Development and partial metabolic characterization of a dietary cholesterol-resistant colony of rabbits

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Abstract A colony of New Zealand white rabbits has been developed which, when fed a cholesterol-supplemented diet, exhibit unusual resistance to hypercholesterolemia and atherosclerosis, disorders usually observed in normal cholesterol-fed rabbits. When resistant rabbits (RT) were fed a normal low cholesterol diet (ND), their plasma lipoprotein patterns were significantly different from those of normal rabbits (NR) fed the same diet. The low density lipoprotein cholesterol (LDL-c)/high density lipoprotein cholesterol (HDL-c) ratio and LDL-c/very low density lipoprotein cholesterol (VLDL-c) ratio were lower in the resistant rabbits. The hydrated density of HDL of the normal-responsive rabbits was greater than that of the resistant rabbits. LDL from resistant rabbits contained a lower proportion of esterified cholesterol and protein than LDL from normal rabbits. Peripheral mononuclear cells from resistant rabbits bound about 30% more ^{125}I-labeled rabbit LDL than mononuclear cells from normal rabbits. These results demonstrate that the plasma cholesterol levels of these animals is at least partly under genetic control and that compositional differences exist between the major plasma lipoprotein classes of normal and resistant rabbits even during the ingestion of low-cholesterol diet. The results indicate that at least a part of the difference in the cholesterolemic responses between the two rabbit groups is due to an enhanced LDL uptake by the mononuclear cells, and presumably by other somatic cells of the resistant group.—**Overturf, M. L., S. A. Smith, D. Hewett-Emmett, D. S. Loose-Mitchell, M. R. Soma, A. M. Gotto, Jr., and J. D. Morrisett.** Development and partial metabolic characterization of a dietary cholesterol-resistant colony of rabbits. *J. Lipid Res.* 1989. **30:** 263–273.

Supplementary key words cholesterol diet • plasma lipoproteins • cholesterolemia phenotypes • pedigree • mononuclear cell metabolism

The extensive inter-individual variability of plasma cholesterol and lipoprotein concentrations among humans and other mammals in their response to changes in dietary cholesterol is well documented. Results from studies on humans maintained under well-controlled (e.g., diet ward) conditions indicate that changes in dietary cholesterol produce widely differing responses in plasma cholesterol (1–6). This heterogeneity in response of plasma cholesterol levels to dietary cholesterol intake has also been observed in experimental animal populations. It was documented first in rabbits (7–9) and subsequently observed in pigeons (10), nonhuman primates (11, 12), rats (13), and mice (14). Regardless of the population or species being studied, most animals responded to a cholesterol-enriched diet with significant hypercholesterolemia and were therefore classified as hyper-responders. However, a small fraction of these populations sometimes exhibited very little change in response to the dietary cholesterol challenge, and were therefore termed hypo- or nonresponders. Although there is substantial evidence that much of the variability in plasma cholesterol response is due to genetic factors (8, 11, 13, 15, 16), mechanism(s) directly responsible are not well delineated.

During the course of a previous study in which a population of New Zealand white rabbits was fed a 0.1% cholesterol-enriched diet for 7 months, a protocol that typically caused severe hypercholesterolemia in these animals, a single male was observed to maintain a plasma cholesterol level that never increased above that measured during the consumption of an essentially cholesterol-free diet (17). In order to determine the metabolic basis by which this animal maintained a normal plasma cholesterol level, we initiated the development of a colony of rabbits that exhibited this phenotypic trait. We have now undertaken a series of systematic studies designed to determine which

Abbreviations: NR, normal-responsive (typical rabbit); IT, intermediate-resistant (rabbit); RT, cholesterol-resistant (rabbit); ND, normal diet; CD, cholesterol diet; HDL, high density lipoproteins (d 1.063–1.210 g/ml); LDL, low density lipoproteins (d 1.006–1.063 g/ml); VLDL, very low density lipoproteins (d < 1.006 g/ml); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; LP, lipoprotein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum.

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metabolic pathways have been altered in such a way as to confer this trait. In this initial report, we describe the inheritance of this trait, and compare the plasma lipoproteins of these hyporesponsive rabbits with lipoproteins of their normo-responding counterparts. We also include a comparison of the metabolism of low density lipoproteins by peripheral mononuclear cells from each type of animal.

MATERIALS and METHODS

Housing and diets

All rabbits, with the exception of does with litters, were caged individually in a room temperature-controlled at 20 ± 2°C and cycled with 12 hr light and darkness. Water was available at all times. Animals were fed a standard laboratory Purina rabbit chow that contained ~60 μg cholesterol per g as determined from $CHCl_3$–CH_3OH 2:1 (v/v) extracts. The 0.1% cholesterol-enriched diet consisted of the same standard chow supplemented with 0.10 g of cholesterol (USP) and 0.5 ml of corn oil per 100 g chow. The cholesterol and corn oil were dissolved in a large volume of chloroform (ACS grade) and mixed with the pellets which had been freed of fines by sieving (18). The saturated pellets were left in a fume hood until the odor of chloroform was not detectable. Rabbits were restricted to 100 g of chow per day until they were 4 months old; thereafter the ration was increased to 150 g/day.

Phlebotomy

Blood (≤30 ml) was drawn with a butterfly infusion set (23 gauge × 3/4) from the central ear artery of calm, unanesthetized animals. Plasma for lipoprotein analysis and genetic marker studies was obtained by collecting blood after a 14-hr overnight fast into disodium EDTA (1 mg/ml), followed by separation of the plasma by low-speed centrifugation. Mononuclear cells were also obtained from blood in the same way.

Breeding stock selection

Dams (2.0–2.5 kg) of a large New Zealand White rabbit breeding colony, from which the progenitor was derived, were selected randomly and tested for susceptibility to hypercholesterolemia after they had ingested the 0.1% cholesterol-enriched test diet for 8 weeks. Plasma cholesterol levels were determined prior to feeding the cholesterol-enriched diet (basal cholesterol level), and at 4-week intervals during consumption of the test diet for 8 weeks. The rabbits from the lowest ten percentiles, determined from the means of 2-month plasma cholesterol concentrations, were used for breeding with the progenitor. The dams were not bred until at least 8 weeks had elapsed since their last cholesterol-enriched diet meal. Progeny were weaned from the dams at 6 weeks after birth, caged individually, and fed the basic chow diet until they were tested for relative dietary-cholesterol responsiveness.

Phenotypes of animals of the experimental pedigree

When the progeny were at least 16 weeks of age their plasma cholesterol levels were determined. They were then fed the cholesterol-enriched chow for at least 8 weeks and their plasma cholesterol levels were determined at 4-week intervals. Typical rabbits from the same colony were obtained randomly and tested for response to the diet. All of these animals were classified as susceptible to hypercholesterolemia as defined below. These rabbits served as control animals.

Classification of relative susceptibility

Rabbits in the pedigree that had been fed the cholesterol-enriched diet were grouped into one of three categories based on their susceptibility to hypercholesterolemia. The categories of assignment were normal-responsive (NR), intermediate-resistant (IT), and resistant (RT). The designation of each primary category was based on a comparison with the mean plasma cholesterol levels of 15 rabbits that were randomly obtained from the large closed colony from which the male RT progenitor was derived.

Specifically, the cholesterol-resistant animals were defined as those whose elevation of plasma cholesterol levels after both week-4 and week-8 of cholesterol-enriched diet consumption were ≥ 1.5 standard deviations below the mean monthly elevations of the normal reference animals. After being fed the cholesterol-enriched diet for 4 weeks and 8 weeks, the reference animals had an increase in mean plasma cholesterol levels relative to the baseline level of 98 ± 34 mg/dl and 201 ± 116 mg/dl (mean ± SD), respectively. Accordingly, rabbits with an increase of <47 mg/dl after 1 month and <27 mg/dl after 2 months were defined as resistant animals. The intermediate-resistant animals were defined as those whose consecutive bimonthly cholesterol level elevations were one standard deviation below the mean monthly elevations of the normal control group. Thus, rabbits with plasma cholesterol elevations <64 mg/dl after 1 month and <85 mg/dl after 2 months of consuming the cholesterol-enriched diet were classified as intermediate-resistant when they did not meet the cholesterol-resistant criteria. Animals that did not meet either the criteria for RT or IT classification were categorized as normal rabbits.

Genetic marker studies

EDTA-treated whole blood was centrifuged at 1240 *g*. The cells were recentrifuged (2×) with isotonic saline and the upper layer (leukocyte-rich) and plasma were stored

(−70°C) for subsequent DNA and protein genetic marker studies. Among the five readily typed polymorphic markers in the blood of New Zealand White rabbits (19), we chose to type for erythrocyte 6-phosphogluconate dehydrogenase (PGD) and adenosine deaminase (ADA), based on our experience from parallel studies. The erythrocyte-rich portion (lower layer) was homogenized and extracted with a mixture of water and toluene. Aliquots of the aqueous phase were subjected to starch-gel electrophoresis, and the alleles were typed using standard histochemical stains for PGD and ADA (19).

Lipoprotein isolation

Sequential density ultracentrifugation of lipoproteins from the plasma of rabbits fed the normal diet was performed as described (20). VLDL was isolated after centrifugation at plasma density (d 1.006 g/ml) for 18 hr, 55,000 rpm, and 4°C in a Beckman L265B ultracentrifuge using a Ti 40.3 rotor. The LDL fraction was isolated at d 1.063 g/ml from the supernatant (18 hr), and the HDL fraction from the d 1.210 g/ml supernatant (40 hr).

Prior to chemical analyses, the lipoprotein fractions were dialyzed against buffer containing 150 mM NaCl, 1 mM EDTA, and 1 mM NaN_3, pH 7.4. The homogeneity of each lipoprotein density fraction obtained by either sequential centrifugation or of pooled fractions from zonal centrifugation, was determined by PAGE (21, 22). Any heterogenous fraction was purified by recentrifugation.

Zonal ultracentrifugation

Isolation of lipoproteins was also performed by zonal ultracentrifugation (23, 24). Total lipoproteins were separated from whole plasma using a nonlinear NaBr gradient (1.0–1.4 g/ml). The VLDL, IDL, and LDL were then analyzed with a linear gradient (1.0–1.3 g/ml); HDL analysis was performed on a nonlinear gradient (1.0–1.4 g/ml). After separation had occurred, the contents of the rotor were pumped out. The absorbance of the eluate at 280 nm was recorded continuously with an ISCO monitor equipped with a quartz flow-through cell.

Lipoprotein iodination

Rabbit LDL (d 1.03–1.05 g/ml) was isolated from blood collected in 0.1% EDTA from normal healthy New Zealand male rabbits by sequential ultracentrifugation as described above. LDL preparations from several animals were pooled, dialyzed against 150 mM NaCl, 0.3 mM EDTA, pH 7.4, and stored at 4°C after sterilization by Millipore (0.45 μm) filtration.

^{125}I-Labeled LDL was prepared using iodine monochloride according to the McFarlane procedure (25). Following iodination, 0.5 M malonate buffer was added to the ^{125}I-labeled LDL to obtain pH 7.4, and free iodine was removed by passage of the ^{125}I-labeled LDL preparation through a Bio-gel (Bio-Rad) P4 column. Samples were then dialyzed against 50 mM Tris, 1 mM EDTA, pH 7.4. The specific activity of the LDL preparation was a nominal 500 cpm/ng of LDL protein. More than 98% of the radioactivity was precipitated by a 15% TCA solution and less than 2% was extracted by chloroform–methanol 2:1 (v/v).

Lipoprotein-deficient serum

The lipoprotein-deficient serum was obtained by ultracentrifugation as described by Goldstein, Basu, and Brown (26), except that fetal bovine serum (FBS) rather than human plasma was used. The final preparation was sterilized by passage through a 0.45-μm filter, adjusted to a protein concentration of 50 mg/ml with 150 mM NaCl, and kept frozen at −20°C until use. The total cholesterol content of the LPDS was 15.0 μg/ml.

Polyacrylamide gel electrophoresis

An electrophoretic procedure that utilizes a sample gel, a spacer gel, and a separating gel for the resolution of HDL, LDL and VLDL (21) was used to determine the relative abundance of different lipoprotein classes in plasma, and to evaluate the homogeneity of ultracentrifuged fractions. The electrophoretic gels were scanned in Pyrex tubes with an ISCO model 1310 photometric gel scanner coupled to a model U5-A optical recorder unit (0.5 mm slit, 580 nm filter). Areas under the peaks were determined by planimetry (22).

Chemical analyses

The chemical composition of the major plasma lipoprotein classes separated by sequential ultracentrifugation was determined. Protein content was measured by the method of Lowry et al. (27), using bovine serum albumin as a standard. Inorganic phosphorus was measured by the method of Bartlett (28) using a factor of 25 to convert the results to estimates of phospholipid. Free cholesterol, cholesteryl ester, and triglyceride were estimated using enzymatic procedures (Boehringer Mannheim Biochemicals, Indianapolis, IN). All samples were assayed in duplicate.

Mononuclear cell isolation

Blood from healthy normal and resistant rabbits was collected under sterile conditions in 0.1% EDTA. After dilution (1:1) with PBS, 10 ml of blood were layered on 3 ml of Lymphoprep (29). Following centrifugation at room temperature, the mononuclear cells were collected and washed four times at 4°C with cold PBS. Cells were finally resuspended in DMEM. Cell viability was determined by the exclusion of 0.4% trypan blue and found to be always greater than 90%, both before and after the 48-hr incubation. The cells were seeded in 35-mm petri dishes at a density of about 2.0×10^6 cells per ml, as determined by cell cytometry, and incubated 48 hr in DMEM supplemented with 10% LPDS, L-glutamine

(0.3 mg/ml), and gentamycin (10 μg/ml) at 37°C in a 90% humidified atmosphere of air–CO_2 95:5. This procedure yielded approximately 2.0–2.5 × 10^7 cells from 20 ml of noncoagulated blood.

Mononuclear cell uptake and degradation of rabbit LDL

Rabbit LDL uptake and degradation by mononuclear cells was determined according to Ho et al. (30). Briefly, after incubating the cells for 48 hr in 10% LPDS, various amounts of ^{125}I-labeled LDL and unlabeled LDL were added and incubations were continued at 37°C for an additional 5 hr. Cells and medium were harvested in siliconized tubes and centrifuged at 1500 rpm for 10 min at 4°C. An aliquot of the supernatant was counted to assay degradation after precipitation of the unreacted iodinated LDL by 10% TCA, and after chloroform extraction of free iodine as described (31).

To assess total cellular uptake of labeled LDL, the cellular pellets were washed four times with cold PBS–BSA (5 mg/ml) buffer, resuspended in 100 μl of iced PBS in microfuge tubes, and centrifuged at 12,000 rpm for 2 min at 4°C. The supernatants were removed and the infranatants containing the pellets were counted.

Radioactivity determinations

^{125}I activity was determined in an LKB 1282 Compugamma gamma-counter (LKB Instruments, Inc., Rockville, MD) with counting to a standard error of less than ± 5%.

Statistical analysis

The comparisons of unpaired data were made using Student's *t*-test. The difference in the values obtained with resistant rabbits versus normal rabbits was judged significant at the $P < 0.05$ level. The data expressed in the figures and table are mean ± standard error of the mean unless otherwise stated.

RESULTS

During the course of previous studies using cholesterol-fed New Zealand White rabbits, one male from a group of 15 animals did not develop hypercholesterolemia while consuming a 0.10% cholesterol-enriched diet (17). When fed a normal chow diet, this animal had a plasma cholesterol level of 48 mg/dl; this remained essentially unchanged during the 7-month period when the rabbit was fed the cholesterol-enriched diet (**Fig. 1**). These levels were similar to those of a group of normal-responsive rabbits fed only normal chow diet; their plasma cholesterol levels had a baseline value of 65 ± 5 mg/dl which decreased to 31 ± 3 mg/dl by the end of 7 months.

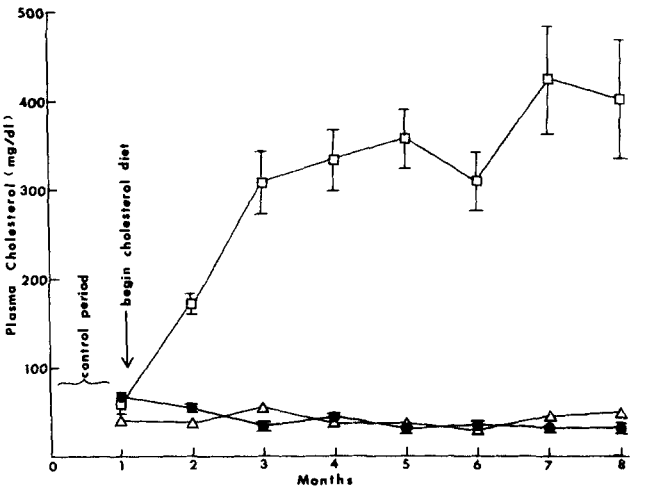

Fig. 1. Cholesterol response of normal and cholesterol-resistant rabbits. The data represent the plasma cholesterol levels of a group of normal rabbits (n = 14) (□) and the progenitor rabbit (△) fed the 0.1% cholesterol-enriched diet (wt/wt), and a group of normal rabbits (n = 15) (●) fed regular low-cholesterol rabbit chow. The averages of duplicate determinations were used to calculate group means ± SEM.

However, when this group of 15 normal-responsive rabbits was fed the cholesterol-enriched diet, their mean cholesterol level rose sharply to ~300 mg/dl over the next 8 weeks, and ultimately to ~400 mg/dl by the end of 7 months.

Physical measurements and blood chemistry determinations performed at regular intervals showed no remarkable differences between the resistant and normal-responsive rabbits when fed normal chow diets for 7 months. Body weight, an index of growth rate, for the normal rabbits increased from 2.03 ± 0.23 kg to 3.53 ± 0.31 kg after 7 months on a 0.1% cholesterol-enriched diet. The resistant animals' weight increased from 2.08 kg to 3.72 kg during the 7-month period on the same diet. There was no significant difference in mean systolic-diastolic blood pressure as determined by the ear-capsule method (32) between the resistant rabbit and normal-responsive rabbits on a normal chow diet or on a cholesterol-enriched diet. Over the course of study, mean arterial blood pressure in both RT and NR rabbits rose from 75 to 85 mm of mercury. Likewise, conventional clinical chemistry profile determinations of 20 serum analytes performed on a SMAC System Biochemical Analyzer demonstrated no significant difference between the resistant and the normal-responsive animals when fed the regular diet.

Pedigree and genetic markers

The progenitor resistant rabbit, designated I-2 in the pedigree thereby signifying the generation and progeny number, respectively (**Fig. 2**), was initially bred to typical does randomly selected from the commercial colony

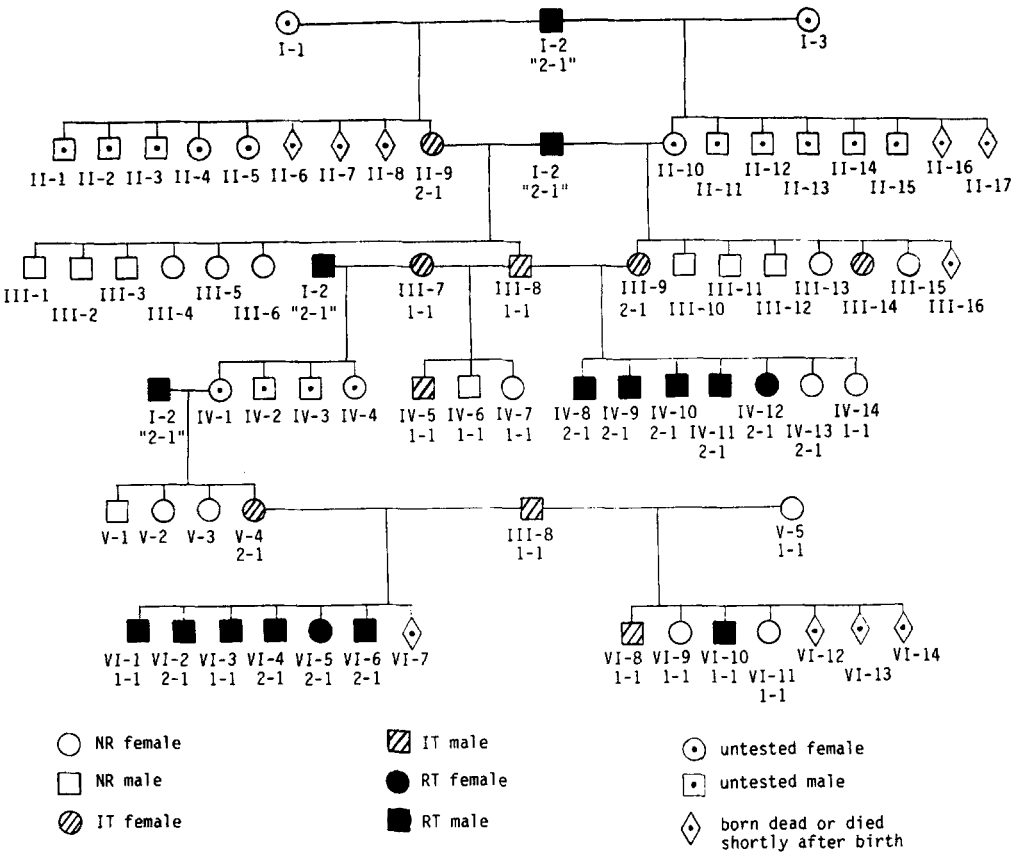

Fig. 2. Primary lineage of pedigree developed from male progenitor cholesterol-resistant rabbit. As indicated, not all progeny were challenged or tested by feeding the 0.1% cholesterol diet. The crosses with the progenitor male (I-2) that failed to lead to resistant progeny are not shown. Three phenotypes were defined on the basis of their cholesterolemic response to the 0.1% cholesterol diet, over a 2-month feeding period, according to statistical parameters (Materials and Methods). The phenotypic designations are: RT, cholesterol-resistant; IT, intermediate-resistant; and NR, normal-responsive. Members of each generation (I-VI) of the pedigree are identified by an Arabic numeral after the Roman numeral designating the generation, e.g., III-8. When determined, the marker phenotype for the polymorphic genetic marker, 6-phosphogluconate dehydrogenase, is shown below the pedigree identification, i.e., 1-1, 2-1, 2-2: the marker phenotype of the progenitor male (I-2) was inferred to be "2-1" based on the phenotypes of his descendents; III-8 (1-1) excludes the phenotype 2-2 for the progenitor male and V-4 (2-1) excludes the possibility that he had phenotype 1-1.

which produced the progenitor. Of the numerous such crosses (only two are shown: I-1 and I-3), only one F_1 sibling (II-9) was classified as intermediate-resistant; however, not all progeny were fed the cholesterol-enriched diet and were thus untested regarding their plasma cholesterol-responsiveness phenotype.

When this rabbit was backcrossed to her sire (I-2), again just one IT rabbit (III-8) was obtained. Two intermediate-resistant rabbits (III-9 and III-14) resulted from backcrossing an untested F_1 doe (II-10) with the progenitor. However, when rabbit III-9 was bred to III-8, four resistant bucks and one resistant doe were produced.

The lower half of the pedigree shows the results of utilizing two randomly obtained does (III-7 and V-5) of a group which, when fed the cholesterol-enriched diet, were in the lowest 10 percentile of plasma cholesterol levels. Significantly, although these rabbits were the lowest in terms of cholesterol responsiveness, one animal (III-7) was classified by our criteria as being only intermediate-resistant, and the other (V-5) as normal-responsive.

When the intermediate-resistant rabbit III-7 (obtained outside the lineage) was bred with I-2, and then an untested F_1 rabbit resulting from that cross was backcrossed to I-2, an intermediate doe (V-4) was produced. This doe, when bred with buck III-8, subsequently produced six resistant progeny; an untested littermate died from trauma shortly after birth.

Adenosine deaminase (ADA; EC 3.5.4.4) and 6-phosphogluconate dehydrogenase (6-PGD; EC 1.1.1.44), two erythrocyte enzymes previously shown to exhibit genetic polymorphism in inbred and wild rabbits (19), were typed from fresh cells. The genetic marker determinations of selected animals in the pedigree demonstrated that although the ADA locus was monomorphic, 6-PGD ex-

hibited two alleles. As a consequence, 6-PGD was used as the informative marker for pedigree verification. Since the progenitor died before it was typed, its 6-PGD genotype was inferred from the pedigree and its heterozygosity at the 6-PGD locus was given the Arabic numbers designation (2-1) and denoted by quotation marks through the pedigree. Examination of the genetic typing of 6-PGD provided support for the accuracy of the pedigree as shown.

Phenotype

After the rabbits were phenotyped according to their cholesterolemia response to the cholesterol-enriched diet, it was observed that those classified as comprising the hypercholesterolemia-resistant group also had significantly lower cholesterol levels when consuming the regular chow diet. There was no statistically significant difference between the responses of the intermediate and normal-responsive group during the 16-week feeding period when they were both consuming regular low-cholesterol rabbit chow (**Fig. 3**). However, after the animals had been fed the cholesterol-enriched diet for 4 weeks, the difference became significant ($P < 0.05$). At the end of the 8th week, the difference between IT and NR group mean values increased due to the increase in the mean response of the normal group. The plasma cholesterol concentration of the resistant group, after being fed the cholesterol-enriched diet for 8 weeks, was not significantly altered from that measured during consumption of the regular diet.

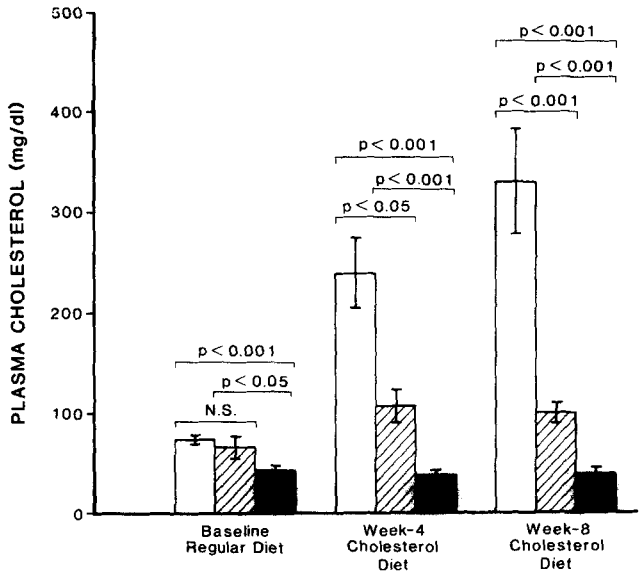

Fig. 3. Effect of the 0.1% cholesterol diet on plasma cholesterol concentrations of the progeny. Until the rabbits were 16 weeks of age they were fed regular rabbit chow. Then the plasma cholesterol concentrations were determined and they were fed the cholesterol diet for 8 weeks, during which time the plasma cholesterol concentrations were measured at the end of each 4-week interval. The changes in plasma cholesterol concentration of the individual animals were used to classify each into one of three groups according to their cholesterolemic response. The total number of rabbits phenotyped in this manner was 34. Of these, 13 were classified as cholesterol-resistant (darkened bar), 7 were intermediate-resistant (striped bar), and 14 were normal-responsive (open bar). All cholesterol determinations were performed in duplicate and the average values were used to calculate group means ± SEM.

Lipoprotein class composition

Densitometric scans were obtained for lipoproteins in the plasma of RT and NR rabbits fed normal chow diet for 32 weeks, and then cholesterol-enriched diet for 8 weeks. During the normal diet feeding period, LDL concentration was higher and VLDL concentration was lower in the normal animals than in the resistant animals (**Fig. 4A, C**). At the end of the 8-week feeding period, there was little change in the distribution of lipoproteins of the RT rabbits (Fig. 4D). In contrast, the distribution of lipoproteins in the NR rabbits on cholesterol-enriched diet was markedly altered; the HDL concentration was greatly reduced and the LDL and VLDL (β-VLDL) concentrations were increased, with the increase in the β-VLDL mass being relatively much greater (Fig. 4B).

Because of the different electrophoretic distributions of lipoproteins observed in the plasma from resistant and normal-responsive animals during consumption of regular chow, evaluation by an independent methodology was deemed appropriate. Pooled plasmas from RT and NR animals that had been on normal diet long enough for their cholesterol concentrations to become normalized were separated into their major lipoprotein classes by zonal ultracentrifugation. The results shown in **Fig. 5A** indicate that HDL was the predominant lipoprotein class, and that there was comparatively more HDL in plasma of the resistant than in the normal animals. Recentrifugation of the LDL/VLDL fraction on a shallow density gradient clearly showed that there was more LDL in the normal rabbits (Fig. 5B). Recentrifugation of the HDL fractions (from Fig. 5A) confirmed the electrophoretic results and revealed that the hydrated density of HDL from the resistant group was detectably lower than HDL from the normals (Fig. 5C).

Lipoprotein chemical composition

The chemical compositions of the major lipoproteins separated by sequential ultracentrifugation of the plasma from five normal and five resistant animals fed regular chow are shown in **Table 1.** There was considerable variability between the lipid and protein constituents in the lipoprotein classes from individuals of each group. Despite this high inter-individual variability, statistically significant differences ($P < 0.05$) in the protein, triglyceride, and cholesteryl ester components of the LDL from the resistant and normal-responsive groups were found. The mean percent abundance of triglyceride in LDL from

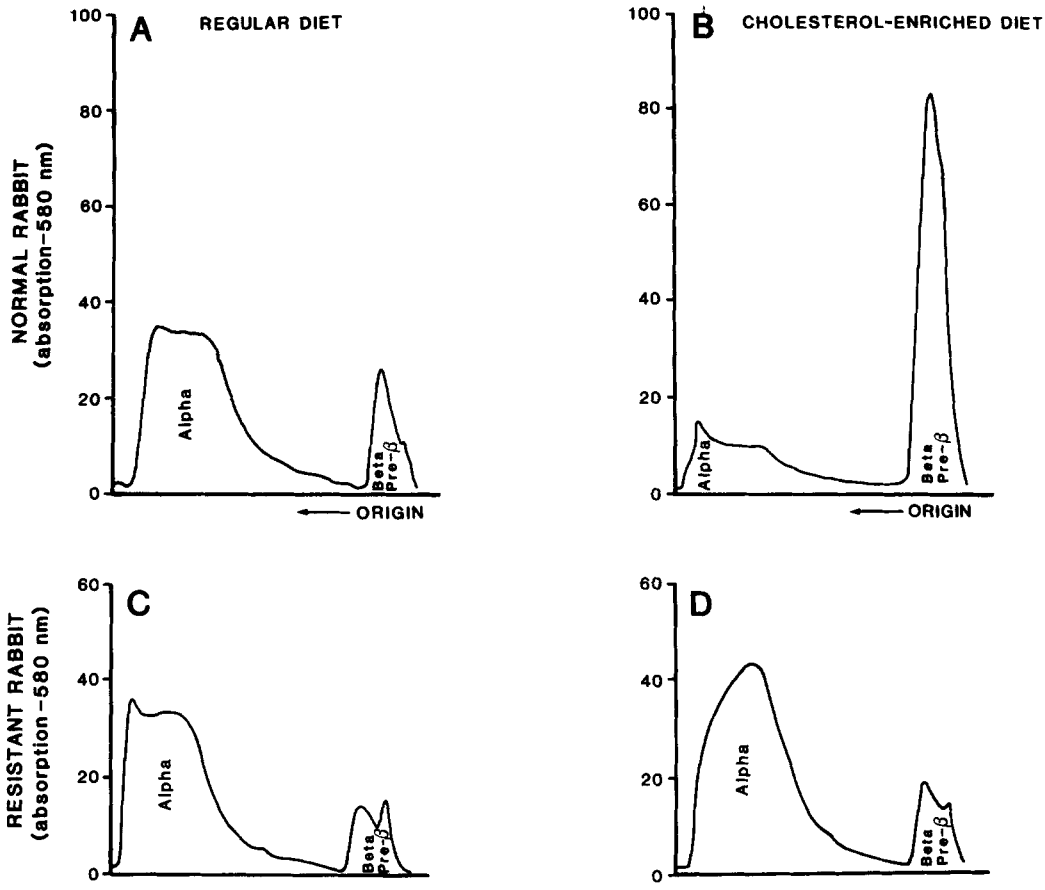

Fig. 4. Tracings of densitometric scans of plasma lipoproteins from a representative normal and a cholesterol-resistant rabbit separated by polyacrylamide gel electrophoresis. Panels A and B show scans from a normal rabbit fed a cholesterol-free diet for 32 weeks, followed by a cholesterol-enriched diet for 2 months, respectively. Panels C and D represent scans of plasma from a cholesterol-resistant rabbit with the same dietary regimen as described for the susceptible animal.

RT animals was 39.4 compared to 27.0 for normal animals. This difference was compensated for by the occurrence of 20.9 and 30.2% cholesteryl ester in the LDL from these respective animal groups.

Mononuclear cell metabolism

Studies were performed to determine whether there was a difference in the metabolism of lipoproteins by peripheral mononuclear cells from resistant and normal-responsive rabbits. The cells were obtained from animals that had eaten only normal chow diet for 32 weeks. Thus, the plasma cholesterol of normal animals had reverted to their control (pre-cholesterol diet) levels. The cells were derepressed by incubation in LPDS for 48 hr prior to the study.

The saturation curves for total incorporation and degradation of pooled normal rabbit ^{125}I-labeled LDL were nearly linear over a 10-hr incubation period. In this case, a 20-fold excess of unlabeled LDL inhibited specific degradation by 75%.

Using parameters established from these studies, the ^{125}I-labeled LDL incorporation and degradation by mononuclear cells from 10 normal-responsive and 10 resistant rabbits (**Fig. 6**) were determined. The cells obtained from the resistant animals demonstrated about 30% higher rate of internalization and degradation of ^{125}I-labeled LDL. The nonspecific incorporation and degradation was similar for the cells from both groups of animals.

DISCUSSION

The major goal of this study was to develop a colony of NZW rabbits that would not develop hypercholesterolemia when fed a cholesterol-enriched diet. The impetus for this study was the discovery of a male rabbit which, unlike the numerous cholesterol-fed rabbits of our earlier studies (17, 33–35), showed no increase of plasma cholesterol concentration in response to the diet. We then decided to de-

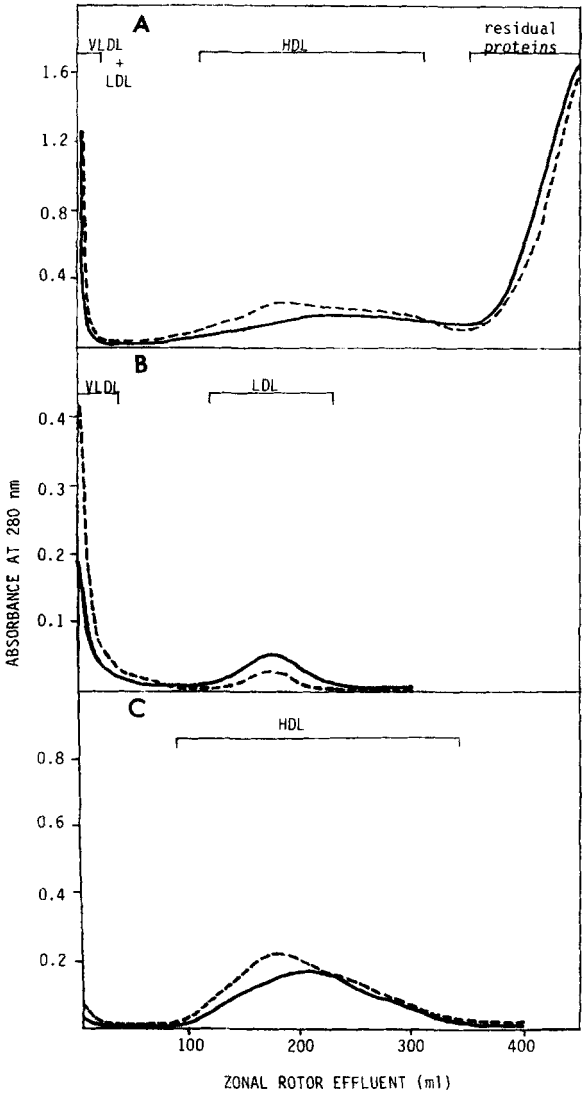

Fig. 5. Zonal rotor effluent patterns of plasma from normal-responsive (—) and cholesterol-resistant (-----) rabbits fed the cholesterol-free diet for 8 months. Ten milliliters of plasma from each of ten rabbits per group were pooled (panel A). Panel B shows the patterns obtained from pooled VLDL-LDL fractions (panel A), and panel C represents the effluent patterns obtained from pooling the HDL fractions (panel A) followed by centrifugation.

termine whether this unusual degree of resistance to dietary hypercholesterolemia exhibited by this rabbit was inherited and if so, to develop and characterize the lipoproteins of a colony of rabbits expressing the trait. A colony consisting of an extended pedigree of cholesterol-resistant (RT) animals would constitute a valuable resource for subsequent studies defining the responsible mechanism(s), and its probable genetic control involved in the complex regulation of dietary cholesterol homeostasis.

By repetitively utilizing the resistant buck in a breeding scheme which included backcrosses and sib matings, a lineage was developed which was comprised of resistant and normal-responsive rabbits, and of rabbits whose cholesterolemic response was intermediate (IT). All of their conventionally measured blood analytes were within normal range. A reduction in the growth rate, which would indicate that the RT-progenitor might have a major malabsorption syndrome or was otherwise unhealthy, was not observed. These observations, together with the demonstration that responsiveness to dietary cholesterol in NZW rabbits is inherited (8) and that inbred strains of JAX rabbits respond differently to dietary cholesterol (36), made it plausible that a pedigree containing resistant rabbits was obtainable.

The progenitor rabbit died at 2.75 years of age, and nine additional resistant rabbits were killed after they had consumed the cholesterol diet for at least 7 months. Of these animals, none had atherosclerotic lesions at necropsy. In contrast, typical rabbits from the progenitor derivative colony, fed the same amount of cholesterol for a comparable period of time, had a mean aortic surface atherosclerotic lesion index of about 50% (35). Other common manifestations of hypercholesterolemia such as cutaneous xanthomas, cholesterol deposits in the eyes, and cholesterol-infiltrated livers, were also absent in the resistant animals.

The pedigree was designed with the fundamental long-term objective of developing a strain of resistant rabbits from a breed which is typically very sensitive to dietary cholesterol. Efforts were made to avoid developing either a multifactorial polygenic resistant strain or inbreeding depression which is known to be a serious problem in rabbits. These included utilizing programs of line breeding by distant backcrossing, linecrossing, and outcrossing. Future development of this pedigree will continue to employ a distant backcrossing strategy which will allow for the promotion of homozygosity at the loci affecting cholesterol-resistance and avoid inbreeding depression which commonly occurs with traditional backcrossing. Our initial intent was to investigate the potential for the inheritance of dietary cholesterolemia in these animals rather than to determine the mode of inheritance. As a result, although the complex mating scheme which utilizes only a small number of animals and outcrossings does not yet permit firm conclusions regarding inheritability or mode of inheritance, the pedigree strongly suggests that "cholesterol-resistance" is a polygenic trait.

Plasma lipoproteins (class and chemical composition)

Since it was found that the resistant rabbits frequently were those which had lower basal diet (regular low-cholesterol rabbit chow) plasma cholesterol levels (< 40 mg/dl), studies were performed to characterize and compare the major plasma lipoprotein classes during basal diet consumption. Electrophoretic mobility analysis, which separates plasma lipoproteins on the basis of

TABLE 1. Percentage composition of lipoproteins from resistant (RT) and normal (NR) rabbits consuming regular diet

Rabbit	Protein	Triglyceride	High Density Lipoproteins: Cholesterol	High Density Lipoproteins: Esterified Cholesterol	Phospholipid
			%		
Cholesterol-resistant rabbits					
RT-1	56.9	5.0	1.4	19.2	17.5
RT-2	50.5	8.2	1.8	19.2	20.4
RT-3	60.1	7.0	1.0	15.0	16.9
RT-4	59.5	10.1	0.2	13.8	16.5
RT-5	62.8	8.8	0.7	13.3	14.3
Mean ± SD	58.0 ± 4.2	7.8 ± 1.7	1.0 ± 0.5	16.1 ± 2.6	17.0 ± 2.0
Normal Rabbits					
NR-1	63.0	8.2	0.9	13.6	14.4
NR-2	56.6	11.6	0.7	12.5	18.6
NR-3	41.8	5.5	3.5	24.4	24.7
NR-4	54.4	9.6	1.7	16.3	17.9
NR-5	58.6	5.5	2.0	18.0	15.9
Mean ± SD	54.9 ± 7.1	8.1 ± 2.4	1.8 ± 1.0	17.0 ± 4.2	18.3 ± 3.5
		Low Density Lipoproteins			
Cholesterol-resistant rabbits					
RT-1	14.6	44.6	5.3	21.7	13.8
RT-2	19.2	37.9	5.6	21.0	16.3
RT-3	15.1	35.8	5.2	22.8	21.1
RT-4	19.0	42.9	5.3	17.4	15.4
RT-5	20.5	36.0	5.4	21.4	16.7
Mean ± SD	17.7 ± 2.4[a]	39.4 ± 3.6[a]	5.4 ± 0.1	20.9 ± 1.8[a]	16.7 ± 2.4
Normal rabbits					
NR-1	20.8	30.2	6.1	29.1	13.7
NR-2	19.1	39.6	4.9	23.0	13.4
NR-3	20.0	11.1	10.5	41.6	16.7
NR-4	22.6	33.9	6.0	24.4	13.0
NR-5	24.4	20.1	6.8	33.0	15.8
Mean ± SD	21.4 ± 1.9	27.0 ± 10.2	6.9 ± 1.9	30.2 ± 6.7	14.5 ± 1.4
		Very Low Density Lipoproteins			
Cholesterol-resistant rabbits					
RT-1	8.7	44.1	0.3	1.7	45.2
RT-2	7.1	65.0	1.8	4.2	21.9
RT-3	9.1	60.8	1.6	5.9	22.6
RT-4	10.8	65.2	2.6	5.5	15.5
RT-5	9.4	64.2	3.1	8.1	15.2
Mean ± SD	9.0 ± 1.2	59.9 ± 8.1	1.9 ± 0.9	5.1 ± 2.1	24.1 ± 11.0
Normal rabbits					
NR-1	7.7	51.8	1.5	5.7	33.4
NR-2	5.9	65.0	2.3	6.1	20.8
NR-3	11.4	34.6	2.3	11.8	39.9
NR-4	8.2	48.1	2.9	8.2	32.6
NR-5	6.0	46.4	1.7	6.4	39.5
Mean ± SD	7.8 ± 2.0	49.2 ± 9.8	2.1 ± 0.5	7.6 ± 2.2	33.2 ± 6.9

[a]Significantly different, $P < 0.05$ compared to the mean of the normoresponsive group.

both charge and size difference, failed to consistently reveal a difference in mobility of the lipoproteins from the two groups of rabbits (NR and RT) during consumption of regular chow. The ratio of LDL-cholesterol to HDL- and VLDL-cholesterol was, however, lower in the resistant rabbits. After both groups were then fed the cholesterol-enriched diet for 8 weeks, consistent substantial differences were observed. In accordance with numerous studies of normal cholesterol-fed NZW rabbits, the VLDL (β-migrating cholesteryl ester-rich VLDL) and LDL were greatly increased, and HDL was decreased in the normal-responsive rabbit. By contrast, there was no

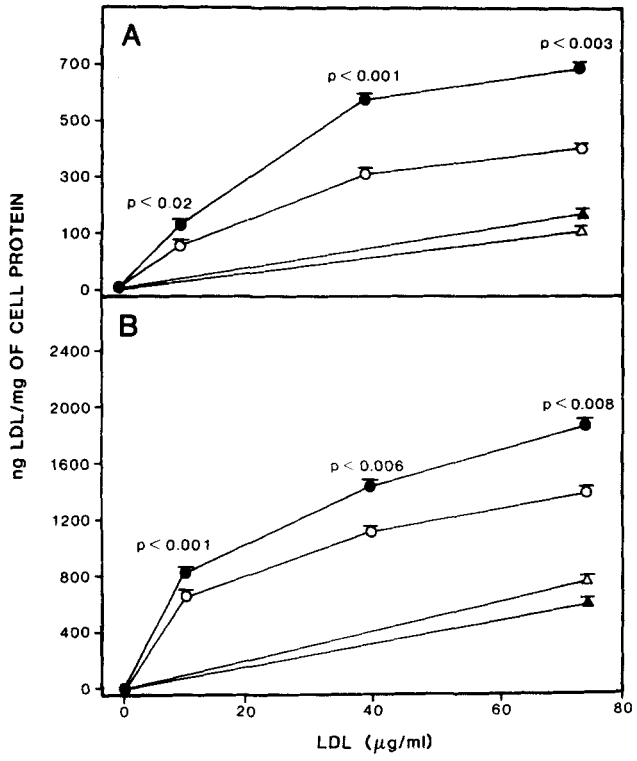

Fig. 6. Dose-dependent ^{125}I-labeled LDL incorporation (panel A) and degradation (panel B) by mononuclear cells from normal (○) and cholesterol-resistant (●) rabbits. Peripheral mononuclear cells were isolated and incubated in media supplemented with 10% lipoprotein-deficient serum for 48 hr at 37°C in an atmosphere of 95% air and 5% CO_2. Normal rabbit ^{125}I-labeled LDL was then added to the media at increasing concentrations, in either the presence or absence of 20-fold excess unlabeled rabbit LDL, and the incubations were continued for 5 hr. The nonspecific incorporation and degradation by the cells from normal-responsive and resistant rabbits are denoted by (△) and (▲) symbols, respectively. Each data point represents the mean ± SEM of values obtained from individual incubations of cells from ten NR and ten RT rabbits.

substantial difference observed in either the mobility or cholesterol content of the lipoproteins in the resistant rabbits.

Zonal ultracentrifugation, using both continuous and step gradients, indicated that during basal diet ingestion there was proportionately more HDL and less LDL in the plasma of the resistant rabbits. In addition, it appeared that the HDL of this group of animals was less dense than the HDL of normal-responsive rabbits. However, there were no statistically significant differences in the chemical composition between the HDL classes from either group of rabbits fed regular chow which could explain the slight disparity in densities indicated by the centrifugation data. On the other hand, there was significantly less esterified cholesterol and protein in the LDL of the resistant animals.

Mononuclear cell metabolism

Because of the sharp divergence of responses exhibited by resistant and normal-responsive rabbits to dietary cholesterol, it was important to determine whether the difference was manifested by LDL receptor activities. To evaluate this possibility, yet avoid traumatizing or killing animals in the pedigree being developed, peripheral mononuclear cells rather than fibroblasts or hepatocytes were used.

When mononuclear cells from normal and resistant rabbits were first derepressed and then provided with labeled rabbit LDL, about 30% more LDL was taken up and degraded by resistant-rabbit cells. This is analogous to the differences reported for mononuclear cells from humans where a negative correlation was found between the increment in plasma cholesterol concentration and the capacity of cells to degrade ^{125}I-labeled LDL after receptor derepression (37).

The findings of the present study demonstrate that a colony of New Zealand rabbits that are strongly resistant to dietary hypercholesterolemia has been developed through selective breeding. Secondly, compositional differences in plasma lipoprotein classes of resistant and susceptible rabbits are present even during the ingestion of regular low-cholesterol diet. Thirdly, the difference in the cholesterolemic responses of the normal-responsive and resistant rabbits appears to be related in part to differences in the capacity of their mononuclear cells, and presumably other peripheral cells, to catabolize LDL. This study represents the first report of a series of ongoing investigations aimed at elucidating mechanisms controlling cholesterolemic response to dietary cholesterol.

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