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Chromosomal Linkage Associated with Disease Severity in the Hydrocephalic H-Tx Rat

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Infantile hydrocephalus results in neurological deficits despite surgical treatment. Fetal-onset hydrocephalus in humans can be caused by developmental abnormalities that are genetic in origin. The H-Tx rat has hydrocephalus with 40% penetrance and a polygenic inheritance. A backcross with Fisher F344 inbred strain produced a total of 1500 progeny with 17.5% hydrocephalus. Of these, only 12.3% had overt disease and the remaining 5.2% had mild disease seen only after fixation of the brain. Disease severity was measured for all affected rats using the ratio of ventricle to brain width. The severity measure confirmed that there are two populations, mild hydrocephalus (M; ratio, <0.4) and severe hydrocephalus (S; ratio, >0.4), with a small overlap. For genotyping, the two populations were each subdivided based on the ratio measure to give a total of four groups of increasing severity. After an initial genome scan with microsatellite markers, all hydrocephalic rats and a subset of 128 normal progeny were genotyped on chromosomes 4, 9, 10, 11, 17 and 19. Rats in the mildest group had association with a locus on chromosome 4 (LOD 2.4), whereas those in the severest group were associated with a locus on chromosome 17 (LOD 3.2). All except the least affected group were associated with a heterozygous genotype on chromosomes 10 and 11 (LOD 4.5 and 3.5, respectively). Chromosomes 9 and 19 had weak linkage to hydrocephalus. The number of hydrocephalus-associated loci carried by each rat correlated with the severity of disease. It is concluded that the severity of hydrocephalus in H-Tx is influenced by different genetic loci.

KEY WORDS: Infantile hydrocephalus; H-Tx rat strain; genetic loci; disease severity.

INTRODUCTION

Infantile hydrocephalus is a condition that occurs at a rate of 0.5–1.5 per 1000 births. It can be defined as an increase in cerebrospinal fluid (CSF) leading to expansion of the cerebral ventricles. Hydrocephalus is associated with brain damage leading to mental and physical disability (Laurence and Coates, 1962). Insertion of a CSF shunt device is the normal treatment for hydrocephalus. Shunt treatment alleviates the condition but is not curative. Children with hydrocephalus have a variety of neurobehavioural deficits and low nonverbal IQ scores (Dennis *et al.*, 1981; Fletcher *et al.*, 1992; Brookshire *et al.*, 1995).

Hydrocephalus has a varied etiology. It may be acquired in the perinatal period through a number of different causes, such as periventricular hemorrhage and meningitis. Infantile hydrocephalus also has a heritable component (Lorber, 1984; Varadi *et al.*, 1988) and a number of familial forms have been reported (Barros-Nunes and Rivas, 1993; Zlotogora *et al.*, 1994). There are a number of rodent models with inherited hydrocephalus (Gruneberg, 1943a; Borit and Sidman, 1972; Sasaki *et al.*, 1983; D'Amato *et al.*,

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1986; Jones *et al.*, 1987; Takeuchi *et al.*, 1987, 1988; Bruni *et al.*, 1988; Peres-Figares *et al.*, 1998). Only one of these has been characterized at the molecular level (Gruneberg, 1943b; Kume *et al.*, 1998). Many rodent models have abnormalities of the cerebral aqueduct (Sasaki *et al.*, 1983; D'amato *et al.*, 1986; Jones *et al.*, 1987; Takeuchi *et al.*, 1987, 1988), as has the H-Tx rat, the subject of this investigation (Jones and Bucknall, 1988).

The H-Tx strain, first described in 1981 (Kohn *et al.*, 1981), originated from an institutional colony of unknown origin and has prenatal hydrocephalus associated with obstruction of the cerebral aqueduct (Jones and Bucknall, 1988). Behavioral testing of severely affected rats at 22 days of age with the Morris water maze has shown that learning deficits exist that are not fully reversed by prior shunt operations (Jones *et al.*, 1995). Other studies have also detected learning deficits (Miyazawa *et al.*, 1997), some of which are improved with shunt treatment (Suda *et al.*, 1994).

From analysis of the strain breeding characteristics and from test crosses, we determined that the H-Tx colony is likely homozygous for hydrocephalus loci and the inheritance appears to be mediated by several genes (Jones *et al.*, 2000, 2001). The colony at the University of Florida has been maintained chiefly by brother–sister mating for 22 generations. The hydrocephalus is expressed with an overall penetrance of 40%, although the frequency is variable between mating pairs and between successive litters. Most cases are severe, such that affected animals die at 4–6 weeks of age. A few rats appear to develop mild ventricular dilatation with a longer survival time. In colonies of this strain maintained elsewhere, two distinct forms have been documented. One is the severe form, similar to that found by us, and the other a mild or compensating form that is less frequent (Kiefer *et al.*, 1998). In one study, 12% of the pups identified with a domed head survived for 2 months and 5% survived to 6 months of age (Miyazawa *et al.*, 1997), and in another 2 of 41 hydrocephalic pups survived for more than 80 days (Kohn *et al.*, 1981). This suggests that genetic factors modify the severity of expression.

To identify genomic regions associated with hydrocephalus in the H-Tx rat, we performed a linkage analysis on the severely hydrocephalic progeny from a backcross using Fisher (F344) rats as the normal strain (Jones *et al.*, 2001). This study identified regions on chromosome (Chr) 11 with significant linkage and regions on Chrs 9, 17, and 19 with suggestive linkage to hydrocephalus. Among the BC_1 progeny, in addition to the 12.3% with severe hydrocephalus already reported, a further 5.2% had a mild form. In this report, we obtained a measure of hydrocephalus severity for all the severely and mildly hydrocephalic progeny and performed a linkage analysis according to severity. The hypothesis tested was that different genetic loci will determine the severity of hydrocephalus in H-Tx. In addition, we report a further chromosomal region on Chr 10 with significant linkage that was not detected in the previous study.

MATERIALS AND METHODS

Animals

For all experiments, the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) were followed and the protocols were approved by the University of Florida Animal Care and Use Committee. H-Tx rats bred at the University of Florida are conventionally housed rats that originated from one of several inbred pairs provided in 1992 by D. F. Kohn, Columbia University, New York. New born pups are inspected for outward manifestation of hydrocephalus within 24 h of birth. Litters are examined again at 7–10 days, at which time both sex and overt phenotype are recorded. Fisher (F344) inbred rats were purchased from Harlan Sprague–Dawley.

Backcross Experiment

Normal F_1 progeny generated from reciprocal H-Tx and F344 matings (17 males and 21 females) were backcrossed to H-Tx rats in both directions. From these matings 1500 backcross progeny (BC_1) were obtained. All BC_1 rats were deeply anesthetized with pentobarbital (60mg/kg i.p.) between 2 and 22 days of age. Liver tissue was removed for freezing and the brains were fixed by intravascular perfusion with 4% paraformaldehyde in phosphate buffer. The brains were excised and sliced in the coronal plane at 1-mm thickness and the slices examined with a binocular microscope.

Phenotypic Analysis

The cerebral ventricles are very small in normal rats and not visible on 1-mm slices. All rats with visible ventricles, however small, were classed as hydrocephalic. For these, the number of slices with visible ventricles was counted. The slice at the level of the

striatum and anterior commissure was selected for measurement (Fig. 1). Using a digital camera (Pixera) and imaging software (Image-Pro, Media Cybernetics), the external diameter of the brain and the diameter of the ventricles were measured at the widest point, and the ratio of ventricles to brain was calculated (Miyazawa *et al.*, 1997). The rats were ordered according to increasing ratio, which was defined as severity and subdivided into four groups (see Results).

DNA Extraction, Amplification, and Analysis

DNA was extracted from frozen liver tissue by standard procedures and amplified using microsatellite markers (Research Genetics) that were polymorphic between the H-Tx and the F344 strains (Jones *et al.*, 2001). Amplification was performed in 19-μl reaction volumes containing 100 ng of DNA, 63 μmol each of dNTPs, 0.17 μmol of SSLP primers, 0.75 U of Taq DNA polymerase (Sigma) in a PCR buffer containing 1.5 m*M* $MgCl_2$ using a thermocycler (PCR Express, Hybaid). The thermocycling protocol was 94.0°C for 2 min, 34 cycles at 94.0°C for 30 s, 50–65°C for 30 s, and 72°C for 45 s, followed by 72°C for 2 min and 25°C for 2 min. The products were separated by electrophoresis on 5% agarose gels stained with ethidium bromide and photographed with UV illumination and a digital camera. Size differences of 10 base pairs or more are resolved by this method. Parental DNA was also tested in the PCR reactions to provide confirmation that the parental rats were each homozygous for the markers but different between the two strains. The genotypes of the BC_1 progeny were scored as H-Tx homozygous if there was one allele or as heterozygous for H-Tx and F344 when two alleles were present.

Genotyping Strategy

Previously, 185 severely hydrocephalic rats were identified from the number of brain slices with dilated ventricles (six or more). These and 128 control rats were genotyped with 110 microsatellite markers with 83% coverage of the rat genome. For this study, a subset of these markers (one or two per chromosome with even distribution) was used to genotype the remaining 79 rats with mild hydrocephalus, identified as having up to four slices with dilated ventricles. For Chrs 9, 11, 17, and 19, where possible linkage was identified in the previous study (Jones *et al.*, 2001), all informative markers were used. In addition, all hydrocephalic and 128 control rats were genotyped with four markers on chromosome 4 and with six markers on Chr 10 (Table 1).

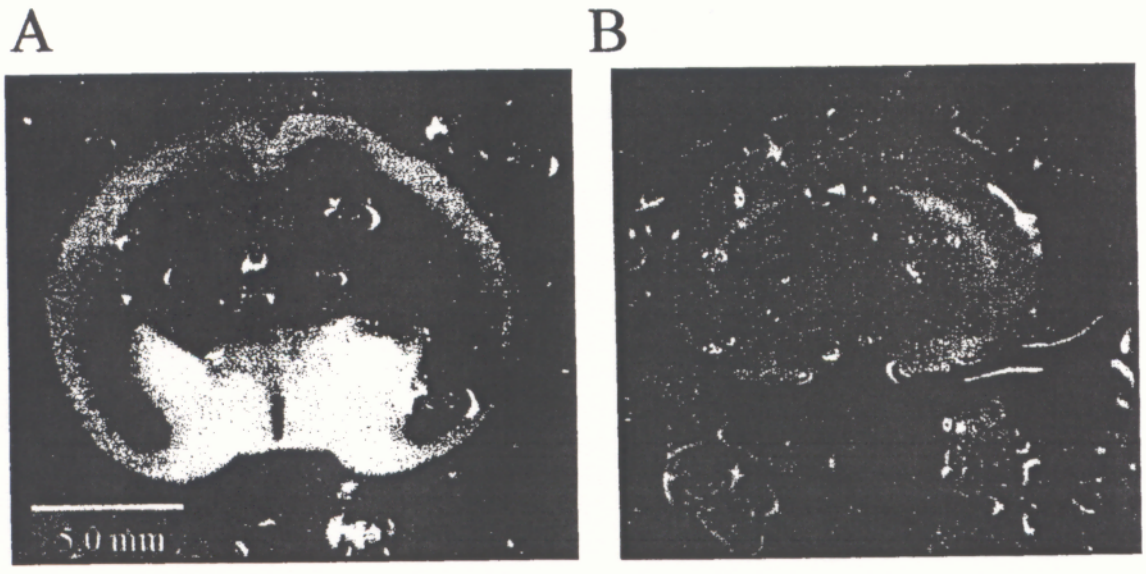

Fig. 1. Photographs of brain slices from two rats at 13 days of age. (A) A severely hydrocephalic rat in group S2 that had 11 slices with dilated ventricles. (B) A rat with mild hydrocephalus with two slices dilated ventricles and a ratio that placed it in group M2. In B ventricular measurement was taken from the sum of the left and right ventricles.

Table I. Genotype Data for Selected Hydrocephalic Groups and a Subset of Control Rats

			Hydrocephalic group(s)[c]		Control (n = 128)[d]				
Chr	Locus[a]	Position[b]	Hom	Het	Hom	Het	χ^2	p	LOD[e]
4			M1 (n = 35)						
	D4Rat2	0.0	24	11	57	70	5.25	.022	
	D4Rat166	37.4/	25	9	60	67	6.42	.011	
	D4Rat141	72.5/87.9	28	6	68	60	8.33	.004	2.4
	D4Rat72	102.2/	18	16	70	58	ns		
9			M1 + M2 + S1 + S2 (n = 260)						
	D9Rat88	5.9/1.1	125	134	61	65	ns		
	D9Rat13	45.5/42.3	152	107	56	68	5.65	.017	2.1
	D9Rat100	63.6/72.3	162	99	59	67	7.45	.006	
	D9Rat2	79.5/	162	97	59	67	7.94	.005	2.1
	D9Rat1	79.5/87.2	162	94	60	66	7.89	.005	
10			M2 + S1 + S2 (n = 225)						
	D10Rat37	34.0/31.6	100	123	74	53	5.31	.021	
	D10Rat133	46.6/52.7	106	118	68	59	ns		
	D10Rat153	51.1/	98	125	71	57	3.88	.049	
	D10Rat13	73.4/76.9	78	148	70	58	12.85	<.001	
	D10Rat135	94.1/	70	155	70	58	17.98	<.001	
	D10Rat2	93.9/	69	153	70	57	18.49	<.001	4.5
11			M2 + S1 + S2 (n = 225)						
	D11Rat28	2.5/4.5	97	128	62	65	ns		
	D11Rat73	8.2/	97	129	64	64	ns		
	D11Rat68	18.5/	96	130	63	63	ns		
	D11Rat6	19.5/	98	128	60	67	ns		
	D11Rat93	29.8/48.5	87	138	68	60	6.35	.012	
	D4Rat178	35.8/53.9	85	140	73	54	11.95	<.001	
	D11Rat46	36.5/	82	144	72	55	12.954	<.001	3.5
	D11Rat91	35.8/56.2	80	145	71	53	14.468	<.001	
	D11Rat89	38.3/57.2	82	142	71	57	11.04	<.001	
17			S2 (n = 92)						
	D17Rat113	11.7/	58	34	65	61	ns		
	D17Rat59	13.9/20.0	53	39	60	67	ns		
	D1Rat83	21.8/27.8	58	33	68	60	ns		
	D17Mit4	28.7/	68	23	61	65	14.1	<.001	3.2
	D17Rat151	32.7/	68	24	65	62	10.63	<.001	
	D17Rat127	34.9/	65	27	67	59	6.09	.014	
	D17Rat42	40.3/	64	28	66	62	6.45	.011	
	D17Rat130	40.9/	63	27	67	60	5.82	.016	
	D17Rat65	47.6/	65	27	62	65	9.56	.002	2.3
	D17Rat154	47.5/	65	27	61	64	9.52	.002	
19			S1 + S2 (n = 186)						
	D19Rat28	2.2/0.0	50	73	26	34	ns		
	D19Rat12	20.3/21.2	80	105	65	62	ns		
	D19Rat9	29.5/	80	106	77	50	8.86	.003	
	D19Rat90	/33.5	79	105	78	50	9.08	.003	2.0

[a] Loci in boldface type are those with peak linkage and used to construct Fig. 4.
[b] SSLP marker position (Whitehead Institute/MIT Center for Genome Research rat mapping project, release 8).
[c] Hydrocephalic groups M1, M2, S1, and S2, are variously grouped depending on chromosome.
[d] Control rats (n = 128) are subset of the total nonhydrocephalic BC_1 progeny (n = 1237).
[e] LOD score calculated by MAPMAKER/QTL.

Data Analysis

Validation of the ventricle-to-brain ratio as a severity measure was performed using linear regression analysis. Chi-square was used to test for linkage between phenotype and genotype within the severity groups and contingency chi-square was used to test for differences between groups. The genotyped data were analyzed with MAPMAKER computer programs (Lander *et al.*, 1987). This is a statistical package developed to map genes controlling quantitative traits in experimental crosses. MAPMAKER/EXP was used to calculate genetic distances for all the markers in a linkage group using the maximum-likelihood map for each chromosome and an automatic error detection function to remove potential genotyping errors. The maximum-likelihood maps were then analyzed with MAPMAKER/QTL, which performs interval mapping and calculates a LOD score (log likelihood of the odds) and the percentage of the variance explained for each locus with significant linkage. MAPMAKER was also used to draw LOD score graphs.

Linear regression was used to test the relation between ventricle-to-brain ratio and the chi-square test was used to compare the number of hydrocephalus-susceptibility alleles between groups.

RESULTS

Analysis of Phenotype

The ventricle:brain ratio ranged from 0.04 in the least affected rat to 0.83 in the most severely affected rat (Fig. 1). Four rats were omitted from the data analysis because the brains became damaged in preparation of the slices. For logistical reasons, it was not possible to prepare the brains using pups all at the same age. Hence, it was important to determine whether the severity ratio was affected by age. Linear regression analysis demonstrated that there was no significant correlation between ratio and age (Fig. 2A), and thus no correction was required for variation in age at fixation (2–22 days of age). On the other hand, there was as expected, a positive correlation between ratio and external brain dimension, between ratio and ventricle dimension (Fig. 2B), and between ratio and number of slices with dilated ventricles ($p < .001$ for all three correlations).

A distribution plot of frequency against ratio (Fig. 3) indicates that there are two partially overlapping populations of hydrocephalic rats: those with mild ventricular dilatation (ratio, 0.04–0.40; $n = 74$) and those

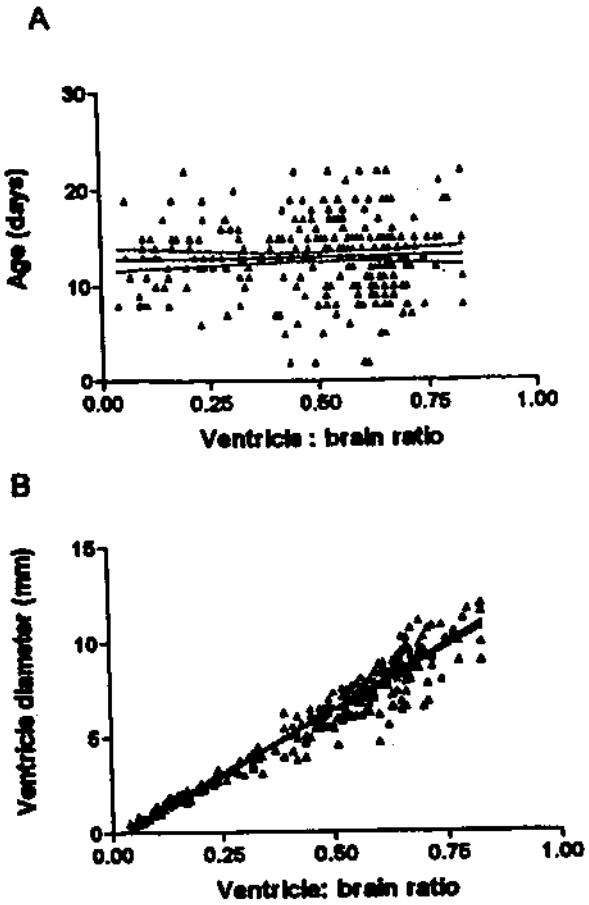

Fig. 2. Regression plot of (A) age against severity (ventricle:brain ratio) and (B) ventricle diameter against ratio. The slope is not significant from zero in A, showing that severity is independent of age. In B, there is a significant gradient ($p < .001$) showing that ventricle diameter is linearly related to severity ratio.

with severe hydrocephalus (ratio, 0.41–0.83; $n = 186$). The original classification of mild or severe hydrocephalus was based on whether or not there was clinically overt hydrocephalus before sacrifice and on the number of slices dilated after fixation. After measurement of the ventricle-to-brain ratio, this was revised for five rats. Three originally designated severe had a ratio in the mild category and two originally mild rats had ratios in the severe group. For genotype analysis, the rats were subdivided according to ratio as follows—0.40–0.20, 0.21–0.40, 0.41–0.60, and 0.61–0.83—to give four groups overall, corresponding to groups M1 ($n = 35$); M2 ($n = 39$); S1 ($n = 94$;) S2 ($n = 92$); respectively.

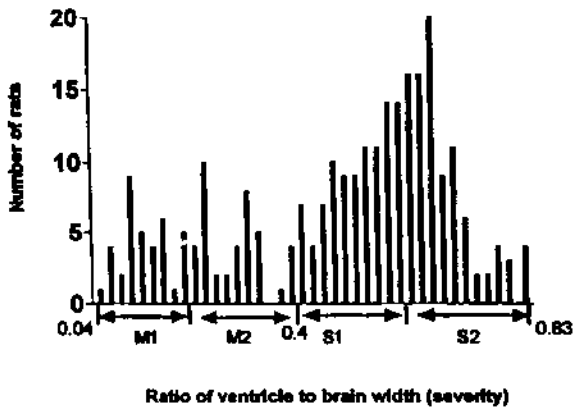

Fig. 3. Distribution plot of numbers of rats against severity as measured by the ventricle-to-brain ratio. There appear to be two populations, one with a severity ratio <0.4 (mild hydrocephalus) and another with a severity ratio >0.4 (severe hydrocephalus), with very little overlap between the two. The subpopulations used for genotype analysis are designated M1, M2, S1, and S2.

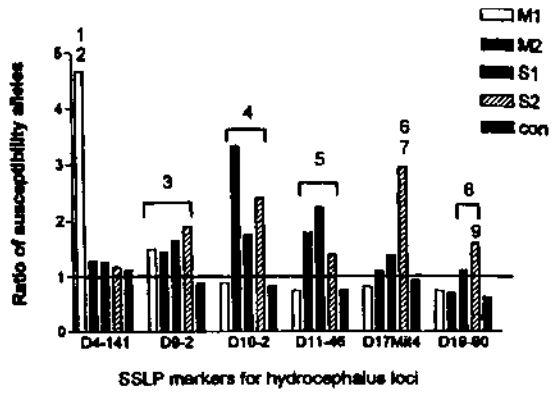

Fig. 4. Histograms of the allele ratios at the peak markers, homozygous:heterozygous for Chrs 4, 9, and 17 and the reverse, heterozygous:homozygous for Chrs 10, 11, and 19, for five groups of rats, M1, M2, S1, S2, and control (con); n = 34, 39, 94, 92, and 128, respectively. (1) M1 significantly different from con, $p < .004$; (2) M1 significantly different from M2, $p < .033$; (3) all four groups significantly different from con, $p < .005$; (4, 5) M2 + S1 + S2 group significantly different from con, $p < .002$; (6) S2 group significantly different from con, $p < .001$; (7) S2 significantly different from S1, $p < .023$; (8) S1 + S2 significantly different from con, $p < .003$; (9) S2 significantly different from con, $p < .02$. LOD score graphs were plotted for each of the following hydrocephalic group combinations: 1, 3, 4, 5, 6, and 8 versus control.

Association with Sex

The percentage of male hydrocephalic pups was 60, 54, 64, and 61 for the M1, M2, S1, and S2 groups, respectively. This was not significant for the two mild groups (n = 74), but there were significantly more males in the two severe groups ($\chi^2 = 5.91, p < .05; n = 186$). For normal progeny the percentage of males was 43. Because there was no significant linkage to the X chromosome, the abnormal sex ratio may be an environmental effect (Jones *et al.*, 2001).

Linkage Analysis

As reported previously (Jones *et al.*, 2001), hydrocephalus was associated with homozygosity for the H-Tx alleles on Chrs 9 and 17. However, on Chrs 11 and 19 hydrocephalus was associated with heterozygosity, that is, hydrocephalus is more likely to occur in animals that have both an H-Tx and an F344 allele. In this analysis a third heterozygous locus was detected on Chr 10. In addition, a homozygous locus with suggestive linkage was detected on Chr 4. The genotype data for selected groups of hydrocephalic rats, together with a subset of 128 control rats, were analyzed separately for each group, using all informative markers on Chrs 4, 9, 10, 11, 17, and 19 (Table I). The marker with the highest significance level on each chromosome was selected for histogram plots of the ratio of susceptibility alleles for each severity group (Fig. 4). The severity groups that had allele ratios significantly different from those of control rats were selected for further analysis with MAPMAKER/QTL. For Chr 4, only the M1 group had a ratio that was significantly different from control ($p < .004$) and the LOD score was 2.4 mapping between marker *D4Rat166* and marker *D4Rat141* (Table I, Fig. 5). This is suggestive linkage only for this small subset of BC_1 progeny with mild hydrocephalus. On Chr 9, all four severity groups were significantly different from control rats ($p < .005$) (Fig. 4) and were combined for a LOD score analysis. There was suggestive linkage (LOD = 2.1) that mapped between *D9Rat13* and *D9Rat100* and there was also another suggestive locus (LOD = 2.1) between *D9Rat100* and *D9Rat2* (Table I, Fig. 5). The first peak was not detected in our previous study in which only severe rats were genotyped (Jones *et al.*, 2001). For Chrs 10 and 11, the severity groups M2, S1, and S2 were combined for analysis since they were significantly different from the control group ($p < .002$); whereas group M1 was similar to control rats (Fig. 4). The peak LOD scores were 4.5 at *D10Rat2* and 3.5 at *D11Rat46* (Table I, Fig. 5). Both loci are at the telomeric ends and are fully significant (Lander and Kruglyak, 1995). For Chr 17, only the most severely affected rats were significantly different from controls

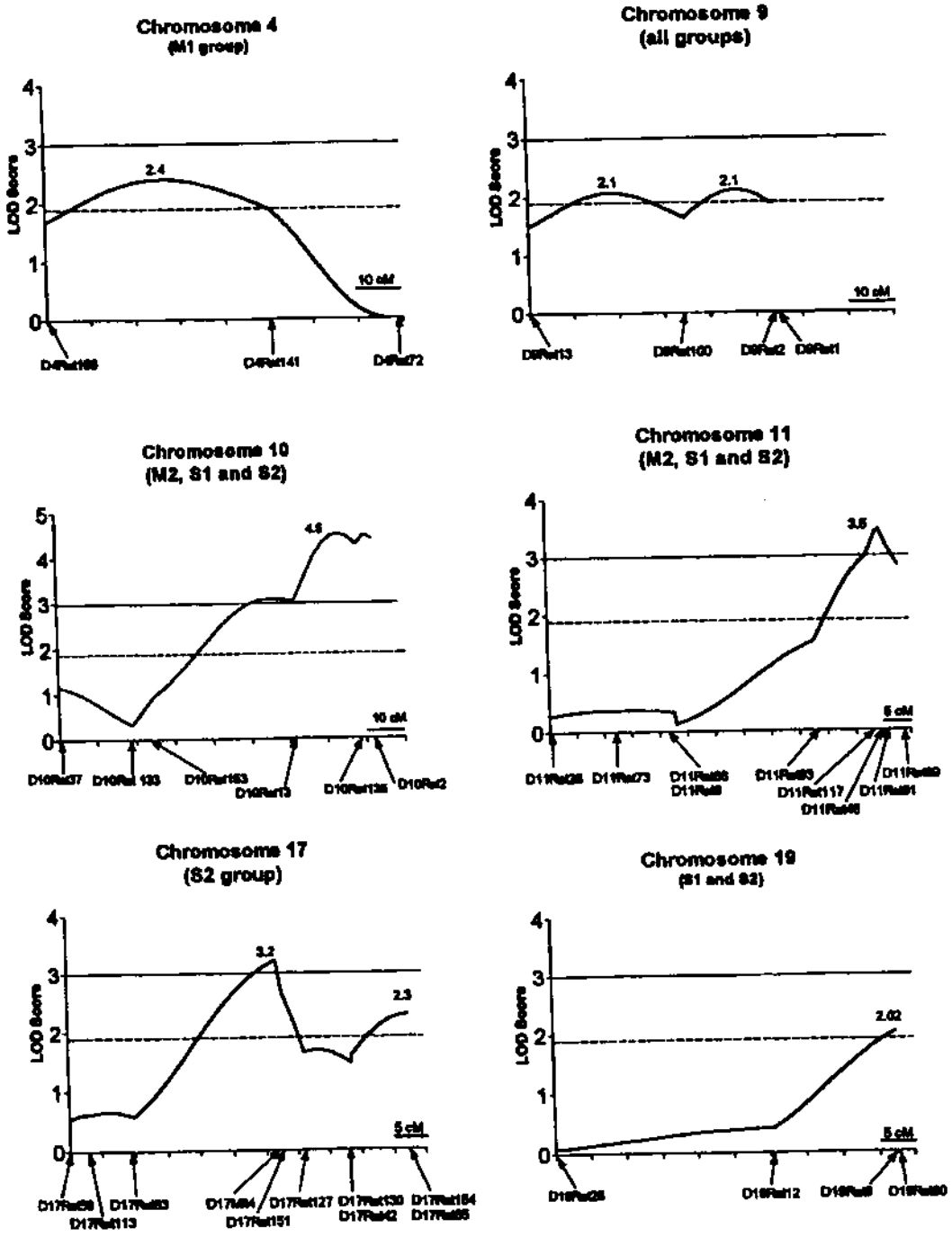

Fig. 5. LOD score plots of the data in Table I as calculated by MAPMAKER/QTL for the six chromosomes with loci linked to hydrocephalus. The plots for the various chromosomes consist of different combinations of severity groups, depending on which groups were significantly different from the control as shown in Fig. 4.

($p < .001$) (Fig. 4), and the peak LOD score was 3.2, which is very close to full significance, mapped to *D17Mit4*. There was also suggestive linkage (LOD = 2.3) at the end of the chromosome at *D17Rat65* (Table I, Fig. 5). On Chr 19, only the two severe groups, particularly S2, were significantly different from control rats ($p < .003$) (Fig. 4), whereas the rats with mild disease were not different from control animals. There was suggestive linkage for severe hydrocephalus (LOD = 2.0) near *D19Rat90* at the telomeric end of the chromosome (Table I, Fig. 5). Looking at individual loci (Figs. 4 and 5), it is clear that the two extremes for hydrocephalus are associated with Chr 4, which is important only for mildly affected rats, and Chr 17, which is important only for the most severe condition.

Multilocus Susceptibility for Hydrocephalus

The number of susceptibility alleles was examined for each rat using the six peak loci that were used to construct Fig. 4. Linear regression analysis of ventricle-to-brain ratio against number of susceptibility alleles for all affected progeny showed a small but significant slope ($p < .05$; not illustrated). This indicates that the severity of hydrocephalus is associated with an increasing number of susceptibility loci. The percentage of rats with each number of alleles was calculated for the mild (M1 + M2) hydrocephalus group, for the severe group (S1 + S2), and for the control group (Fig. 6). There was no hydrocephalic rat with none of the six susceptibility alleles and there was no control rat that had all six alleles. The highest percentage for the mild group was three and four alleles (31%), with a steep decline in numbers for five and six alleles (12 and 1.5%, respectively). The severe group also had the highest percentage with three and four alleles (26%), but in addition, there were more rats with five and six alleles (24 and 5.6%, respectively) than in the mild group. The control group, on the other hand, had most rats in the two allele category (33%), with a steady decline above two alleles to zero at six alleles. Both hydrocephalic groups, M1 + M2 and S1 + S2, have curves that are shifted to the right compared to that of the control group and the curve for the severe group is more right-shifted that that for the mild group (Fig. 6).

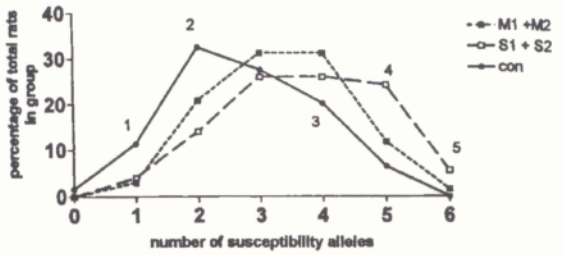

Fig. 6. The percentage of rats from the mild, severe, and control groups plotted against the number of susceptibility alleles calculated for each rat. There were no hydrocephalic rats with zero alleles and there were no control rats with all six alleles, although there is considerable overlap of the graphs. For the control rats (con), the largest percentages were in the two and three allele categories. For the mild group (M1 + M2), the largest percentage relative to the severe group (S1 + S2) was for rats with two, three, and four alleles. For the severe group, the largest percentage relative to the mild and control groups was for rats with five and six alleles as shown by a shift in the graph to the right. (1) Control significantly different from M1 + M2, $p < .01$, and from S1 + S2, $p < .05$; (2) control significantly larger than S1 + S2, $p < .001$; (3) control significantly less than M1 + M2, $p < .05$; (4) S1 + S2 significantly greater than control, $p < .001$, and greater than M1 + M2, $p < 0.01$; (5) S1 + S2 significantly greater than the other two groups, $p < .05$.

DISCUSSION

In a previous report we determined that severe hydrocephalus in H-Tx rats is associated with a locus on Chr 11 and possibly with three more loci on Chrs 9, 17, and 19 (Jones *et al.*, 2001). Extension of the earlier mapping has now revealed an additional locus on Chr 10. In addition, there was a second group of individuals that had a mild form of hydrocephalus which was not included in the previous study. Designation of these animals as mild or severe was previously based on overt appearance and on the number of brain slices showing dilated ventricles after fixation. For the present study, a more precise measure of severity, based on the ratio of ventricle width to brain width, was obtained for all animals with dilated ventricles. This demonstrated that there was a continuous scale ranging from the mildest to the most severe and, also, that there appear to be two populations that correspond largely to the two original classifications, with a small overlap between them. Since the brains were not all removed at the same age, the question was addressed whether the younger rats would have more severe hydrocephalus if sacrificed at an older age. Although we cannot provide a direct answer, there is evidence from the regression analysis (Fig. 2A), which showed no significant increase in severity with age, and some of the youngest pups had ratios that placed them in the severe group. However, it is not possible to predict how many of these progeny would have survived beyond the 4–6 weeks, reported for severely hydrocephalic rats (Kohn *et al.*, 1981; Jones and Bucknall, 1988; Wada, 1988).

Hydrocephalus has not been reported for the parental F344 strain, and examination of the brains from 92 individuals has confirmed that it is a nonhydrocephalic strain (Jones *et al.*, unpublished observations). Test crosses between F344 and H-Tx did not produce any F_1 rats with hydrocephalus. However, a test cross between H-Tx and LEW inbred rats did produce a small percentage of hydrocephalic pups, showing that the trait can be dominant under some conditions (Jones *et al.*, 2000). F344 was selected for the backcross on the basis of the F_1 phenotype.

In our H-Tx parental rats used for the backcross, the incidence of severe hydrocephalus was 40%, but these rats were not examined for mild disease which appeared to be rare. However, mild dilatation has been observed by others in H-Tx at a frequency of about 5% of all hydrocephalic offspring (Kohn *et al.*, 1981; Wada, 1988; Miyazawa *et al.*, 1997). In this backcross, the frequency of severe hydrocephalus was 12% and an additional 5%, or 30% of all affected animals, had mild disease. Hence, genetic influences from the normal strain have reduced the frequency of severely affected rats and, also, altered the relative frequency of the two forms.

The genotyping results produced a number of interesting features. The significant association of the least hydrocephalic rats with a locus on Chr 4, which was not present in any other group including control rats, was a surprise. The LOD (2.4) was only suggestive but there were only 34 rats in this group. In contrast, the locus observed previously associated with Chr 17 was very significant for the most severe rats, with almost no effect for any other group. The LOD for the peak locus on Chr 17 was 3.2, showing that by selecting for the most severe animals (S2), the score increased from that obtained previously for all severely affected rats (LOD 2.4) (Jones *et al.*, 2001). The same S2 group also showed a second possible locus on Chr 17 at the telomeric end (LOD 2.3) which had not been observed in our previous study. Clearly, the peak locus on Chr 17 is important for severe ventricular dilatation. Chromosome 11 was previously shown to have a hydrocephalus locus associated with heterozygosity (Jones *et al.*, 2001). In this study, the heterozygosity was associated with all hydrocephalic groups except the least affected, M1. The LOD increased from 3.1 previously to 3.5 when M2, S1, and S2 were analyzed together. Hence, the addition of M2 rats increased the significance for a hydrocephalus-associated allele on Chr 11. However, the highest LOD score (4.5) was obtained for the newly discovered locus on Chr 10 with hydrocephalus also associated with heterozygosity. Two remaining loci that have suggestive linkage are on Chr 9, where there is a similar level of significance for all severity groups, and on Chr 19, a heterozygous locus that is associated with severe hydrocephalus only. Altogether, the identified loci account for about 20% of the variance. This appears to be low for a trait that is strongly inherited.

In addition to the heritable component, there is evidence for environmental and maternal influences in the H-Tx strain. It was reported previously that the frequency of overt hydrocephalus is lower in the first litter than in subsequent litters, suggesting a maternal effect that has yet to be investigated (Jones *et al.*, 2000). Another observed maternal effect is that the frequency of hydrocephalic offspring in the backcross progeny is significantly higher if the mating is H-Tx female × F_1 male than if it is the reverse (Jones *et al.*, 2001). All severity groups have an excess of males, although this is more marked in the most severely affected groups. An excess of males with hydrocephalus was reported previously in our colony (Jones *et al.*, 2000) and also in earlier publications on H-Tx (Kohn *et al.*, 1981; Wada, 1988). There is a possibility that pathogens are affecting expression because the conventionally housed colony on which these data were based was known to carry *Mycoplasma pulmonis*. More recently, we obtained SPF H-Tx rats and the percentage of affected male offspring is about 50, although this still has to be confirmed with a larger data set (Jones *et al.*, unpublished observations). It is still possible that other genetic effects such as imprinting and mitochondrial transmission are responsible for some of these phenomena, but currently there is no evidence to support this.

From a previous analysis of the breeding characteristics of H-Tx, it was concluded that there is likely to be more than one susceptibility locus controlling the expression of hydrocephalus. This study has shown that, in addition to loci found previously [*i.e.*, one significant locus on Chr 11 and three loci on Chrs 9, 17, and 19 suggestive for hydrocephalus (Jones *et al.*, 2001)], there is an additional significant locus on Chr 10 and one on Chr 4 that is associated only with mild ventricular dilatation. This locus appears to have the effect of opposing other susceptibility loci by being unfavorable for the development of severe disease. The rats with mild ventricular dilatation did not have overt hydrocephalus at the time of euthanasia. Hence, it is possible that they would have survived to adulthood. The susceptibility locus on Chr 17, on the other hand, appears to code for a factor that exacerbates the condition and it is very close to a LOD score for full significance, for the most severe

rats only. The marker *D17Mit4* maps to the gene *Chrm3*, coding for the muscarinic M3 receptor (Jacob *et al.*, 1995), making it a possible candidate gene for this locus. This separation of loci according to severity suggests that different processes work together in some instances and against each other in other circumstances, to determine outcome. In addition, the strongest loci are associated with heterozygosity (Chrs 10 and 11), which suggests that alleles from the "normal" strain may increase the risk for hydrocephalus expression.

This study has identified hydrocephalus-associated loci that affect disease severity. Loci affecting severity have been observed in other rodent models of human disease, particularly those that are inflammatory or autoimmune diseases. For example, rats with collagen-induced arthritis have four loci that contribute to disease severity (Remmers *et al.*, 1996) and mice with infection-induced arthritis have two loci that affect the severity of histopathological lesions (Weis *et al.*, 1999). Other examples with one or two severity loci in mouse models are lupus-like nephritis (Santiago *et al.*, 1998), dextran sulfate-induced colitis (Mahler *et al.*, 1999) and experimental allergic encephalomyelitis (Teuscher *et al.*, 1999).

In conclusion, it seems likely that the same basic biochemical processes lead to hydrocephalus in all the affected groups but that the disease severity, as measured by the degree of ventricular dilatation, is determined by a complex combination of hydrocephalus-susceptibility alleles, genetic background, and environmental effects. In the future, these phenomena will be further analyzed by separating the different loci into congenic strains and by investigating potential candidate genes that map to the chromosomal regions with significant linkage to hydrocephalus.

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