Expression and Linkage of Genes for X-linked Hemophilias A and B in the Dog

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The linkage distance on the X chromosome between the genes for hemophilia A (classic hemophilia) and B (PTC deficiency, Christmas disease) was estimated directly by breeding two strains of dogs, each segregating for a different type of hemophilia. Gene expression was determined by bioassays of plasma factor VIII (antihemophilic factor) and factor IX (PTC, Christmas factor). Double heterozygotes in repulsion for both hemophilia A and B could be readily identified by intermediate plasma levels of both procoagulants. There was no evidence of a tendency toward preferential inactivation of the paternally derived X chromosome, and the procoagulant levels showed that random inactivation had occurred at both loci. When double heterozygotes were bred against normal males or males with hemophilia A and B, the progeny that resulted indicated that the genes recombined freely. Thus, the genes are at least 50 map units apart. The phenotypes of five new hemophilic genotypes are described as a result of the various crossbreedings, including males with double hemophilia AB. When both hemophilia genes are in the coupling phase, there is evidence of increased intrauterine or neonatal lethality in males. The data from this study, along with that on gene linkage of human hemophilia A and B, provide support for the thesis of homology of the X chromosome during speciation.

IT HAS BEEN RECOGNIZED for some time that hemophilia A (classic hemophilia) and hemophilia B (PTC deficiency, Christmas disease) are separate X-linked diseases whose genic loci are probably nonallelic.[1] Several studies have been undertaken in an attempt to determine the linkage of the two hemophilia genes. These studies have been indirect, however, since well-documented families in which both of the relatively rare hemophilia genes were segregating have not been encountered.[2] Instead, families have been examined in which one or the other of the hemophilia genes was segregating with another X-linked gene, either color blindness[3] or Xg blood groups.[4,5] In the color blindness-hemophilia studies,[3] it was concluded that the hemophilic loci were at least 38 map units apart, and more likely at least 50. In the Xg blood group-hemophilia studies,[4,5] neither the hemophilia A nor hemophilia B locus was closely linked to that of blood group Xg. Linkage studies of

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hemophilia A with glucose-6-phosphate dehydrogenase (G-6-PD)[6] and vitamin D-resistant rickets,[7] but not with hemophilia B, have been carried out in humans, as has the linkage between carpal subluxation and hemophilia A in the dog.[8]

Both hemophilia A[9] and hemophilia B[10] occur in dogs. The availability of these animals has allowed a direct study of the linkage between the two hemophilia genes by crossbreedings. Dosage compensation has long been established for the hemophilia loci, and the presumptive identification of both hemophilia A and B heterozygotes has been demonstrated.[11,12] Each type of heterozygote had plasma factor VIII (antihemophilic factor, AHF) or factor IX (PTC, Christmas factor) levels, respectively, reduced to about 50% of the normal level, with no overlap in values between heterozygotes and normal females. The identification of heterozygotes early in life permitted a relatively efficient breeding program. In the F_1 generation of the cross between the two lines of hemophilia A and hemophilia B animals, females doubly heterozygous for both hemophilia A and B with the genes in repulsion would be expected. In the F_2 generation when the double heterozygotes in repulsion are bred, the frequency of recombination would be directly proportional to the linkage distance. This paper describes the results of a direct linkage study along with data on expression of the genes.

MATERIALS AND METHODS

Hemophilia A and B dogs from the North Carolina and Albany colonies were used for the crossbreeding experiments. To produce double heterozygotes in repulsion, hemophilia A dogs from three strains and hemophilia B dogs from two strains were used as follows:

(1) For the cross, hemophilia A Carrier (^aXX) with hemophilia B hemizygote (X_bY), hemophilia A dogs came from the North Carolina Irish setter strain and hemophilia B dogs came from the Guelph cairn terrier strain.[10,13] There were four matings of this type.

(2) For the cross, hemophilia B carrier (X_bX) with hemophilia A hemizygote (^aXY), a cairn terrier strain and a coonhound strain of hemophilia B were mated with a beagle strain and a mongrel strain of hemophilia A, respectively.

(3) For the cross of a female hemophilia A homozygote ($^aX^aX$) with a hemophilia B male (X_bY), the Irish setter hemophilia A strain and the cairn terrier hemophilia B strain were used.

The phenotypes of the animals for the hemophilia genes were established by bioassays for plasma factor VIII and for factor IX, with a modified one-stage partial thromboplastin time procedure,[14,15] either nonactivated or kaolin-activated. Substrate for the procoagulant assays consisted of pooled plasma from hemophilia A and hemophilia B dogs, respectively. A standard plasma pool obtained from 20 randomly chosen normal dogs, ten of each sex,

Table 1. Crosses to Yield Double Heterozygotes for Hemophilia A and Hemophilia B: Genes in Repulsion

Matings			Genotype						
Type		Number	Males			Females			Totals
Sire	Dam		^aXY	X_bY	XY	^aXX	X_bX	^aXX_b	
X_bY	^aXX	4	5	—	3	—	1	7	16
^aXY	X_bX	2	—	—	3	6	—	4	13
X_bY	$^aX^aX$	1	3	—	—	—	—	8	11
Totals		7	8	—	6	6	1	19	40

was assigned values of 100% for quantitation of factor VIII and factor IX, respectively. The normal control animals and hemophiliacs came from the inbred colonies of dogs maintained at North Carolina and Albany. Plasma factor VIII and IX levels of ten normal female dogs had ranges of 68%–200% and 67%–130%, respectively, with means of 102% and 95%. Plasma factor VIII and IX levels of ten normal male dogs had ranges of 70%–150% and 66%–160%, respectively, with means of 103% and 97%.

Plasma VIII and IX levels were determined during the first weeks of life and at several intervals as the animals matured. The animals from different litters that were stillborn or died before phenotyping was completed are not included in the data.

RESULTS

Females Doubly Heterozygous for Hemophilia A and B, F_1 Generation

The first experiment was to produce females doubly heterozygous for hemophilias A and B, in repulsion. The breeding data are shown in Table 1. Only one anticipated phenotype was not observed (X_bY males from the $^aXY \bullet X_bX$ mating) and no phenotype was observed that was not anticipated The deviations from expectation were slight and explicable as fluctuations in a small sample.

The coagulation data for the double heterozygotes are shown in Fig. 1. The figure shows that these animals were clearly of intermediate phenotype with respect to both factors. Factor VIII ranged from 12% to 64% (mean of 36%), while factor IX ranged from 19% to 64% (mean of 45%).

Genetic Recombination of Hemophilia A and B

Once doubly heterozygous adult females were available, they were bred to determine the recombination frequency among the progeny. The sires were of the three phenotypes: normal, hemophilia A, and hemophilia B. The results from 15 litters, with 78 tested progeny, are shown in Table 2. The male:female ratio was 33:45 ($\chi^2 = 2.45$; $p > 0.10$). From these matings, four male genotypes are possible, and all were observed. The crossover ratio was 15:18 in males, suggesting a deficiency of doubly hemophilic males of (^aX_bX) from the $XY \bullet {}^aXX_b$ and $^aXY \bullet {}^aXX_b$ matings.

There are nine possible female genotypes from the three mating types as listed in the footnote to Table 2. All were observed among the 45 females. The crossover ratio was 26:19 among females. Neglecting the possibility of double crossover, there was also coupling of the genes in one double heterozygote from the cross with a normal sire (Table 2, first listed cross). In that case, females with both hemophilia genes on the same X chromosome occurred in each of the three types of matings.

The ratio of crossovers to noncrossovers for all progeny, both male and females, of the double heterozygotes is 41:37. This is not so close to the maximum 50% frequency of 39:39 that the linkage distance is too great to be measured.

Reliability of Genotyping of Double Heterozygotes by Bioassay for Factors VIII and IX

The reliability of the bioassay method for establishing presumed genotypes was assessed by breeding tests of eight females of the F_1 generation diagnosed

Table 2. Matings of Females Doubly Heterozygous for Hemophilia A and Hemophilia B

Matings			Males				Females			
Type		Number	Noncrossover		Crossover		Noncrossover		Crossover	
Sire	Dam		Genotype	Number	Genotype	Number	Genotype	Number	Genotype	Number
XY	$^{a}XX_{b}$	1	^{a}XY	1	$^{a}X_{b}Y$	—	^{a}XX	1	XX	2
			$X_{b}Y$	1	XY	1	$X_{b}X$	—	$^{a}X_{b}X$	1
^{a}XY	$^{a}XX_{b}$	3	^{a}XY	1	$^{a}X_{b}Y$	—	$^{a}X^{a}X$	1	$^{a}X_{b}X^{a}$	1
			$X_{b}Y$	2	XY	6	$^{a}X_{b}X$	1	$X^{a}X$	4
$X_{b}Y$	$^{a}XX_{b}$	11	^{a}XY	6	$^{a}X_{b}Y$	3	$^{a}XX_{b}$	11	$^{a}X_{b}X_{b}$	7
			$X_{b}Y$	7	XY	5	$X_{b}X_{b}$	5	XX_{b}	11
Totals		15		18		15		19		

Males: XY, normal; ^{a}XY, hemophilia A; $X_{b}Y$, hemophilia B; $^{a}X_{b}Y$, double hemophilia AB.

Females: XX, normal; ^{a}XX, hemophilia A carrier; $X_{b}X$, hemophilia B carrier; $^{a}X_{b}X$ or $^{a}XX_{b}$, double hemophilia AB carrier $^{a}X^{a}X$, hemophilia A; $X_{b}X_{b}$, hemophilia B, $^{a}X_{b}X^{a}$, hemophilia A, also hemophilia B carrier; $^{a}X_{b}X_{b}$, hemophilia B, also hemophilia A carrier

Table 3. Genotypes of Progeny of Semiobligate Carriers of Both Hemophilia A and B

Double Heterozygote X^aX_b (Dog No.)	Genotype of Sire	Progeny							
		Hemophilic						Nonhemophilic	
		Males			Females			Males	Females
		^aXY	X_bY	^aX_bY	^aXaX	$^aXaX_b$	$^aX_bX_b$	Genotypes (Number)	
I. Double heterozygotes nonobligate for hemophilia A gene*									
1	X_bY	1	—	—	—	—	—	—	X_bX (1)
2†	X_bY	—	2	—	—	—	—	XY (5)	XX (2) X_bX (1) ^aXX_b (2)
3	^aXY	1	—	1	—	—	—	XY (2)	—
4	^aXY	—	1	—	1	1	—	XY (1)	^aXX (2)
5	^aXY	1	—	—	—	—	—	XY (2)	^aXX_b (1)
II. Double heterozygotes nonobligate for hemophilia B gene‡									
6	X_bY	—	—	1	—	—	1	—	^aXX_b (3) X_bX (4)
7†	^aXY	2	2	—	—	—	—	XY (5)	XX (1) ^aXX_b (2)
8	XY	1	1	—	—	—	—	XY (1)	XX (2) ^aXX (1) ^aXX_b (1)

Double heterozygotes from mating, $^aXX \cdot X_bY$, line 1, Table 1.
†Two litters.
‡Double heterozygotes from mating $X_bX \cdot {}^aXY$, line 2, Table 1.

as double heterozygotes. The findings are summarized in Table 3. The positive diagnosis would be confirmed by both hemophilia A and B appearing in the progeny. Five of the females (section I, Table 3) were obligate carriers for hemophilia B only. Hemophilia B appeared in litters of three of the five females, with the remaining two females having assay-positive hemophilia B carriers. Hemophilia A appeared in litters of four of the five females. The fifth female had a bioassay-positive hemophilia A carrier.

Three of the females (section II, Table 3) were obligate carriers for hemophilia A. Hemophilia A and B appeared in the litters of all three animals.

A false negative diagnosis would be indicated by unexpected phenotypes. Breeding studies were carried out on one of the females, presumptively X_bX (line 1, Table 1), whose factor IX level was 51% and factor VIII level was 105%. This dog was bred three times to a hemophilia B male and had three litters. From this there were five hemophilia B females, one hemophilia B male, but no hemophilia A offspring. All of these data indicate that genotyping of females for double heterozygosity of bioassay is highly reliable.

DISCUSSION

The ratio of crossovers to noncrossovers between the hemophilia A and hemophilia B phenotypes, shown in Table 2, demonstrates unequivocally that in meiotic division in the female there is free genetic recombination between the two loci. This implies that the loci, although both are on the X chromosome, are widely separate. These data from direct linkage studies in the dog compare favorably with and appear to confirm the indirect linkage studies in the human,[3] which likewise pointed to widely separate genetic loci for the two hemophilia genes.

A number of comparative studies of the X chromosome in eutherian mammals have been made. It has been suggested that in the various mammalian species the X chromosome, unlike the somatic chromosomes, is morphologically identical or at least very similar. The X- chromosome, on the basis of size, comprises about 5%–6% of the haploid set of chromosomes. Comparison of the X chromosomes of man and dog showed them to be similar in these respects.[16] This morphologic homology is extended for these two species to genic composition in respect to the mutant genes for hemophilia A and B. Our present study on linkage of these two genes in the dog, combined with the human linkage data,[3] would support the thesis that linkage relationships between individual genes on the X chromosome are conserved quantitatively during speciation in mammals.

The ability to diagnose heterozygosity in the female carriers of hemophilia A and B by bioassays early in life was of great help in carrying out this study. Previous data had indicated that carriers of hemophilia A or hemophilia B had reduced levels of plasma procoagulants, factors VIII and IX, respectively, a prediction of the Lyon hypothesis.[17] The findings for singly heterozygous females were extended to doubly heterozygous females. As with singly heterozygous females, there was no overlap with normal females in the plasma levels of either of the antihemophilic factors. The mean values for the plasma antihemophilic procoagulants, factors VIII and factor IX, in the F_1 generation

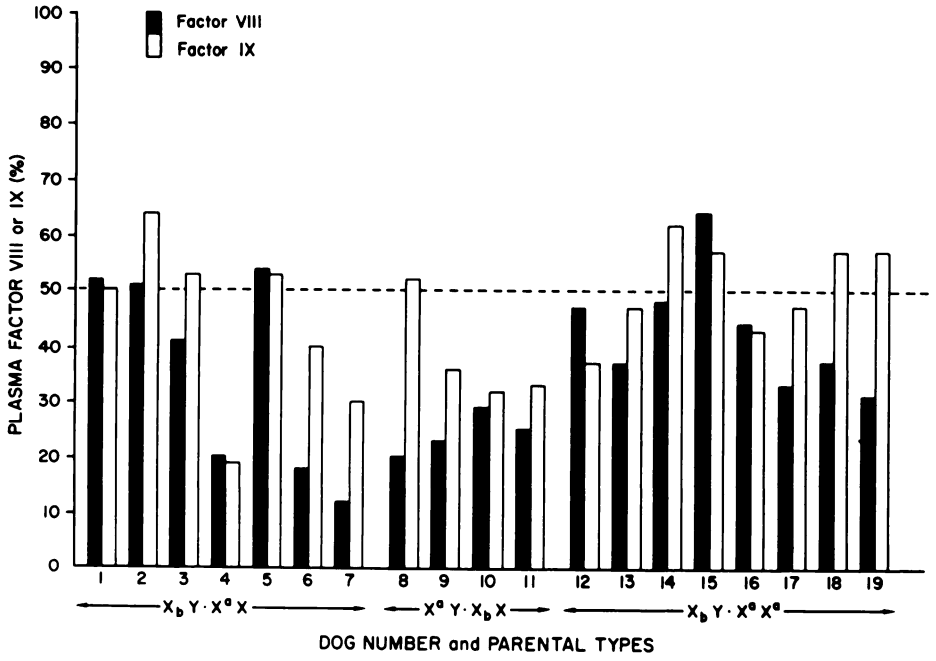

Fig. 1. Plasma factor VIII and IX levels in double heterozygotes for hemophilia A and B, derived from indicated parental genotypes.

double heterozygotes, were 36% and 45%, respectively. These levels are believed to be related to the dosage compensation mechanism by which normal males and females, with an X chromosome ratio of 1:2, have the same plasma levels of factors VIII and IX. A number of models for dosage compensation due to inactivation of one X chromosome of the pair have been proposed.[18] According to the Lyon hypothesis, X-inactivation is random in an embryonic cell, and once identified, the same X chromosome continues to be inactive in subsequent cell divisions. On a random basis, the expected values for plasma factors VIII and IX in the double heterozygotes would each be 50% and their sum would be 100%. There are several possibilities for the deviation of the values found, whose mean and sum were below that expected. Inherent limitations in bioassays and the sample size may be important, or the deviations may be related to the manufacture of the two procoagulants by separate cell populations. Each population derived from different cell clones may have a different mix of active maternal and paternal X chromosomes that could vary independently of each other and thus account for the values observed.

Another possibility that can be examined in our data is that the paternal X chromosome is the late replicating one of the X-pair and hence is inactive, as in marsupials.[19,20] Whether there may be a tendency for the sex chromatin to be of paternal origin in eutherian mammals is currently being debated.[21,22] Mules and hinnies, crosses between the donkey and the horse, in which the source of the X chromosome, maternal or paternal, can be identified, appear to show preferential inactivation of the paternal X chromosome.[21] Inspection

of the data on factor VIII and XI levels in the F_1 double heterozygotes (Fig. 1) shows that the paternal and maternal genes are both expressed and approximately equally so. Fifteen of the 19 heterozygous animals were of hemophilia B paternal parentage. The mothers were either hemophilia A heterozygotes or homozygotes. The mean factor IX level was 39% and the mean factor VIII level was 47%, the reverse of what would be expected if there were a preferential paternal X-inactivation. There were only four double heterozygotes of hemophilia A paternal parentage, and their mean factor VIII level was only moderately greater than the mean factor IX level, 38% compared to 24%. The variance between the groupings in Fig. 1 was not significantly different from each other. These data thus suggest that X-inactivation was indeed random, without respect to the paternal or material source of the X-chromosome.

Prior to this study, there were only three known genotypes for each of the hemophilia genes, the heterozygotes (^aXX and X^bX) and the male and female hemophilia phenotypes (^aXY, $^aX^aX$,[23] X_bY, X_bX_b[24]). With the free recombination occurring in the breeding of the double heterozygotes, several new hemophilic genotypes made their appearance as a result of crossing-over. These included males with double hemophilia AB (^aX_bY), females presumably with double heterozygosity in coupling (^aX_bX), and hemophilia A and hemophilia B females who were also carriers of the other hemophilia gene ($^aX_bX^a$ and $^aX_bX_b$). Including the double heterozygotes with genes in repulsion, five new hemophilic genotypes were identified in this study.

The question of a greater chance of lethality for ^aX_bY animals is raised by the data for males in Table 2. With no measurable linkage between the two hemophilic loci, a 1:1:1:1 ratio for normal, hemophilia A, hemophilia B, and hemophilia AB males, respectively, would be expected. There was actually a considerable deficiency in the number of hemophilia AB animals found; the ratio was 12:8:10:3. This deficiency was significantly different ($p < .05$) from that expected if there were no lethality factor.

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