casein diets. The organs examined were free of amyloid deposits. All animals survived to termination of the experiment.

Growth and weight data. Figure 1 shows the growth curves of the control animals. All animals gained weight during the experiment. However, the animals fed the powdered high and low casein diets showed an initial weight loss during the first week of the diets and did not gain as much during the course of the experiment as the animals fed the commercial pelleted food. During the ninth week the high casein animals showed a sudden weight loss which cannot be fully explained; it is possible that this sudden loss may have been due to an inadvertent temporary insufficiency of food presented to the animals during this week.

At termination of the experiment the body weights of animals fed the high and low casein diets were significantly lower than the body weights of the animals fed the commercial pelleted food (table 1), but were not significantly different from each other.

The mean weight of spleens from animals fed the low casein was significantly lower than the mean spleen weights from the animals fed the commercial pellets and the high casein diet; the mean spleen weights of the latter 2 groups were not significantly different (table 1).

The mean liver weights from the 3 dietary groups were all significantly different, being highest in the animals fed the

Fig. 1 Growth in body weight of injected and control mice fed various diets.
TABLE 1
Comparison of organ and body weights of control and amyloid animals fed various diets

<table>
<thead>
<tr>
<th>Diet groups</th>
<th>Commercial pellet (^2)</th>
<th>High casein</th>
<th>Low casein</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10 animals)</td>
<td>(10 animals)</td>
<td>(10 animals)</td>
<td></td>
</tr>
<tr>
<td>Body wt, g</td>
<td>Control animals</td>
<td>37.97</td>
<td></td>
<td>29.23</td>
</tr>
<tr>
<td></td>
<td>Injected animals</td>
<td>27.87</td>
<td></td>
<td>25.7</td>
</tr>
<tr>
<td>Spleen wt, mg</td>
<td>113.5</td>
<td>111.0</td>
<td></td>
<td>113.5</td>
</tr>
<tr>
<td>Liver wt, mg</td>
<td>1846.5</td>
<td>1543.5</td>
<td></td>
<td>1846.5</td>
</tr>
<tr>
<td></td>
<td>Injected animals</td>
<td>1785.7 (96%)</td>
<td>1860.8 (120.5%)</td>
<td>1036.7 (87.5%)</td>
</tr>
</tbody>
</table>

1 Those values for each parameter on the same horizontal line are not significantly different; those on different horizontal lines are significantly different from all others of same parameter.
2 Old Guilford Mouse and Rat Breeder Diet.
3 Percentage increase over control weight.

Commercial pellets and lowest in those fed the low casein (table 1).

Experimental animals

Animals receiving subcutaneous injections of casein showed no macroscopic pathologic changes during the course of the experiment other than those expected as a result of the induction of amyloidotic disease. In general, the animals appeared less hardy and less well groomed than those not given caseinate injections. Typical signs of illness in the mouse, such as ruffled coat and lessened activity, were noted only in mice that died during the course of the experiment, and then only for a few days immediately preceding death. The injection sites showed no ulcerations nor evidence of infectious processes during the course of the experiment; however, at autopsy, some fibrosis was noted at these sites.

Significantly fewer animals fed the high casein diet survived until termination of the experiment than survived among those fed the commercial pellet and low casein diets (table 2). Although more animals survived of those fed the low casein diet than of those fed the commercial pellet diet, the difference was not statistically significant. Most of the deaths in all groups occurred during the last 2 weeks of the experimental period. All injected animals that survived until termination of the experiment showed macroscopic and microscopic evidence of amyloidosis in the liver, spleen and kidneys except for one animal fed the low casein diet; and that animal showed no evidence of amyloid disease.
AMYLOIDOSIS — EFFECT OF DIET

TABLE 2
Survival of control and caseinate-injected animals fed various diets

<table>
<thead>
<tr>
<th>Animals</th>
<th>Diets</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Commercial</td>
<td>Low casein</td>
</tr>
<tr>
<td></td>
<td>pellet 2</td>
<td></td>
</tr>
<tr>
<td>10/10 3</td>
<td>10/10</td>
<td>—</td>
</tr>
<tr>
<td>Casein-</td>
<td>14/20</td>
<td>18/20</td>
</tr>
<tr>
<td>injected</td>
<td>6/20</td>
<td>&lt; 0.02</td>
</tr>
</tbody>
</table>

1 Number alive on same horizontal line not significantly different.
2 Old Guilford Mouse and Rat Breeder Diet.
3 Number surviving until termination over number at beginning of experiment.

Autopsies were performed on many of the animals that died. However, tissue samples were not taken from all these animals because in many cases postmortem changes were too far advanced. Grossly, the spleens of all these animals were quite enlarged and the kidneys exhibited some degree of lobulation. Microscopic study showed that the sampled spleens, livers and kidneys showed some degree of amyloidosis, the severity of the disease being, on a subjective basis, greatest in animals fed the high casein diet. No other signs of pathology were observed. The exact cause of death could not be determined, but may have been the result of renal failure brought on by amyloid deposits in the kidney. The histologic features of the amyloid deposits in these animals as well as those that survived until termination of the experiment were similar to those described by others (9–11).

Growth and weight data. The growth curves of the injected animals are shown in figure 1. The animals fed the purified diets showed an initial small weight loss during the first week; after this all animals showed growth similar to that of the control animals for 5 to 6 weeks. After this initial growth period, weight loss occurred in all injected animals and generally continued to termination of the experiment. The sudden gain of weight of the injected animals fed high casein during the ninth week is correlated with the fact that a major number of deaths occurred in this group at this time, and only the less affected, higher weight animals contributed to the weight mean plotted here.

At termination of the experiment there was no significant difference in body weights among the injected dietary groups (table 1).

Among the injected animals the mean spleen weight of the animals fed low casein was significantly lower than the mean spleen weights of those fed commercial pellet and high casein diets; the latter 2 groups showed no significant difference in mean spleen weights (table 2). The mean spleen weights of the injected animals are substantially higher than those of the control animals in each dietary group, but the same interdiet relationship exists in both the control and the injected animals (table 1).

The mean liver weight of the injected animals fed low casein was significantly lower than that of those fed the commercial pellet and high protein; the latter 2 groups showed no significant difference in mean liver weights (table 1). Whereas the mean liver weight of animals fed high casein was significantly lower than that of those fed commercial pellets in the uninjected control groups, there was no difference between the two in the injected groups. Further, the injected animals fed

TABLE 3
Chalkley scores; 1 quantitation of amyloid in spleens and livers from animals fed various diets

<table>
<thead>
<tr>
<th>Commercial</th>
<th>High casein</th>
<th>Low casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pellet 2</td>
<td>(14 animals)</td>
<td>(6 animals)</td>
</tr>
<tr>
<td>&quot;hits&quot; 3/1000 total count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>243 (24.3%)</td>
<td>217.3 (21.7%)</td>
</tr>
<tr>
<td>Liver</td>
<td>80.0 (16.0%)</td>
<td></td>
</tr>
<tr>
<td>28.1 (5.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2 (2.2%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Counts for parameters on same horizontal line not significantly different. Counts on different horizontal lines significantly different from all others of same parameter (P < 0.01); percentage of total volume of the organ occupied by amyloid substance in parenthesis.
2 Old Guilford Mouse and Rat Breeder Diet.
3 "Hits" refer to the number of times a pointer in the ocular of the microscope impinges on amyloid material.
high casein showed a higher mean liver weight than the uninjected control animals, and those fed commercial pellet and low casein diets showed a slightly lower mean liver weight (table 1).

Amyloid quantitation. The data derived by the Chalkley method for the quantitative morphologic analysis of tissues is presented in table 3. The proportion of amyloid in the spleens of the 3 dietary groups was not significantly different, ranging from 24.3% of the total spleen volume in the animals fed commercial pellets to 19.9% in those fed low casein. Figure 2 shows representative amyloid deposits in the spleen of an animal fed the commercial pellet diet.

There was a significant difference in the proportion of amyloid in the livers of the 3 dietary groups (table 3). The animals fed high casein showed the greatest proportion of amyloid and those fed the low casein, the least. Figure 3 shows representative amyloid deposits in the livers of animals fed the commercial pellet and high casein diets.

No effort was made to quantitate the amyloid volume of the kidney due to the difficulty in obtaining tissue sections which would give adequate sampling of this organ. Such data would be of value, however, and efforts are being made to overcome these difficulties. Amyloid deposits occurred in the distal two-thirds of the papilla, interstitially between the tubules, especially in the inner cortical region, and in the vascular portion of the glomeruli. In many cases, necrosis of the papilla and cortical lesions occurred, as previously described (12, 13). Figure 4 shows a kidney severely affected by amyloidotic disease.

General staining characteristics. Splenic, hepatic and renal amyloid deposits exhibited the same staining characteristics. Staining with Leib's crystal violet method (pH 1.5 to 1.9) rendered amyloid “metachromatic” in color; however, rinsing
Fig. 3 Representative amyloid deposits in livers from animals fed (A) high casein diet, and (B) Old Guilford laboratory ration. Note the greater amount of amyloid in the liver of an animal fed the high casein diet. H&E × 60.
Fig. 4 Kidney severely affected by amyloidotic disease. Note the bland necrosis of the papilla and the wedge-shaped areas of tubular involvement in the cortex. H&E × 12.5.

the slides in water removed the metachromasia, leaving amyloid colorless. The same stain applied at pH 2.5 to 6.00 gave amyloid a "metachromatic" reaction which was water-fast. The induced amyloid material stained not at all with thioflavin-T; weakly with Congo red and was moderately birefringent after Congo red staining.

DISCUSSION

The results of this experiment indicate that variation of a single protein, casein, in the diet can affect the course of experimentally induced amyloid disease in mice under the conditions outlined. Neither the high casein nor the low casein diets prevented the development of amyloid disease. In general, however, a high casein diet tended to favor the development of amyloid disease as compared with a standard commercial ration of "normal" protein content. Although the results suggest that a low casein diet may inhibit somewhat the development of amyloid disease, the evidence is not conclusive.

Chalkley scores used to "quantitate" the proportion of amyloid in the organs of the animals clearly substantiate the above interpretations with respect to liver amyloid in the 3 diet groups (table 3). The scores indicate there is no difference in the proportion of splenic amyloid among the 3 groups. Examination of organ weight data (table 1), however, indicates that the same trend exists in the spleen as well as the liver. The spleens of the animals fed high casein increased more in weight as a result of the injections than those of animals fed the commercial food (table 1, 118.5% versus 77.2% increase). The spleens of the injected low casein animals increased the least in weight (55.8%). The liver weights of the high casein injected animals increased considerably over those of the control high casein animals, whereas those of the commercial food and low casein-fed animals actually weighed less than those of the respective control animals. The interpretations are strengthened because the least survival of animals...
occurred in the injected high casein animals and the greatest survival in the low casein injected animals.

The manner in which the high and low casein diets exert their influence in the development of amyloid disease is not readily apparent. The low casein diet may lessen amyloid development because the diet itself is deficient in protein. The similarity of growth curves of both the normal and injected animals fed the low casein and commercial food diets contradict this interpretation and indicate that no overt nutritional deficiency was present in animals fed the low casein diet. The lack of lymphoid atrophy, the presence of fair amounts of body fat and the general good health of the control animals fed the low casein diet substantiate this interpretation.

Since the sole protein in the high and low protein diets was casein, the alteration in amyloid disease may represent a specific protein effect, that is, a qualitative rather than a quantitative effect on the part of the dietary protein. Pertinent to this point is that the diets used by Grayzel et al. (2) which favored amyloid production were, in general, notably high in milk products (up to 60% of the diet), whereas those in which amyloid induction was delayed or lessened were considerably lower in milk products (19%). In contrast, the diets of Ku and Simon (1) contained no milk products, yet the low protein diet decreased the incidence of amyloid disease as compared with a diet containing more normal protein content. Another argument against the quality of the protein being the major factor in the dietary effects on amyloid disease noted in this experiment is that in adult mammals proteins of dietary origin are rapidly hydrolyzed during digestion and few peptides or intact proteins are absorbed through the intestinal wall (14) into the portal circulation. Unfortunately, the experimental design is such that the role of protein quality cannot be eliminated as a possibility in causing the observed effects.

It might be argued that it is the variation of the quantity of carbohydrate rather than of protein in the diet that is responsible for the observed differences in amyloid production. However, this is rather doubtful for the following reasons. A diet very low in carbohydrate would be expected to bring about a rather large increase in amyloid substance; Jaffe (4) noted no amyloid production in mice fed a low carbohydrate diet consisting of dried beef heart powder mixed with water and a little yeast to make a dough. A diet very high in carbohydrate should greatly diminish the production of amyloid substance; Grayzel et al. (2), using a high carbohydrate stock diet containing 3 parts whole powdered milk and 2 parts ground white bread, produced amyloid earlier in mice than any other of their diets. Chemical analysis of isolated amyloid fibrils (15) indicated that amyloid contains little carbohydrate (4.6%), but a great deal of protein (15% nitrogen). Moreover, it is known that animals can synthesize sufficient carbohydrate for their needs from protein and fat provided these substances are not deficient.

Although the carbohydrates of the high and low casein diets are different in type (sucrose and starch), it is difficult to visualize that this difference would affect the outcome of the experiment. Starch and sucrose are eventually converted by the organism to glucose, the primary carbohydrate utilized by the body tissues. Thus, the interpretation that protein level is the factor affecting amyloid production in this experiment appears to be justifiable.

It is difficult to compare the dietary effects on experimental amyloid disease reported here with those in studies of other workers. Diets reported in the literature are composed of various combinations of natural and semipurified as well as exceedingly variable and different foodstuffs (1, 2, 4). Comparison is made even more complicated by the use of mice of different strains, ages and sex by these investigators. Although casein injections were used to induce amyloidosis in all these studies, the amount injected, the concentration of the casein solution, and the schedule of injections varied greatly. Finally, the means of determining the degree of amyloid disease varied among investigators; some used the percentage of mice showing amyloid disease as an end point, and others used the time of appearance of amyloid deposits.
The experiment reported here, as well as studies by other investigators, serve to point up the importance of nutrition in relation to experimental amyloid. Of even greater importance is the relation of nutrition to amyloid disease in humans. Although the incidence of so-called secondary amyloid disease has decreased with the advent of antibiotics, the primary or idiopathic forms are being more widely recognized. The relationships of nutrition to experimental and human amyloidosis remain to be fully delineated.

LITERATURE CITED