# GENETIC HETEROGENEITY OF RABBIT ALPHA-1-ANTITRYPSIN

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ABSTRACT

Sixteen inbred or partially inbred strains of rabbits were investigated for electrophoretic and quantitative variations of alpha-1-antitrypsin (A-1-AT). We found interindividual differences in the electrophoretic A-1-AT patterns as well as quantitative differences in the concentrations of A-1-AT and the serum trypsin-inhibiting activity.

Three electrophoretic phenotypes were distinguished: M, P and MP. M was characterized by a predominant anodal A-1-AT band, and P had a major cathodal component. The MP pattern can be explained by the occurrence of the M and P components in the same serum due to heterozygosity.

The P pattern was associated with an A-1-AT concentration of approximately 56% of that in sera with the M phenotype. The levels of A-1-AT in sera with the MP phenotype were intermediate between those in M and P types.

In addition to the type-specific quantitative variation, we found a quantitative sexual dimorphism of a moderate degree: Female rabbits had A-1-AT concentrations 16% less than males.

ALL mammalian sera that have been investigated so far contain several protease inhibitors. One major inhibitor of trypsin, chymotrypsin, and pancreatic, as well as leukocytic, elastase is known as alpha-1-antitrypsin (A-1-AT) or alpha-1-protease inhibitor (HEIMBURGER, HAUPT and SCHWICK 1971). This inhibitor has been isolated and partially characterized from several common laboratory species, including the rabbit (KUEPPERS 1972; KOJ *et al.* 1978). Electrophoretic heterogeneity has been observed, but interindividual differences have so far not been investigated.

With the recent availability of inbred or incipient inbred strains of rabbits such an approach was possible. We report here our observations of genetic heterogeneity of A-1-AT in inbred strains of rabbits. This heterogeneity is expressed in a difference of electric charge as well as in the quantity of A-1-AT present in peripheral blood. The genetic nature of the difference is clearly demonstrated in several intercrosses and backcrosses between rabbits of different strains and interstrain hybrids.

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## MATERIALS AND METHODS

*Serum samples*: Rabbits (*Oryctolagus cuniculus*) used for the strain comparisons were mature healthy adults obtained from The Jackson Laboratory colony. Strains included were AC/J, ACEP/J, AX/J, AXBU/J, B/J, C/J, OS/J, WH/J, X/J, III/J, IIIC/J, III/*cd*J, IIIEP/J, IIIVO/J, IIIVO/*ah*J and IIIVO/*vpt*J (Fox 1975; Usher *et al.* 1982). Progeny from the various crosses were a minimum of 6 weeks of age when sampled. All rabbits were fed Purina Rabbit Chow Checkers 20 with *ad libitum* water. Blood samples were collected in the morning either by cardiac puncture or by ear bleeding (Hoppe, Laird and Fox 1969). After clotting, initially at room temperature and then at 4°, the samples were centrifuged. Sera were stored at −60° and only shortly before use at −15°. For the strain comparisons serum samples were obtained from three males and three females of each of the 16 strains.

*Isolation of rabbit A-1-AT*: Rabbit A-1-AT was prepared by several chromatographic steps (James and Cohen 1978). A 50–75% ammonium sulfate precipitate was redissolved and subjected to chromatography on Cibacron Sepharose (Pharmacia) to remove albumin (Travis and Pannell 1973). The eluted material was subsequently chromatographed on QAE Sepharose columns using a buffer containing 0.05 M Tris and 0.18 M NaCl, pH 8. Then, it was chromatographed again on QAE Sepharose, equilibrated and eluted with 0.005 M sodium phosphate and 0.1 M NaCl, pH 6.5.

This procedure resulted in a preparation of rabbit A-1-AT that was electrophoretically pure.

Antiserum was produced in a goat by three subcutaneous injections of 1 mg of rabbit A-1-AT in 1 ml of normal saline solution emulsified with 1 ml of complete Freund's adjuvant.

Crossed immunoelectrophoresis of rabbit sera was performed essentially as described by Laurell (1965). The electrophoresis in the first direction was for 3 hr at 200 V. The electrophoresis in the second direction into the antiserum containing gel was for 90 min at 200 V. Agarose was 1.25% in 0.1 M barbital buffer containing 0.007 M $CaCl_2$, pH 8.65, for both runs.

Radial immunodiffusion was performed according to the method of Mancini, Carbonara and Heremans (1965). The standard was pooled rabbit serum that was composed of equal amounts of 64 serum samples from the different strains. The results were expressed as percent of the amount of A-1-AT in the standard.

The trypsin-inhibiting activity was determined by a semiautomated method described before (Kueppers and Mills 1983) except that rabbit sera were used in a 2% dilution.

Bovine trypsin was from Boehringer-Mannheim; it was active site titrated (Chase and Shaw 1967) and found to be 66% active.

## RESULTS

There was a good correlation between immunologically detected A-1-AT and the functional trypsin-inhibiting activity. The correlation coefficient was $r = 0.92$ ($y = 0.22 + 3.5x$). Table 1 lists the mean trypsin-inhibiting activities and A-1-AT concentrations of sera from the individual strains.

There was a substantial difference between male and female values in most strains, but the difference was often not statistically significant. There was, however, a clear trend for the male values to be somewhat higher than the female values. The only exceptions were strains III/J, IIIC/J and IIIEP/J that showed slightly but not significantly higher trypsin inhibition values in females.

When the values of all males were compared with those of all females a highly significant ($P < 0.001$) difference was found; the values of females were lower by 0.086 ± 0.052 mg/ml of trypsin inhibition and 18.1 ± 14% of A-1-AT by immunologic determination. Expressed on a relative scale, the mean trypsin-inhibiting activity in female sera was 87 ± 9.7% of that in male sera; the A-1-AT concentration in females was 84 ± 10.3% of that in males.

TABLE 1

*Trypsin-inhibiting activity and A-1-AT concentration of inbred strains of rabbits*

| Strain | Male | | Female | | Male-female differences[c] | | Phenotype |
|---|---|---|---|---|---|---|---|
| | Trypsin inhibition | A-1-AT[b] | Trypsin inhibition | A-1-AT[b] | $D_1$ | $D_2$ | |
| AC/J | 0.735 ± 0.029 | 138 ± 18 | 0.660 ± 0.022 | 118.8 ± 7.4 | 0.075 (0.02) | 19.2 (NS) | M |
| ACEP/J | 0.780 ± 0.030 | 126.6 ± 3.3 | 0.644 ± 0.070 | 103.9 ± 12.1 | 0.136 (0.036) | 22.7 (0.036) | M, MP |
| AX/J | 0.771 ± 0.118 | 149.6 ± 12.4 | 0.622 ± 0.097 | 127.3 ± 4.1 | 0.149 (NS) | 22.3 (0.04) | M |
| AXBU/J | 0.723 ± 0.107 | 139.4 ± 27.7 | 0.636 ± 0.110 | 92.2 ± 22.2 | 0.087 (NS) | 47.2 (NS) | M |
| B/J | 0.665 ± 0.019 | 109 ± 2.7 | 0.626 ± 0.099 | 101 ± 13.5 | 0.039 (NS) | 8 (NS) | M |
| C/J | 0.656 ± 0.042 | 107.7 ± 13.8 | 0.605 ± 0.079 | 94 ± 23 | 0.051 (NS) | 13.7 (NS) | M |
| OS/J | 0.764 ± 0.004 | 126 ± 13.8 | 0.636 ± 0.073 | 100.6 ± 16 | 0.128 (0.039) | 25.4 (NS) | M |
| WH/J | 0.761 ± 0.047 | 122 ± 13 | 0.662 ± 0.046 | 93.6 ± 8.8 | 0.099 (NS) | 28.4 (0.034) | M |
| X/J | 0.826 ± 0.052 | 154.3 ±11.6 | 0.645 ± 0.03 | 107.4 ± 7.9 | 0.181 (0.01) | 46.9 (0.007) | M |
| III/J | 0.561 ± 0.012 | 101.3 ± 18 | 0.628 ± 0.097 | 101.6 ± 2.1 | −0.067 (NS) | −0.3 (NS) | M, MP |
| IIIC/J | 0.455 ± 0.036 | 64 ± 1.6 | 0.456 ± 0.072 | 59.1 ± 7.1 | −0.001 (NS) | 4.9 (NS) | P |
| III/*cdJ* | 0.702 ± 0.029 | 138 ± 18 | 0.660 ± 0.022 | 118.8 ± 7.4 | 0.042 (NS) | 19.2 (NS) | M |
| IIIEP/J | 0.678 ± 0.048 | 110.4 ± 23.6 | 0.641 ± 0.163 | 113.5 ± 29 | 0.036 (NS) | −3.1 (NS) | P, MP |
| IIIVO/J | 0.638 ± 0.019 | 84.7 ± 1.3 | 0.499 ± 0.129 | 76 ± 25.2 | 0.139 (NS) | 8.7 (NS) | P |
| IIIVO/*ahJ* | 0.600 ± 0.074 | 84.8 ± 12.7 | 0.460 ± 0.011 | 62.4 ± 3.3 | 0.140 (0.01) | 22.4 (0.04) | P |
| IIIVO/*vptJ* | 0.579 ± 0.148 | 80.2 ± 31 | 0.441 ± 0.055 | 76.4 ± 20.2 | 0.138 (0.03) | 3.8 (0.04) | P |

[a] Expressed in milligrams of trypsin inhibited by 1 ml of serum.

[b] Expressed as percent of standard serum pool.

[c] $D_1$ and $D_2$ are the differences between the serum trypsin-inhibiting activity (columns 1 and 3) and A-1-AT concentration (columns 2 and 4), respectively, of males and females. The $P$ value is in parentheses; NS = not significant ($P > 0.05$).

Crossed immunoelectrophoresis revealed that A-1-AT was heterogeneous in electric charge. There were always two components of apparent immunological identity in the postalbumin region. In most samples the anodal component was present in higher concentrations than the cathodal one: The ratio of concentrations in the anodal peak to that of the cathodal component ranged from 1.2 to 2.5. This pattern we labeled "M." In a few samples the cathodal peak had a concentration of A-1-AT three to four times higher than the anodal one. This pattern we labeled "P" (see Figure 1).

Table 1 shows that the lowest trypsin inhibition and A-1-AT values were associated with the P patterns, whereas high values were found in samples with M precipitates. Most strains showed an M or P pattern but not both.

An apparent exception were strains III/J and IIIEP/J that contained some samples with an M pattern and others with a P-like precipitate; that was, however, atypical in that the ratio of anodal to cathodal peak was approximately 0.8 instead of 0.25 to 0.3 as was typical for the P pattern.

Strains IIIC/J, IIIVO/J, IIIVO/*ah*J and IIIVO/*vpt*J had P precipitates. All other strains had M or MP patterns. The mean A-1-AT concentration for M males was 131.5 ± 20 and 110 ± 18% for M females and 0.74 ± 0.12 and

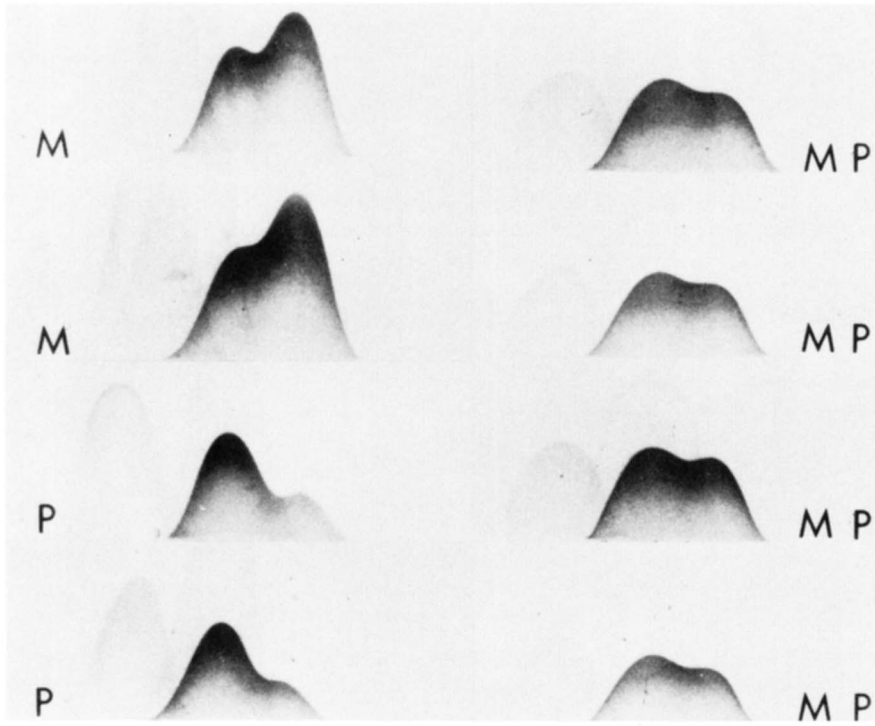


FIGURE 1.—Crossed immunoelectrophoresis of rabbit A-1-AT. The three phenotypes M, P and MP, are indicated. The MP patterns are from offspring of crosses of parents known to be homozygous for $Pi^M$ and $Pi^P$, AX/J and IIIVO/*ah*J, respectively. Anode is at right. The faint immunoprecipitates toward the cathode are due to other serum proteins.

0.69 ± 0.09 mg of trypsin inhibited/ml of serum for males and females, respectively. The values for P male animals were 70.7 ± 14% and 0.54 ± 0.06 mg/ml and for females 65.2 ± 12% and 0.49 ± 0.06 mg/ml, respectively. The difference between values for M and P animals was significant ($P < 0.001$). MP animals had intermediate concentrations: For males 93.4 ± 13% and 0.608 ± 0.053 mg/ml and for females 87.4 ± 11% and 0.595 ± 0.052 mg/ml.

Because of the apparent strain specificity of the electrophoretic patterns, we set up crosses to test whether the patterns were genetically determined. Twenty-six crosses were set up from which a total of 124 offspring were obtained. The phenotypes of parents and offspring are listed in Table 2.

When both parents had the A-1-AT phenotype M in the two matings of AX/J × AX/J, all offspring had the M phenotype. In three crosses of IIIC/J × IIIVO/J, both parents and offspring had the phenotype P.

When AX/J (M) and IIIVO/*ah*J (P) were crossed, all offspring had a pattern that was intermediate between M and P with the second more cathodally migrating peak always more concentrated than the anodal peak (Figure 1). Clearly, this was the phenotype associated with heterozygosity. When these animals were intercrossed and the $F_2$ generation was tested we observed segregation of M and P patterns. From ten matings we obtained 51 offspring; 17 showed an M pattern, 17 had an MP type and 17 were P. This distribution is not significantly different from the expected Mendelian ratio of 12.75: 25.5:12.75 ($P > 0.05$), assuming that M and P are determined by alleles at one locus. From seven backcross matings (AX/J × IIIVO/*ah*J) (MP) × (IIIVO/*ah*J) (P) we obtained 36 offspring, 21 with an MP type and 15 with a P phenotype. This outcome was also in good agreement with the expected ratio of 18:18, again assuming control by two alleles at one locus. The distribution pattern was unaffected by the sex of the progeny.

## DISCUSSION

A-1-AT is a predominant protease inhibitor in rabbit serum. The good correlation ($r = 0.92$) between the functional inhibition values and the immunologically determined concentrations of A-1-AT shows that in both assays the same trypsin inhibitor is measured. Other protease inhibitors are, however, present that account for a mean of 0.22 ± 0.13 mg of trypsin inhibited/ml of serum as can be calculated from the regression equation of functional and immunological values given in RESULTS.

For the immunological measurements we chose to express all values as fractions of a normal pool. This was done because we were not certain that our A-1-AT preparations were of high enough purity to be suitable as a correct standard. Crossed immunoelectrophoresis of whole rabbit serum using our antiserum showed two weak immunoprecipitates in addition to the major precipitation peak due to A-1-AT (Figure 1). On radial immunodiffusion these two additional precipitates were hardly visible so that they did not interfere with the measurements of the A-1-AT precipitation ring.

In our initial screening we tested a total of 96 individual serum samples from 16 inbred strains for trypsin-inhibiting activity. It became clear that we

TABLE 2

*Crosses between several strains*

| | Parents[b] | | Progeny | |
|---|---|---|---|---|
| $N^a$ | Dam | Sire | Male | Female |
| 2 | AX/J (M) | AX/J (M) | 4 M | 4 M |
| 3 | IIIC/J (P) | IIIVO/J (P) | 9 P | 5 P |
| 4 | AX/J (M) | IIIVO/*ah*J (P) | 6 MP | 9 MP |
| 10 | [AX/J × IIIVO/*ah*J] (MP) | [AX/J × IIIVO/*ah*J] (MP) | 8 M, 4 MP, 7 P | 9 M, 13 MP, 10 P |
| 7 | IIIVO/*ah*J (P) | [AX/J × IIIVO/*ah*J] (MP) | 10 MP, 7 P | 11 MP, 8 P |

[a] $N$ = number of matings producing a litter.
[b] A-1-AT phenotype in parentheses.

were dealing with two types of quantitative differences: (1) There was the difference between male and female sera. In most strains males had higher inhibition values than females, but the difference was often not statistically significant. However, when the values of all samples were combined the trypsin-inhibiting activity in male samples was significantly higher than that in female samples ($P < 0.001$). This sexual dimorphism was less pronounced than that recently described in mice (KUEPPERS and MILLS 1983). Male mice have an A-1-AT concentration approximately twice as high as that of females. In humans, males and females have the same concentration of A-1-AT (GANROT 1972). (2) There was also a quantitative difference between strains. The trypsin inhibition values, as well as the immunologically determined A-1-AT concentrations of strains IIIC/J, IIIVO/J, IIIVO/*ah*J and IIIVO/*vpt*J for males and females, were significantly lower ($P < 0.001$) than the values of all other strains individually or combined. The low concentration coincided with the P electrophoretic pattern in all samples. By electrophoresis in agarose or starch gels A-1-AT migrates as two major bands in the postalbumin region. Both bands have inhibitory activity for several proteases, but their relative affinity for some proteases apparently differs (KOJ and REGOECZI 1981). Immunologically, both components are indistinguishable, as was reported earlier (KUEPPERS 1972; KOJ *et al.* 1978) and confirmed in the present report (Figure 1).

The occurrence of different electrophoretic types in various strains suggested that the patterns were genetically determined. All breeding experiments, summarized in Table 2, were consistent with the genetic hypothesis that the M and P phenotypes of A-1-AT are controlled by two alleles at an autosomal locus. Among 124 offspring of five different mating types there was not a single exception: (1) When both parents had either an M or a P pattern all offspring showed the same parental pattern. (2) Matings of one parent with an M type and the other parent with a P phenotype produced offspring with a precipitation pattern that differed from both parental phenotypes. We assumed this to be the heterozygous MP phenotype. It was characterized by a double precipitate that was slightly unequal, with the cathodal peak always more concentrated than the anodal one. The ratio of areas under the anodal peak to that under the cathodal peak was between 0.8 and 0.85 in this pattern. This characteristic precipitate can also be produced by crossed immunoelectrophoresis of a mixture of equal amounts of sera with M and P patterns, further evidence that the MP pattern is indeed the phenotypic expression of heterozygosity. (3) Intercrosses of $F_1$ animals with MP phenotypes and backcrosses of $F_1$ animals to the P phenotype produced offspring that demonstrated the appropriate segregation for M and P (Table 2). The results of all crosses support the interpretation that the observed A-1-AT phenotypes are indeed under genetic control and that the genes that determined the M and P phenotypes are codominant alleles at a single autosomal locus. In analogy to the nomenclature used for human A-1-AT we propose to refer to the locus as *Pi* (for protease inhibitor) and to use superscript letters when referring to specific alleles: $Pi^M$, $Pi^P$ (FAGERHOL and GEDDE-DAHL 1969). The currently known phenotypes are M, MP and P.

One aspect that has been of great interest to us was the lower trypsin-inhibiting activity and lower concentration of A-1-AT associated with the P phenotype. The highest concentration and inhibiting activity was associated with the M phenotype, the P type had the lowest activity and A-1-AT concentration and the MP type had intermediate concentrations.

The molecular nature of the genetic difference between P and M A-1-AT is presently unknown. A single amino acid substitution is likely, but other possibilities cannot be ruled out. In human A-1-AT there are several single amino acid substitutions known that also lead to lower serum concentrations of the substituted protein (for review see KUEPPERS 1978). Obviously, detailed structural studies will have to determine the chemical basis of the two phenotypes.

The amino acid and carbohydrate composition of the two bands that are characteristic of A-1-AT regardless of genetically determined phenotype has been studied by KOJ *et al.* (1978), but a chemical difference has not yet been identified. In analogy to human A-1-AT this within-phenotype microheterogeneity is probably due to differences in the carbohydrate moiety (YOSHIDA and WESSELS 1978). The finding that the two components differ in catabolic rate (REGOECZI, KOJ and LAM 1980) supports this assumption because it is known that changes in composition of oligosaccharide side chains of glycoproteins often bring about substantial differences in their half life (MORELL *et al.* 1968) due to altered affinity to receptor sites on cell surfaces.

This newly identified mutation of A-1-AT in rabbits may have several applications in genetics and experimental biology. It may be a convenient genetic marker in a common laboratory animal about which much physiological information is available but less biochemical genetic data are known.

Genetic animal models of specific protein deficiencies have been useful in the past, notably in the complement and blood coagulation system but also in other groups of proteins. At present we do not have an animal with a genetic A-1-AT deficiency, a condition that leads to pulmonary emphysema in humans (ERIKSSON 1965).

The mutation that we have described here is clearly associated with A-1-AT concentrations almost half of that present in rabbit strains with the highest A-1-AT levels like AC/J, AX/J and X/J. Whether a moderate reduction of the A-1-AT concentration has any physiological consequence and may be useful in experimental studies will have to be shown. It will be important to determine which rabbit proteases are inhibitable by rabbit A-1-AT. It will then be possible to determine which systems may be studied with this mutation.

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