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Rabbit serum esterase genotyping and relationship to serum cholesterol response and basal serum HDL cholesterol level

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Introduction

When animals are fed with cholesterol-rich diets certain individuals respond with only a small increase in serum cholesterol concentration (*hyporesponders*), whereas others develop a marked hypercholesterolemia (*hyperresponders*) This phenomenon, which appears to be under genetic control, has been well-established in various animal species, and also in humans (*Beynen et al.* 1987).

After feeding a cholesterolemic diet to inbred strains of rats and rabbits it was found that differences in the increase of the serum cholesterol level was often associated with genetically determined differences in serum esterase patterns. The cholesterolemic response of rats was low in 6 out of 7 inbred strains displaying an esterase zone with high anodal mobility (which in rats is called ES-1), whereas absence of the enzyme was associated with the development of a high degree of hypercholesterolemia after cholesterol feeding in 2 out of 3 inbred strains (*Van Zutphen & Den Bieman* 1981a). Similar results were obtained in 6 inbred strains of rabbits. Hyporesponsive rabbit strains displayed a fast anodal serum esterase band after electrophoresis (called EST-2F' or EST-2f here), but the hyperresponders did not (*Van Zutphen & Fox* 1977). This association of hyporesponsiveness to dietary cholesterol with the Est-2 genotype, was also observed in hybrids between New Zealand White and Vienna White rabbits (*Van Zutphen et al.* 1981b). The Est-2 locus of the rabbit is assumed to be homologous with the ES1 locus in the rat (*Van Zutphen & Den Bieman* 1988).

The genetics of rabbit esterases has been studied extensively. Nine loci (Es-1, Es-2, Es-3, Est-1, Est-2, Est-3, Est-4, Est-5 and Est-6) have been identified. Six of these loci were found to be linked in Linkage Group VI (LG VI). Two clusters can be distinguished in LG VI: Es-1,2 and Est-1,2,4,6. The distance between these two clusters of esterase loci is 10.6 cM. Es-3, Est-3 and Est-5 segregate independently (*Fox* 1994).

To further investigate the relationship between esterase loci and cholesterol metabolism in the rabbit backcrosses and a F_2 of the hyporesponsive IIIVO/JU and hyperresponsive AX/JU inbred strains were made. The progeny of these crosses were genotyped for Est-2 and Es-1, and the basal serum HDL cholesterol level as well as the serum cholesterol response after a cholesterol-rich diet of all animals were determined.

Materials and Methods

Experimental procedures:

To determine whether alleles of the Est-2 and/or Es-1 locus cosegregate with serum cholesterol response or basal serum HDL cholesterol level, three segregating populations, derived from inbred IIIVO/JU and AX/JU rabbits, were used: progeny from a F_2, progeny form a backcross to the IIIVO/JU strain (= BC_1) and progeny form a backcross to the AX/JU strain (= BC_2). AX/JU is

a dietary cholesterol susceptible (hyperresponding) strain and IIIVO/JU is a dietary cholesterol resistant (hyporesponding) strain (*Van Zutphen & Fox* 1977). Furthermore, IIIVO/JU and AX/JU rabbits have high and low basal serum HDL cholesterol levels, respectively (*Van Lith et al.* 1996). Details on the experimental procedures (animals, housing, diets, blood sampling and serum cholesterol determinations) are described elsewhere in (*Van Lith et al.* 1996).

Electrophoretic and staining techniques:
The serum esterase genotypes were determined by discontinuous horizontal starch gel electrophoresis. Electrophoresis of serum was carried out as previously described (*Van Zutphen* 1974). The gel buffer was composed of 14.4 mM Tris, 5.3 mM citric acid, and 7.6 mM boric acid. LiOH (3.0 M) was used for adjusting the pH (6.5). The electrode buffer consisted of 0.38 M boric acid. LiOH (3.0 M) was added to achieve pH 8.0. After electrophoresis the gels were stained for esterase activity using the azo dye procedure as described previously (*Van Lith et al.* 1991); α-naphthylacetate was used as substrate and Fast Blue BB as the coupling salt. IIIVO/JU rabbits display the EST-2f' zone and are homozygous for the Es-1^a genotype, whereas AX/JU rabbits have no fast anodal serum esterase zone and are homozygous for the Es-1^b genotype (*Van Zutphen & Fox* 1977, *Van Lith et al.* 1992b).

Table 1. Calculation of the expected genotypic ratio in pooled male and female rabbits.

		Genotype[1]		
Cross	Number	A/A	A/I	I/I
		Male rabbits		
BC_1	26	0	13	13
BC_2	44	22	22	0
F_2	65	16.25	32.5	16.25
Total	135	38.25	67.5	29.25
	[n = 14	*3.967* :	*7.000* :	*3.033*]
		Female rabbits		
BC_1	31	0	15.5	15.5
BC_2	32	16	16	0
F_2	77	19.25	38.5	19.25
Total	140	35.25	70	34.75
	[n = 14	*3.525* :	*7.000* :	*3.475*]

[1] A = AX/JU-allel, I = IIIVO/JU-allel.

Statistical analyses:
For both sexes and for each trait (serum cholesterol response or basal serum HDL cholesterol level) the backcross and F_2 data were pooled and ranked from low to high. Then, as a primary analysis, 14 animals with "low" values for the trait and 14 animals with "high" values for the trait were identified and genotyped for the Est-2 and Es-1 locus. Genotyping of the 10% extremes of the phenotypic distribution is a strategy recommended by *Lander & Botstein* (1989). It was tested if there was a possible deviation in the "low" and "high" rabbits from the calculated, expected Mendelian ratio's (see Table 1). A deviation might imply genetic linkage. Additional analysis involving comparison of the phenotypes after grouping by genotype has also been performed. If the Est-2 or Es-1 locus and the trait are segregating independently, the trait values will be equally distributed among the two homozygote and the heterozygote genotypes.
Results for the two traits are presented as means±SEM. The Kolmogorov-Smirnov one-sample test was used to check normality of these data. All results within groups were found to be normally distributed. Deviation from the expected Mendelian ratio for each locus and for each trait in the "low" and "high" rabbits was tested using the chi-squared test. The significance of the difference between the segregating genotype groups was calculated by two-way analysis of variance with gender and genotype as factors. Homogeneity of the variances was tested using Bartlett's test. The variances were similar. For the two-way analysis of variance $P = 0.001$ was chosen as a critical limit for detection of linkage in order to reduce type I error (false positive linkage). In all other cases, the probability of a type I error <0.05 was taken as a criterion of significance. All statistical analyses were carried out according to *Steel & Torrie* (1981) using the SPSS PC+ computer program (*SPSS Inc.* 1990).

Table 2. Distribution of esterase genotypes (Est-2 and Es-1) among hyporesponding and hyperresponding rabbits[1].

Locus	Gender	Phenotype	(Mean±SEM)	Genotype[2] A/A	A/I	I/I	Chi-square	(and P)
Est-2	Males	Hypo	(11.6±0.5)	2	7	5	2.3	(0.325)
		Hyper	(45.7±1.6)	2	8	4	1.4	(0.490)
	Females	Hypo	(17.2±0.6)	3	3	8	8.3	(0.016) *
		Hyper	(53.0±1.3)	4	8	2	0.8	(0.659)
Es-1	Males	Hypo	(11.6±0.5)	2	10	2	2.6	(0.271)
		Hyper	(45.7±1.6)	3	8	3	0.4	(0.827)
	Females	Hypo	(17.2±0.6)	3	3	8	2.5	(0.289)
		Hyper	(53.0±1.3)	4	9	1	2.4	(0.301)

[1] Serum cholesterol response (mM) is calculated as the difference between serum total cholesterol concentration at day 35 and day 0.
[2] A = AX/JU-allel, I = IIIVO/JU-allel.
* Significant deviation from expected [3.525 : 7 : 3.475]-ratio in females (see Table 1).

Table 3. Serum cholesterol response of pooled backcross and F_2 rabbits for the different genotype groups[1].

Locus	Gender	Genotype[2] A/A	A/I	I/I	Significance[3]
		Serum cholesterol response (mM)			
Est-2	Male	26.9±1.4 (39)	27.1±1.3 (63)	25.6±2.3 (29)	S
	Female	35.8±1.6 (36)	34.5±1.8 (46)	31.4±1.4 (53)	
Es-1	Male	28.0±1.5 (36)	25.5±1.4 (71)	25.5±1.9 (25)	S
	Female	35.7±1.7 (34)	35.1±1.4 (66)	30.2±1.6 (40)	

[1] Serum cholesterol response (mM) is calculated as the difference between serum total cholesterol concentration at day 35 and day 0. Results are expressed as means ± SEM. Some serum samples failed to give a conclusive genotype, hence the number of rabbits typed varied slightly with each locus. Number of animals per genotype-group is indicated in parentheses.
[2] A = AX/JU-allel, I = IIIVO/JU-allel.
[3] Significance (P<0.001) based on two-way analysis of variance (S: effect of sex).

Results and Discussion

Serum cholesterol response:

Table 2 shows the Est-2 and Es-1 genotypes of hyporesponding and hyperresponding rabbits. Except for the Est-2 locus in the hyporesponding females, there were no significant deviations from the expected ratio's. Two-way analysis of variance applied to the genotype groups also failed to show a clear-cut evidence for genetic association between the esterase loci and serum cholesterol response (Table 3).

Basal serum HDL cholesterol level:

In Table 4 the distribution of the Est-2 and Es-1 genotypes among the rabbits with low or high basal serum HDL cholesterol levels is given. The

Table 4. Distribution of esterase genotypes (Est-2 and Es-1) among rabbits with low and high basal serum HDL cholesterol level[1].

Locus	Gender	Phenotype	(Mean±SEM)	Genotype[2]			Chi-square	(and P)
				A/A	A/I	I/I		
Est-2	Males	Low	(29±1)	7	6	1	3.8	(0.148)
		High	(69±1)	3	4	7	6.7	(0.035) #
	Females	Low	(25±1)	8	3	3	8.0	(0.018) *
		High	(66±1)	0	5	9	12.9	(0.002) *
Es-1	Males	Low	(29±1)	7	6	1	3.8	(0.148)
		High	(69±1)	3	3	8	10.7	(0.005) #
	Females	Low	(25±1)	6	5	3	2.4	(0.305)
		High	(66±1)	0	3	11	22.1	(<0.001) *

[1] Basal serum HDL cholesterol concentration (at day 0) is expressed as the percentage from serum total cholesterol concentration (at day 0).
[2] A = AX/JU-allel, I = IIIVO/JU-allel.
\# Significant deviation from expected [3.967 : 7 : 3.033]-ratio in males (see Table 1).
* Significant deviation from expected [3.525 : 7 : 3.475]-ratio in females (see Table 1).

Table 5. Basal serum HDL cholesterol level of pooled backcross and F_2 rabbits for the different genotype groups[1].

Locus	Gender	Genotype[2]			Significance[3]
		A/A	A/I	I/I	
		Basal serum HDL cholesterol level (%)			
Est-2	Male	46±2 (39)	50±1 (63)	58±2 (29)	S
	Female	39±2 (36)	46±2 (46)	51±1 (53)	G
Es-1	Male	45±2 (36)	51±1 (71)	59±2 (25)	S
	Female	39±2 (34)	46±1 (66)	52±2 (40)	G

[1] Basal serum HDL cholesterol concentration (at day 0) is expressed as the percentage from serum total cholesterol concentration (at day 0). Results are expressed as means ± SEM. Some serum samples failed to give a conclusive genotype, hence the number of rabbits typed varied slightly with each locus. Number of animals per genotype-group is indicated in parentheses.
[2] A = AX/JU-allel, I = IIIVO/JU-allel.
[3] Significance (P<0.001) based on two-way analysis of variance (S: effect of sex; G: effect of genotype at marker locus).

results indicate that both Est-2 and Es-1 are associated with the basal serum HDL cholesterol level. Two-way analysis of variance clearly confirmed this association (Table 5). Thus, these data indicate that a genetic factor affecting basal serum HDL cholesterol level is located in LG VI of the rabbit. This might be one (or both) of the esterase loci. The physiological function of esterases in the serum of vertebrate animals is still poorly understood, although there is some evidence that serum esterases are involved in triglyceride and cholesterol metabolism (*Kutty* 1980,

Table 6. Linkage homology between rabbit LG VI, rat chromosome 19 (RNO 19), mouse chromosome 8 (MMU 8) and human chromosome 16 (HSA 16) (Based on *Ceci* 1994, *Doggett & Callen* 1995, *Van Zutphen & Den Bieman* 1988 and *Yamada et al.* 1994).

Marker name	Rabbit LG VI	Rat RNO 19	Mouse MMU 8	Human HSA 16
Mitochondrial uncoupling protein		UCP	Ucp	
Cell surface alloantigen		RT2	Ea1	
Moloney leukemia virus 34			Mov34	MOV34
Esterase		ES2	Es1	
Carboxylesterase-1			Ces1	CES1
Esterase	Es-1	ES10	Es6	ESB3
Esterase		ES3	Es22	
Metallothioncin-1			Mt1	MT1A
Metallothionein-2			Mt2	MT2A
Metallothionein-3			Mt3	MT3
Glutamic-oxaloacetic transaminase 2			Got2	GOT2
Cadherin 1			Cdh1	CDH1
Esterase	Est-2	ES1	Es2	
Esterase	Est-6		Es7	
P-cadherin			Cadp	PCADL1
Lecithin-cholesterol acyltransferase			Lcat	LCAT
Haptoglobin		HP	Hp	HP
Chymotrypsinogen B1		CTRB	Ctrb	CTRB
Musculoaponcurotic fibrosarcoma oncogene			Maf	MAF
Adenine Phosphoribosyl-transferase			Aprt	APRT
Cadherin 3			Cdh3	CDH3
Angiotensinogen		AGT	Agt	AGT
Recessive yellow (= extension)	e		e	

Patel et al. 1990, *Van Lith et al.* 1992a, *Van Lith & Beynen* 1993).

However, it is also possible that one or more genes linked to the esterase loci are responsible for the observed association. Comparative gene mapping in the human, mouse, rat and rabbit has revealed evidence for considerable conservation of gene order during mammalian evolution. Linkage homology has been shown for rabbit LG VI, rat chromosome 19, mouse chromosome 8 and human chromosome 16 (Table 6). Interestingly, human chromosome 16 and mouse chromosome 8 contain the gene LCAT. This gene codes for the enzym lecithin-cholesterol acyltransferase (LCAT), which plays a major role in HDL cholesterol metabolism (*Jonas* 1991). *Meijer et al.* (1993) described that serum LCAT-activities in AX/JU rabbits fed on a low-cholesterol diet were significantly lower than in IIIVO/JU rabbits. Based on homology one might speculate that in the rabbit the LCAT-locus is also on LG VI and thus might be the gene responsible for the different basal serum HDL cholesterol levels in rabbits.

Conclusion

In conclusion, the present study does not provide evidence for the previous found association between the Est-2 locus and the serum cholesterol response in rabbits, but the results suggest a genetic linkage between LG VI loci and the locus or loci for basal serum HDL cholesterol levels. In order to identify these loci, further genetic analysis with more markers on rabbit LG VI is warranted.

Summary

Previous studies have indicated that esterases might be involved in the serum cholesterol response in rabbits. The question addressed in this study is whether in rabbits esterase loci of Linkage Group VI (LG VI) are genetically linked with the serum cholesterol response to dietary cholesterol or the basal serum HDL cholesterol level. For this purpose the Est-2 and Es-1 genotypes of rabbits in segregating populations derived from a cross between IIIVO/JU (hyporesponder and high basal serum HDL cholesterol level) and AX/JU (hyperresponder and low basal serum HDL cholesterol level) rabbits were determined. The segregating populations were fed a cholesterol-rich diet for 35 days. Both the Est-2 and Es-1 alleles failed to cosegregate with the serum cholesterol response, whereas a highly significant cosegregation was found with the basal serum HDL cholesterol level. It is concluded that one or more genes of LG VI are regulating the basal serum HDL cholesterol level in rabbits.

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