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Regression of casein and cholesterol-induced hypercholesterolaemia in rabbits

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Summary

Hypercholesterolaemia was induced in rabbits by feeding semipurified diets containing soy protein plus cholesterol (0.8 g/kg) or casein for four weeks. Subsequently for a period of six weeks, some of the rabbits were transferred to diets containing soy protein while others continued to receive the hypercholesterolaemic diets to which was added a mixture of amino acids (g/kg feed; glycine, 3.9; arginine, 6.9 and alanine, 1.6). Such additions increased the concentration of these amino acids in the casein diet to that in the hypocholesterolaemic soy protein diet.

The cholesterol levels in the serum of the rabbits transferred to the soy protein diets declined rapidly, becoming significantly different from animals remaining on the hypercholesterolaemic diets after only three days. Serum cholesterol levels comparable to those in rabbits fed soy protein throughout the entire experiment were reached after about two weeks. The addition of the amino acids tended to reduce the concentration of cholesterol in the serum of the rabbits made hypercholesterolaemic by feeding the diets containing either casein or cholesterol. However, the effect reached significance only with the diet containing casein to which amino acids were added and then only at one time point.

Six weeks after the cholesterol-fed animals were transferred to the cholesterol-free soy protein diet or to the diet containing the additional amino acids, apo E disappeared from the IDL_1 ($1.006 < d < 1.012$ g/ml) and IDL_2 ($1.012 < d < 1.019$ g/ml) fractions, but not from the VLDL fraction. Both with the cholesterol-free soy protein diet and the cholesterol diet fortified with amino acids, cholesterol in the VLDL fraction was reduced to values seen in animals fed the soy protein diet throughout the entire experimental period. The amount of cholesterol in the IDL and LDL fractions was decreased only in the soy protein group.

Replacement of casein by soy protein, or the addition of amino acids to the casein diet did not induce the disappearance of apo E from the IDL or VLDL fractions. Only the soy protein diet lowered the amount of cholesterol in the VLDL and LDL fractions appreciably.

It is concluded that the amount of apo E present in both IDL and VLDL does not invariably correlate with the level of serum cholesterol in rabbits. This study also indicates that the hypercholesterolaemic nature of casein resides only partially in the fact that it contains relatively low proportions of glycine, alanine and arginine compared with soy protein.

Zusammenfassung

In der vorliegenden Arbeit wurde bei Kaninchen eine Hypercholesterinämie erzeugt, indem halbgereinigte Diäten für 4 Wochen verabreicht wurden, die entweder Sojaeiweiß + Cholesterin (0,8 g/kg) oder Kasein enthielten. Anschließend wurden folgende Untergruppen gebildet: Ein Teil der Kaninchen bekam weiterhin die Ausgangsdiäten, während jeweils zwei weitere Gruppen eine Zulage der Ami-

nosäuren Alanin, Arginin und Glycin bekamen, bzw. eine halbgereinigte Diät mit Sojaisolat als Proteinträger erhielten. Der zweite Teil der Versuchsperiode umfaßte einen Zeitraum von 6 Wochen.

Bereits 3 Tage nachdem die Tiere von der hypercholesterinämischen Diät auf die Sojadiät umgestellt waren, hatten sie einen signifikant niedrigen Cholesterinspiegel im Serum. Die Werte der Kontrolltiere, die während der gesamten Versuchsperiode eine Sojadiät erhielten, wurden zwei Wochen nach der Umstellung erreicht. Die Zulage der Aminosäurenmischung zur Kasein- sowie zur Cholesterindiät hatte in beiden Fällen nur in der Tendenz einen hypocholesterinämischen Effekt.

Nach sechs Wochen der hypocholesterinämischen Periode war in der IDL_1-Fraktion ($1{,}006 < d < 1{,}012$ g/ml) und in der IDL_2-Fraktion ($1{,}012 < d < 1{,}019$ g/ml) der Gruppe, die in der ersten Periode Sojaisolat + Cholesterin und in der zweiten Periode Sojaisolat bekam, Apo E elektrophoretisch kaum nachweisbar. In der VLDL-Fraktion ($d < 1{,}006$ g/ml) hingegen war Apo E nicht sichtbar von der Cholesteringruppe verschieden. Dasselbe gilt für die Gruppe, die eine Zulage der Aminosäurenmischung erhalten hatte. In beiden Gruppen war jedoch der Cholesteringehalt der VLDL-Fraktion auf Konzentrationen gesunken, wie sie auch die Soja-Kontrollgruppe aufwies. Die Cholesterinkonzentration in den IDL- und LDL-Fraktionen war nur in der Sojagruppe gesunken.

Der Ersatz von Kasein durch Sojaisolat oder die Zulage einer Mischung der genannten Aminosäuren zum Kasein hatte nicht zur Wirkung, daß Apo E wesentlich in den Fraktionen VLDL und IDL zurückgebildet wurde, obwohl in der VLDL- und LDL-Fraktion die Cholesterinkonzentration der Gruppe, die Soja nach Kasein bekam, reduziert war.

Aus den Ergebnissen wurde die Schlußfolgerung gezogen, daß der Gehalt an Apo E und die Konzentration des Cholesterin in den Lipoproteinen sehr geringer Dichte (VLDL) und mittlerer Dichte (IDL) nicht vollständig voneinander abhängig sind, wie frühere Untersuchungen (28) hätten erwarten lassen. Die Ergebnisse zeigen weiterhin, daß die Zulage der Aminosäuren Alanin, Arginin und Glycin zu Kasein in diesen Mengen und in dem hier beschriebenen Zeitraum nur in der Tendenz, nicht aber statistisch gesichert zur Senkung des Cholesteringehalts führt.

Key words: dietary protein, dietary cholesterol, rabbits, serum cholesterol, serum lipoproteins, apoproteins

Introduction

It is well known that hypercholesterolaemia and atherosclerosis in rabbits can be produced easily by cholesterol as well as casein in the diet. In contrast to casein, soy protein gives low levels of serum cholesterol (6, 11, 19, 32). Earlier studies from this laboratory have shown, that casein- and cholesterol-induced hypercholesterolaemias develop in a similar way: there is first a marked increase of cholesterol in the LDL_1 ($1.019 < d < 1.040$ g/ml) and later in the VLDL fraction ($d < 1.006$ g/ml) with considerable amounts of apoprotein E in fractions with $d < 1.019$ g/ml (28).

The hypocholesterolaemic effect of soy protein can be explained partly on the basis of its amino acid composition. Several studies have been done to investigate the effect of different proteins (9, 11, 33, 35) and amino acids or amino acid combinations (12, 14, 15). Investigations have demonstrated that the addition of those amino acids which are poorly represented in casein when compared with soy protein, namely alanine, arginine and glycine, lower the serum cholesterol level (12). However, it is not clear whether the amino acids are effective by correcting an amino acid imbalance or if they have a cholesterol-lowering effect *per se*.

The main object of this investigation was to examine the regression of hypercholesterolaemia when casein and cholesterol-induced hypercholesterolaemic rabbits are transferred to a normocholesterolaemic diet containing soy protein. In addition, the effect on serum cholesterol concentration of transferring rabbits on the hypercholesterolaemic diets to similar diets fortified with a mixture of alanine, arginine and glycine was studied. At the end of the experimental period of six weeks, cholesterol in the lipoprotein fractions was determined and a qualitative examination of apoprotein E in the lipoproteins with density <1.040 g/ml was also made.

Materials and methods

Animals and diets

In this experiment, male rabbits of the New Zealand White strain were used. The animals were kept individually in cages with wire mesh bases constructed of galvanized steel in a room with controlled lighting (14 h/day), constant temperature (18 °C) and humidity. On arrival in the animal house, the rabbits, which were aged about 9 weeks, were maintained on commercial rabbit pellets (Trouw and Co. N.V., 3881 LB Putten, The Netherlands) for 2 weeks. Subsequently 85 animals were kept on a semisynthetic diet containing soy protein for 2 weeks. At the end of this period the animals were allocated to 3 dietary groups based on their serum cholesterol concentrations and body weights which were about 700 g. These groups were fed semipurified diets containing either soy protein with or without added cholesterol or casein. The rabbits of the groups which received cholesterol or casein developed a hypercholesterolaemia during the pre-experimental period of 4 weeks; these animals were reallocated to groups on the basis of their serum cholesterol concentrations and body weights as illustrated in figure 1. At this stage (week 8) each of the 6 groups consisted of 10 animals. The experimental period continued for a further 6 weeks. Throughout the experiment, a control group of 7 animals was maintained on the diet containing soy protein.

The semipurified diets contained either casein (acid casein, DMV BV, 5466 BA Veghel, The Netherlands) or soy isolate (Promosoy-100, Chemurgy Division, Central Soy Company Inc., Chicago, Ill. 60639/U.S.A.) as a protein source. The composi-

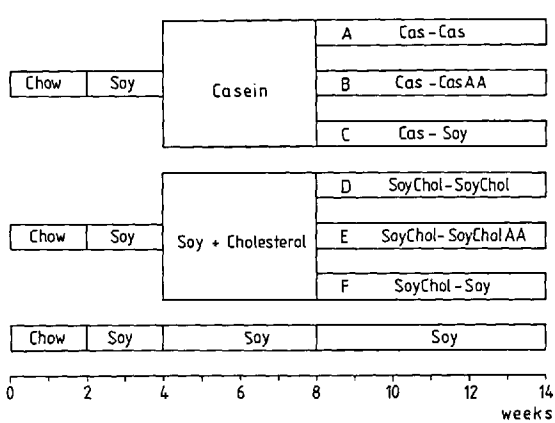

Fig. 1. Experimental design. Pre-experimental period: 4–8 weeks; experimental period: 8–14 weeks.

tion of the semipurified diets was as follows (g/kg feed): casein, 210 (or soy isolate, 208 and methionine, 2); maize starch, 170; dextrose, 210; sawdust, 178 (in the soy protein diets, 180); coconut fat, 90; soybean oil, 10; molasses, 50; dicalcium phosphate, 29; sodium chloride, 8 (in the soy protein diets, 6); magnesium carbonate, 3; magnesium oxide, 2; potassium bicarbonate, 18; vitamin premix, 12; mineral premix, 10. The composition of the vitamin and mineral premixes has been described (37). The 0.08 % cholesterol in the soy protein diet containing cholesterol was added at the expense of sawdust. The diet containing casein and additional amino acids (g/kg feed: glycine, 3.9; arginine, 6.9 and alanine, 1.6) referred to as Cas-CasAA (fig. 1) contained the same concentration of these amino acids as the soy protein diet (Soy). The same amount of these amino acids were added to the cholesterol-supplemented soy protein diet (SoyChol). This diet is referred to as SoyChol-SoyCholAA. Thus SoyChol-SoyCholAA contains more of these amino acids than not only casein, but also the other soy protein diets.

Food was provided each day at 9.00 a.m. on a restricted basis, the rabbits on the semipurified diets receiving 70 g/day, which is equivalent to 1.02 MJ/day. Most rabbits consumed all their food within 4 h. Water was provided *ad libitum.*

Sampling of blood and analytical methods

On days 1, 3, 7, 15, 29, 42 of the experimental period, which began at week 8 (fig. 1), samples of blood were taken from the marginal ear vein of the rabbits between 8.00 a.m. and 10.00 a.m. after the removal of any remaining food at 4.00 p.m. the previous day. Cholesterol in serum was measured enzymatically (25) using the kit (catalase method) supplied by Boehringer Mannheim GmbH, West Germany. As cholesterol standards, three calibrated sera with low, medium and high cholesterol concentrations were used; the cholesterol concentration of these sera was determined by the method of Abell et al. (1).

For separating lipoproteins by ultracentrifugation, the method of Terpstra et al. (34) was used. Samples of serum from each of the experimental groups was pooled, and the following 7 density classes (g/ml) were isolated, using an SW-41 rotor (Beckman Inc., Palo Alto, CA 94304, U.S.A.): VLDL ($d < 1.006$); IDL_1 ($1.006 < d < 1.012$); IDL_2 ($1.012 < d < 1.019$); LDL_1 ($1.019 < d < 1.040$); LDL_2 ($1.040 < d < 1.063$); HDL_1 ($1.063 < d < 1.125$); and HDL_2 ($1.125 < d < 1.21$). Protein in the lipoprotein fractions was estimated by the method of Lowry et al. (21) as modified by Markwell et al. (22), and cholesterol as described above.

The apoprotein composition of the lipoprotein fractions was examined by SDS-polyacrylamide gel electrophoresis. After ultracentrifugation the fractions were dialyzed at 4 °C, exhaustively against a solution containing 5 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.27 mM EDTA. After dialysis the lipoprotein fractions were freeze-dried and subsequently delipidated by ether/ethanol treatment (27). The delipidated lipoproteins were dissolved in 2 % SDS (w/v) and the protein concentration estimated as described above. Aliquots of the apoprotein mixtures were boiled for 1 min in a solution of 6.7 % (v/v) glycerol, 3.3 mM dithiothreitol, 1.5 % SDS and 0.015 % bromophenol blue. Aliquots (containing 10 µg protein) were subjected to SDS-polyacrylamide gel electrophoresis (31) using a 2.4 % stacking and a 10 % separating gel. After electrophoresis the gels were stained in a solution of 0.26 % Coomassie brilliant blue G250 in 50 % (v/v) methanol and 10 % acetic acid and destained in a mixture of 10 % methanol and 10 % acetic acid. Subsequently, the gels were photographed. Estimation of apoprotein content in the different fractions was done semiquantitatively by visualization. The molecular weight of the apoproteins was estimated by the use of standard proteins of known molecular weight obtained from Sigma, namely transferrin (M_r 80,000), albumin (M_r 68,000), γ-globulin (M_r 54,000; M_r 23,000) and ovalbumin (M_r 43,000).

Results

Body weight

At the end of the pre-experimental period (week 8, fig. 1), the body weights of the rabbits expressed as means ± S. E. were 2334 ± 52.0, 2277 ± 60.8, and 2282 ± 87.0 g on the casein, soy protein + cholesterol, and soy-protein diets, respectively. These values were not statistically different. The body weights at the end of the experiment ranged from 2863 ± 52.8 to 3026 ± 53.4 g. The body weight gains were not statistically different with the exception of the groups Cas-Soy and SoyChol-SoyChol. Both groups had a 16 % higher body weight gain during the experimental period ($P<0.05$ and $P<0.01$, respectively), when compared to the group fed soy protein throughout the entire experimental period.

Serum cholesterol

The data in figures 2 and 3 show the development of a hypercholesterolaemia during the pre-experimental period (weeks 4–8, fig. 1) as a result of feeding cholesterol or casein. The serum cholesterol values at the beginning of this period were 1.69 ± 0.06 mmol/l. The cholesterol concentrations at the beginning of the experimental period (week 8, fig. 1) was 3.58 ± 0.43 (n = 30) for the animals which had been on casein and 3.18 ± 0.23 mmol/l (n= 30) for the animals which had been on cholesterol. The rabbits on soy protein had a serum cholesterol concentration of 2.09 ± 0.28 mmol/l at this time, which decreased to a level of about 1.66 ± 0.15 mmol/l on day 3 of the experimental period and then remained very constant. After 3 days of the experimental period, the cholesterol-lowering effect of soy protein was already significant in both hypercholesterolaemic groups ($P<0.05$).

Values corresponding to those of the control group were reached after 15 days in animals of the SoyChol-Soy group (fig. 3) and after 28 days in the

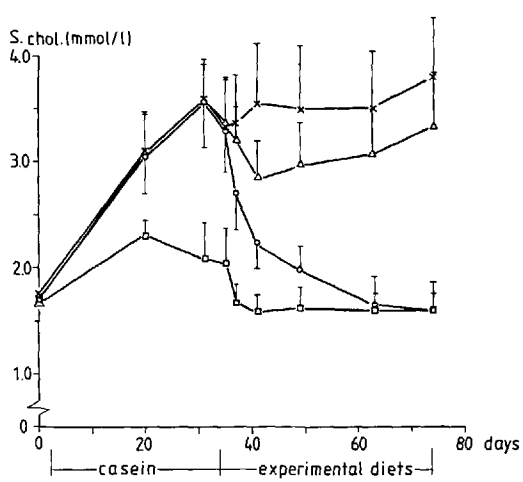

Fig. 2. Serum cholesterol concentrations (mean ± SE) in rabbits fed the semi-purified diets described in figure 1: ×, Cas-Cas; △, Cas-CasAA, ○, Cas-Soy; and □, Soy-Soy. Day 0 in this figure corresponds to week 4 in figure 1.

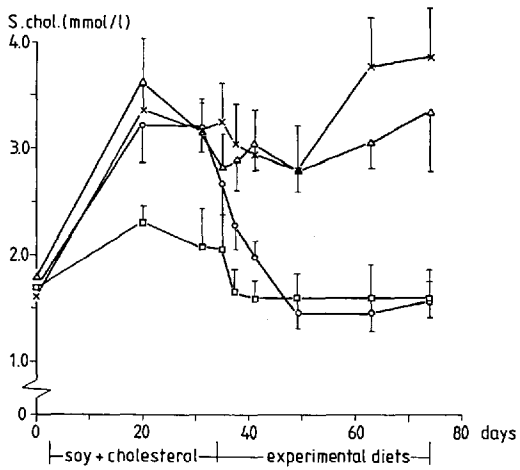

Fig. 3. Serum cholesterol concentration (mean ± SE) in rabbits fed the semipurified diets described in figure 1: ×, SoyChol-SoyChol; △, SoyChol-SoyCholAA; ○, SoyChol-Soy; and □, Soy-Soy. Day 0 in this figure corresponds to week 4 in figure 1.

Cas-Soy group (fig. 2). There was a small but consistent serum-cholesterol-lowering effect of dietary amino acids when they were added to the cholesterol-containing soy diet (fig. 3); however, this effect was not statistically significant. Only at one time point, namely after 7 days, the addition of amino acids significantly reduced the casein-induced hypercholesterolaemia (fig. 2). Throughout the entire experiment, the cholesterol-lowering tendency of amino acids was most pronounced when added to the casein diet.

Table 1. Concentration of cholesterol (mmol/l of whole serum) in serum lipoprotein fractions of rabbits fed semipurified diets.

Lipoprotein fraction	Diet code						
	Cas-Cas	Cas-CasAA	Cas-Soy	SoyChol-SoyChol	SoyChol-Soy CholAA	Soy Chol-Soy	Soy
VLDL (d<1.006)	0.40	0.35	0.31	0.63	0.30	0.30	0.29
IDL_1 (1.006<d<1.012)	0.14	0.10	0.10	0.17	0.19	0.07	0.09
IDL_2 (1.012<d<1.019)	0.27	0.38	0.23	0.41	0.51	0.16	0.20
LDL_1 (1.019<d<1.040)	1.15	1.00	0.40	1.57	1.28	0.39	0.32
LDL_2 (1.040<d<1.063)	0.16	0.10	0.05	0.18	0.13	0.05	0.05
HDL_1 (1.063<d<1.125)	0.62	0.54	0.48	0.40	0.46	0.38	0.37
HDL_2 (1.125<d<1.210)	0.39	0.25	0.27	0.30	0.37	0.28	0.25
VHDL (d>1.210)	0.10	0.10	0.10	0.08	0.09	0.09	0.08
Whole serum	3.25	2.91	1.99	4.04	3.66	1.75	1.73
Recovery (%)	99	97	97	93	91	98	95

The lipoproteins were separated by density-gradient ultracentrifugation from pooled sera (n = 8 or 9) at the end of the experimental period. The densities (d) of the lipoprotein fractions are expressed in g/ml. For experimental design, see figure 1.

Cholesterol content of lipoproteins

Table 1 documents the distribution of cholesterol between lipoprotein fractions of pool sera from rabbits fed the different diets. Blood samples were obtained at the end of the experimental period. Supplementation of the soy protein diet with cholesterol (0.08 %, w/w) caused an increase in the amount of cholesterol in lipoprotein fractions with density <1.063 g/ml. Most of the excess serum total cholesterol can be attributed to the increase in the cholesterol content of the LDL_1 fraction ($1.019<d<1.040$). Cholesterol feeding did not clearly affect the cholesterol concentration of the HDL fractions.

An increase in the amount of cholesterol in the lipoproteins with lower densities was also seen after the replacement of soy protein by casein in the diet (Cas-Cas, table 1). Most of the increase in serum total cholesterol in casein-fed animals was also located in the LDL_1 fraction. Contrary to when cholesterol was included in the diet, the casein-containing diet induced an increase in the amount of cholesterol in the HDL_1 and HDL_2 fractions when compared to the diet containing soy protein.

The cholesterol distribution between lipoproteins of the animals transferred from either the casein or the cholesterol-supplemented soy protein diet, to the soy protein diet resembled closely that of the animals fed soy protein during the entire experimental period (table 1). In the animals transferred from casein to soybean protein, the cholesterol concentration in the HDL_1 fraction was still increased, possibly reflecting cholesterol transport from expanded tissue pools to the sites of degradation and excretion.

Amino acid supplementation of the casein and cholesterol diet tended to reduce the levels of LDL_1-cholesterol (table 1). A striking decrease was seen in the amount of cholesterol in the VLDL fraction of animals fed the cholesterol diet with added amino acids. Enrichment of the casein diet with the amino acid mixture tended to lower the cholesterol content of the HDL_1 and HDL_2 fractions. As shown above in figures 2 and 3, the addition of amino acids to the hypercholesterolaemic diets tended to lower serum total cholesterol levels.

Apoprotein E in lipoproteins with density <1.040 g/ml

The final apoprotein pattern observed on SDS-polyacrylamide gel electrophoresis of the lipoprotein fractions is shown in figure 4. As we have discussed earlier (28), the main changes in apoprotein composition in casein or cholesterol-induced hypercholesterolaemia occur in the fractions with densities <1.040 g/ml. Therefore, only apoproteins of those fractions have been studied.

In animals fed diets containing soy protein, only trace amounts of apo E are found in the lipoproteins with lower densities (28). Hypercholesterolaemia, induced by either casein or cholesterol, is associated with drastically elevated amounts of apo E in the VLDL and IDL fractions (ref. 28; fig. 4). As shown in figure 2, there was a marked reduction of cholesterol in the casein-induced hypercholesterolaemia when soy protein was fed for 6 weeks. However, this was accompanied by only a slight decrease in the amount of apo E in the VLDL and IDL_1 fractions, wheras the

amount of apo E in the IDL_2 fraction was not affected at all (fig. 4C). As might be anticipated, inclusion of the amino acids, arginine, glycine, and alanine, in the casein diet did not significantly alter the apoprotein composition of the VLDL and IDL fractions (fig. 4B).

When cholesterol supplementation of the soy protein diet was discontinued, serum cholesterol levels rapidly declined, and baseline values were reached after 15 days (fig. 3). After six weeks, apo E had almost completely disappeared from the IDL_1 and IDL_2 fractions, but not from the VLDL fraction (fig. 4F). Contrary to what might be expected, enrichment of the SoyChol diet with amino acids had essentially the same effect (fig. 4E).

Changes in apoproteins with a molecular weight of about 70,000 were also observed. An increase in the content of these proteins was seen in the IDL_1 and IDL_2 fractions when rabbits were transferred from the cholesterol-supplemented to the cholesterol-free soy diet (fig. 4F) or when amino

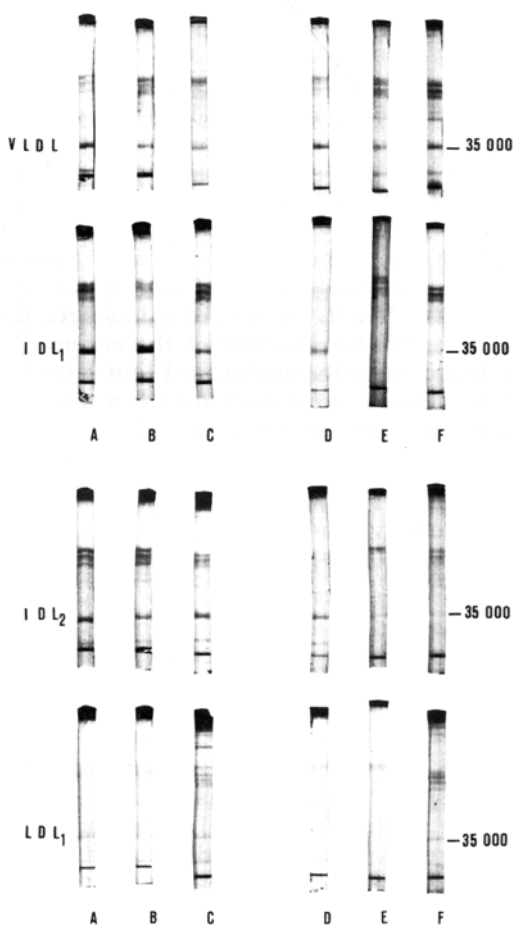

Fig. 4. SDS-gel electrophoresis patterns of apoproteins in 10 % polyacrylamide gels. Lipoproteins were isolated at the end of the experiment from pooled sera of rabbits fed the semipurified diets described in figure 1: A, Cas-Cas; B, Cas-CasAA; C, Cas-Soy; D, SoyCholSoyChol; E, SoyCholSoyCholAA; and D, SoyChol-Soy. The location of apoprotein E is indicated by its molecular weight (35,000).

acids were added (fig. 4E). Feeding a cholesterol-free soy protein diet also caused these high molecular weight proteins to increase in the LDL_1 fraction (fig. 4C and F). One of these proteins could be albumin (M_r 68,000) contaminating the lipoprotein fractions, but at present we have no information about the other proteins present.

Disussion

In these further studies (cf. ref. 28) on the comparison between casein- and cholesterol-induced hypercholesterolaemia in rabbits, the level of cholesterol in the diet (0.08 %, w/w) was chosen so that serum cholesterol concentrations in both types of hypercholesterolaemia were similar. We feel that this is a prerequisite in comparative studies on the mechanisms underlying the different hypercholesterolaemias.

As shown in table 1, much of the excess serum cholesterol in the cholesterol-fed rabbits is carried in the LDL_1 fraction. At more elevated levels of serum cholesterol, a substantial part of the cholesterol is also transported in the IDL and VLDL particles, especially the so-called β-VLDL particles (26, 29). When rabbits are fed cholesterol-containing diets, the production of lipoproteins with densities < 1.040 g/ml overwhelms their clearance by the liver. There is no compensatory increase in bile acid formation (10), and the number of hepatic lipoprotein receptors becomes suppressed (17, 30). Feeding casein to rabbits reduces the faecal excretion of steroids compared to those on soy protein (5, 13), an effect paralleling, if not preceeding, the elevation of serum cholesterol (5). Subsequently, the number of hepatic LDL receptors decreases (8), which aggravates the degree of hypercholesterolaemia and may further reduce biliary steroid output. Thus the accumulation of serum cholesterol in the casein-fed rabbit is probably the result of a decreased clearance of plasma cholesterol. In the cholesterol-fed animal, on the other hand, the hypercholesterolaemia is, most likely, initiated by an increased production of β-VLDL particles, which are believed to be chylomicron-remnants (26). The number of apo B-containing LDL particles probably increases because the apo E containing β-VLDL particles bind with a higher affinity to the hepatic receptors, and therefore diminish LDL uptake.

Withdrawal of cholesterol from the diet causes a rapid fall in serum cholesterol levels (fig. 3). Although the degree of hypercholesterolaemia in the cholesterol- and casein-fed rabbits was similar, transfer back to the soy protein diet effected a slower return to baseline values in the casein-fed animals (fig. 2). Possibly, tissue cholesterol pools remain expanded somewhat longer in casein-fed rabbits, which is suggested by the fact that the high levels of HDL-cholesterol induced by casein persist after replacement of casein by soy protein (table 1). Since HDL is generally considered to transport peripheral cholesterol to the liver for excretion, increased hepatic cholesterol pools induced by casein (2) may not readily decrease. In rabbits made hypercholesterolaemic by dietary cholesterol, hepatic cholesterol levels have been shown to decline rapidly after feeding a cholesterol-free diet (17). The size of the hepatic cholesterol pool may be inversely related to the number of LDL receptors and consequently to

clearance of circulating LDL (cf. ref. 30). However, further studies are required on this point as well as on the question why HDL cholesterol levels are not affected by the inclusion of cholesterol in the diet (table 1; ref. 26, 28, 29), but increased by casein (table 1; ref. 2, 33, 35).

The amino acid composition of dietary protein plays an important, though still poorly understood, role in determining serum cholesterol concentrations in rabbits (32). The lysine : arginine ratio has been suggested to be directly associated with the level of serum cholesterol (18), but this suggestion seems untenable (7, 36). Possibly, the effects of amino acids interact with other components of the semipurified diets. We have shown that dietary salts (3) and fats (4) also affect the hypercholesterolaemic response to casein-containing diets. In our laboratory it was found that the addition of a mixture of arginine, alanine and glycine to a casein-containing diet partially counteracts the hypercholesterolaemic response (12, 16). In the present study we found only a cholesterol-lowering tendency by these amino acids when added to the casein diet so as to increase the levels to those found in soybean protein. This apparent discrepancy with earlier studies (12, 16) may be explained by the fact that we added less glycine and alanine to the diets. It is possible that a more prolonged study would have shown a significant serum cholesterol reduction (cf. ref. 16). Addition of the amino acid mixture to the cholesterol-supplemented soy diet did not affect serum cholesterol levels during the first 15 days (fig. 3). However, this observation should be interpreted with caution, since serum cholesterol in the animals on the diet containing soy protein plus cholesterol fell slightly during this period. The increase in serum cholesterol in the rabbits fed cholesterol was somewhat prevented by the three amino acids (fig. 3). Although the present study provides no substantial evidence for a hypocholesterolaemic action of glycine, alanine and arginine *per se* (i.e., they do not significantly reduce cholesterol-induced hypercholesterolaemia in rabbits), a consistent cholesterol-lowering effect was induced by these amino acids, and this deserves further investigation.

Feeding casein (23, 28) or cholesterol (20, 28, 29) to rabbits elevates the content of both cholesterol and apo E in VLDL and IDL. The question was examined as to the fate of apo E during regression of both types of hypercholesterolaemia. Several unexpected and unexplainable findings emerged from this study in this regard. Replacement of casein by soy protein completely abolished the elevated cholesterol levels in VLDL and IDL after six weeks (table 1), but appreciable amounts of apo E were still present (fig. 4C). Withdrawal of cholesterol from the soy protein diet supplemented with cholesterol also reduced the amount of cholesterol in VLDL and IDL to levels found in animals fed the diet without added cholesterol throughout the entire experimental period (table 1). Apo E almost disappeared from the IDL, but not from the VLDL fraction (fig. 4F). It could be suggested that apo E disappears from IDL first and then from VLDL. That apo E did not disappear from the IDL fraction in the animals fed casein followed by soy protein (fig. 4C) could possibly be explained by the fact that the reduction of hypercholesterolaemia in these animals did not occur as fast as that in the animals made hypercholesterolaemic by feeding them casein. We are not aware of data on the half-life

of apo E in VLDL of rabbits, but the half-life for total VLDL protein, which ranges from several minutes to several hours depending on the degree of hypercholesterolaemia (23, 24), is too short to substantiate the aforementioned suggestion. On the other hand, we do not know what effect our diets would have on the turnover rate of apo E. Furthermore, fortification of the diet containing soybean protein plus cholesterol with amino acids, does not affect cholesterol in IDL, but it reduces the apo E content of IDL (fig. 4E). Thus in one dietary group (SoyChol-SoyCholAA) high cholesterol and low apo E in IDL was observed, whereas in other groups (SoyChol-Soy and Soy) low cholesterol and low apo E or low cholesterol and high apo E were seen (Cas-Soy). Thus it may be concluded that apo E metabolism is not invariably linked to cholesterol metabolism. It should be stressed that we have only qualitatively estimated the concentration of apo E in the lipoprotein fractions by visual inspection of SDS-polyacrylamide gels. It will be necessary to substantiate our observations by direct quantitation of apo E.

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