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Glucocorticoid-induced polycystic kidney disease—A threshold trait

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Glucocorticoid-induced polycystic kidney disease—A threshold trait. Administration of hydrocortisone acetate (250 mg/kg) to newborn mice caused polycystic kidney disease (PKD) of varying proportions in each of 18 different inbred strains; none of the injected controls were affected. All kidneys were histologically examined and scored for degree of cyst formation using a semi-continuous (0 to 4+) grading scheme. Results suggested that this condition is a multifactorial threshold trait. For each strain, estimates of the mean and standard deviation of normally distributed liability were determined by maximum likelihood methods. Concomitant analyses showed: 1) a significant environmental effect related to drug source; 2) a variation in thresholds ranging from 0.94 (N = 46) for the B10.M strain to −0.71 (N = 297) for the C57B1/6J strain; and 3) three groups of strains with different susceptibility to PKD. These results are consistent with a multifactorial basis for susceptibility to PKD. Quantitative analysis of thresholds and liability distributions reveals that genetic, environmental and random elements all contribute to the expression and extent of the cystic trait.

Renal morphogenesis in rodents persists into the early postnatal period (1 to 3 weeks) so that teratogenic induction of tubular malformations is possible before and after birth [1]. A proportion of mice, rats, hamsters and rabbits, will develop polycystic kidney disease (PKD) in response to postnatal administration of exogenous glucocorticoid hormone [2–5].

In both humans and other mammals with naturally occurring renal cysts, the various forms of PKD have been considered as single gene disorders with mendelian modes of inheritance [6]. Our preliminary studies of glucocorticoid-induced PKD in inbred mice showed that the proportions of affected animals varied from strain to strain [2] and were inconsistent with simple Mendelian ratios. This paper describes our hypothesis of the genetic basis of PKD and the formal analysis in 18 inbred strains of mice. The results indicate that susceptibility to induction of PKD by hydrocortisone acetate (HCA) is multifactorial and that cystic change is a threshold trait.

Methods

Experimental protocol

Breeding pairs from inbred, congenic and mutant strains of mice (Table 1) were obtained from either Jackson Laboratories, (Bar Harbour, Maine, USA) or private breeding stocks. They were maintained under the guidelines outlined by the Canadian Council of Animal Care [7], as inbred isolates in our animal quarters. The room was kept at 20°C with alternating 12 hours of light and dark. For each strain, sufficient pups to maintain a breeding stock were excluded from the study. The remaining one day old newborns (2069 mice) were weighed and injected intramuscularly with HCA (250 mg/kg body weight) in the hindquarter using a 27 gauge needle. Mice injected with a similar volume of normal saline and uninjected animals served as controls ($N > 500$).

Animals from the initial survey of 18 strains were studied at various ages between 4 and 14 days. Thereafter, all animals were studied on day 5, as this was found to be the optimal interval for induction of cystic changes. After sacrifice, the kidneys were removed and placed in Trump's fixative [8]. The tissue was processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The microscopic slides of the kidney were independently read by two observers. Sections without any demonstrable change were scored as 0 and cystic changes were scored on an ascending scale of 1+ to 4+, as shown in Figure 1.

Initially, all nine strains received Cortef^{R} Acetate, a preparation of hydrocortisone acetate (HCA) manufactured by the Upjohn Co. of Canada (Montreal, Quebec). Because the Upjohn Company discontinued the drug, a change was made to Hydrocortone^{R} acetate, a preparation from Merck Sharp & Dohme (Canada). Preliminary analysis revealed different responses to the two drug preparations, so that subsequent results with the Merck Sharp & Dohme product (MSD study group) and the Upjohn product (UPC study group) were analyzed separately. Information provided by the manufacturers suggested that the components of both preparations were identical, but concentrations of non-active ingredients in one of them were not available to us.

Analysis of proportions

Proportions of affected mice in the UPC group were compared using a likelihood ratio test [9], and strains were further classified using a technique for detecting a change in a sequence of proportions [10]. Using the method of Elston [11], intraclass correlations (r_I) were calculated for each study group as estimates of heritability. These estimates, based on the ratio of the

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Table 1. PKD in 18 strains of inbred mice[a]

Strain	Proportion UPC	Proportion MSD
B10.G[c]	2/29	
B10.WB[c]	8/46	
B10.M[c]	4/28	
YBR/WiCv-*Eg*^{o}[b,d,f]	13/63	
BALB/c	18/76	
BALB/B		2/25
WB/ReJ[g]	2/7	
DCH-*TfmBlo*[d,f]	21/63	
SK/Cam[g]	3/9	
DBA/2J	21/58	6/41[e]
C57BL/6J-*bg*[b,d]	6/17	
C57BL/6J-*W*^{v}[b,d]	31/80	
CBA/CaJ	64/132	13/59[e]
DD[f]	85/153	51/189[e]
SB/Le	16/23	
C3H/HeJ	226/316	90/238[e]
C57BL/6J[b]	226/297	37/89[e]
A/J	25/31	

[a] MSD and UPC refer to the different manufacturers (Merck Sharp Dohme and Upjohn, respectively) of hydrocortisone acetate.
[b] Single gene mutation on the C57BL/6J strain
[c] Strains congenic with C57BL background
[d] Defined single gene phenotypes (see text)
[e] Significantly different proportions (MSD vs. UPC)
[f] The *Eg*^{o} mutation was kindly supplied by V. Chapman (Roswell Park Memorial Institute, Buffalo, NY). The DD strain was from J. Schlom (NIH, Bethesda, MD). The DCH-*TfmBlo* is from the private stocks of one of us [39].
[g] Estimates of proportions may not be robust because they are based on only 1 litter.

additive component of the among-strain to total variance, would tend to be inflated if used to estimate the heritability of the trait in a random mating population instead of among inbred strains.

Time-series analyses [12] were used to study variation of the trait over time in strains with sufficient sample size (CBA/CaJ, DD, C3H/HeJ and C57BL/6J). These were subgrouped by month of birth, season, generation, number of pregnancies, and litter size.

Threshold analysis

A threshold trait can be defined as one which is only manifested when the total contribution of the genetic and environmental factors reaches a critical amount called the threshold. The combination of these factors is considered to be a variable, termed liability, and for the purpose of analysis, is assumed to have a normal distribution (normal probability density function) [13]. This concept has been extended to include multiple thresholds when increasing expression of a trait is manifested as discrete changes in the phenotype [14, 15]. Conceptually, the underlying, normally distributed liability maps into the discrete phenotypic distribution [16].

In our experiments, increasing expression of PKD was continuous, but had been grouped into quantified categories. It was postulated by one of us that such categorization of a trait could be treated as an empirical multinomial sampling of a truncated continuous distribution, the truncation point being the threshold. Using the graded scores of cystic change as multinomial categories, the hypothesis that their distribution was consistent with an underlying normal distribution was tested. There were sufficient numbers of mice for detailed analysis in 9 out of 18 strains sacrificed on day 5.

To fit the empirical distribution to a normal probability density function, the following log likelihood function, (with mean, m, and variance, s^2)[1] up to an additive constant, was formulated and optimized to obtain maximum likelihood estimates of the mean and variance for each strain receiving either the UPC or MSD preparation:[2]

$$l(m,s^2) = n_0 \log \int_{-\infty}^{\frac{0-m}{s}} \frac{1}{\sqrt{2\pi}} e^{-\frac{x^2}{2}} dx + \sum_{i=1}^{3} n_i \log \int_{\frac{i-1-m}{s}}^{\frac{i-m}{s}} \frac{1}{\sqrt{2\pi}} e^{-\frac{x^2}{2}} dx + n_4 \log \int_{\frac{3-m}{s}}^{\infty} \frac{1}{\sqrt{2\pi}} e^{-\frac{x^2}{2}} dx$$

where log = $\log_e$ and n_i is the number of mice in the score category i+ (i = 0, 1, . . . , 4). From these results, estimates of the expected values for each score were calculated. In both study groups the goodness-of-fit with a normal distribution was determined for each strain, for pooled subgroups of strains, and for the pool of all strains, using likelihood ratio tests comparing the observed and expected scores [18]. Homogeneity of distributions within the defined subgroups was tested using likelihood ratio tests [9]. Estimates of the thresholds (with approximate 95% confidence intervals) in units of standard deviation from the means are given by the relation: t = −m/s.

Results

None of the 500+ controls demonstrated any evidence of cystic change. On the other hand, HCA administration induced PKD in a varying proportion of all 18 strains (Table 1). Of the four strains on the C57B1/6J background, the three single gene mutations [beige (−*bg*), spotting (−W^{v}); and glucuronidase negative (−Eg^{o}) were less affected than the parent strain. Mutant homozygotes, heterozygotes and wild-type animals were not distinguished in the analysis of these mutations. In a related manner, the B10 congenic strains were minimally affected as compared to the C57BL/6J animals, although they also derive from the same C57BL background.

There were statistically significant differences among the proportions of affected animals in the UPC study group (D = 132.74: P = 0.0, 9d.f.). The nine strains could be classified into three distinct subgroups (Fig. 2). The B10.M strain, the only member of Subgroup I, was more resistant than any of the other strains, with less than 7% of the animals affected. Four strains (comprising Subgroup II) with moderate resistance (<50%

[1] Given by

$$f(m,s^2) = \frac{1}{\sqrt{2\pi s^2}} e^{\frac{-(x-m)^2}{2s^2}}$$

where x is any value in the domain of the distribution [9].
[2] Computerized optimization of this function was achieved using a conjugate gradient method [17]. A Fortran program was written for this purpose and supplemented with NAG library subroutines (VAX 8800 computer Digital Equipment Corporation).

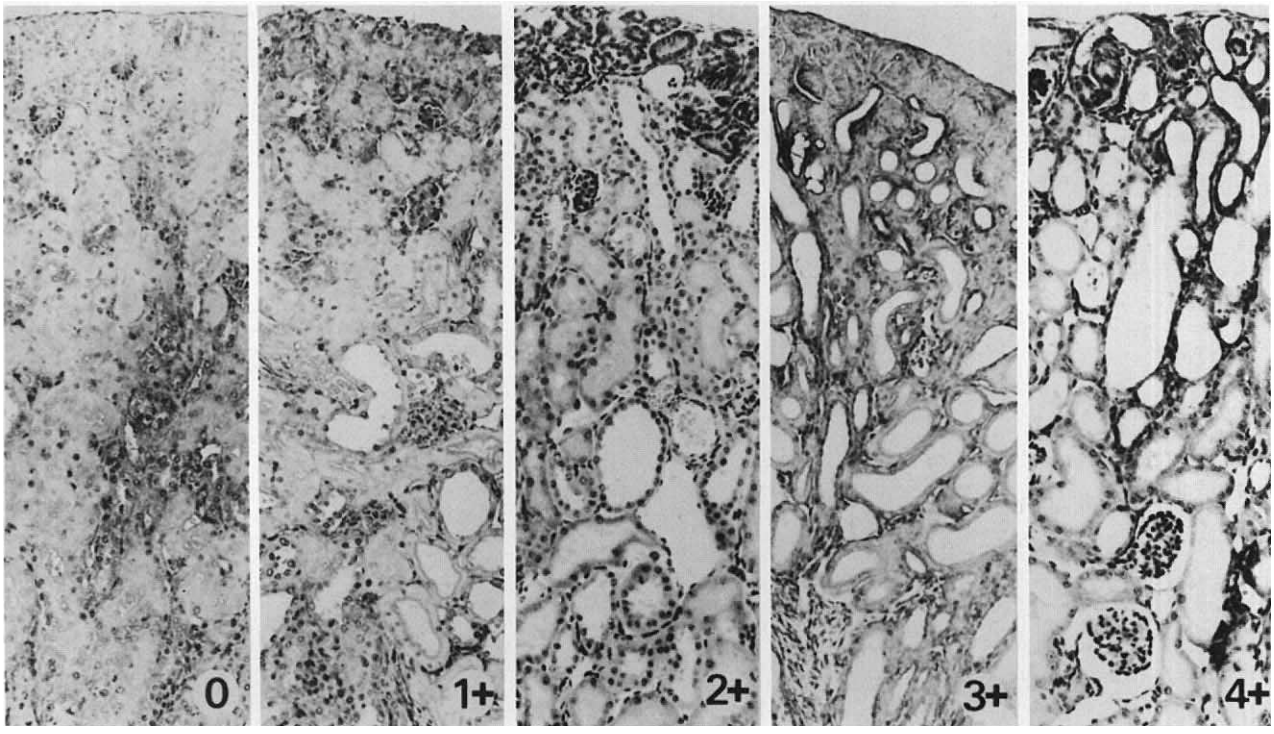

Fig. 1. *A composite photomicrograph of kidneys demonstrating a measurement scale of severity of 0 to 4+.* The left frame shows a normal (0) kidney while each successive frame represents 1+, 2+, 3+, 4+ cyst development. Cystic changes are present in proximal, distal and collecting tubules, the distal changes being relatively more prominent as severity increases. Glomerular tufts appear normal, but there is occasional dilatation of Bowman's space. Various immature nephron forms can be seen in the outer cortex as these are differentiating organs (H & E: each × 90).

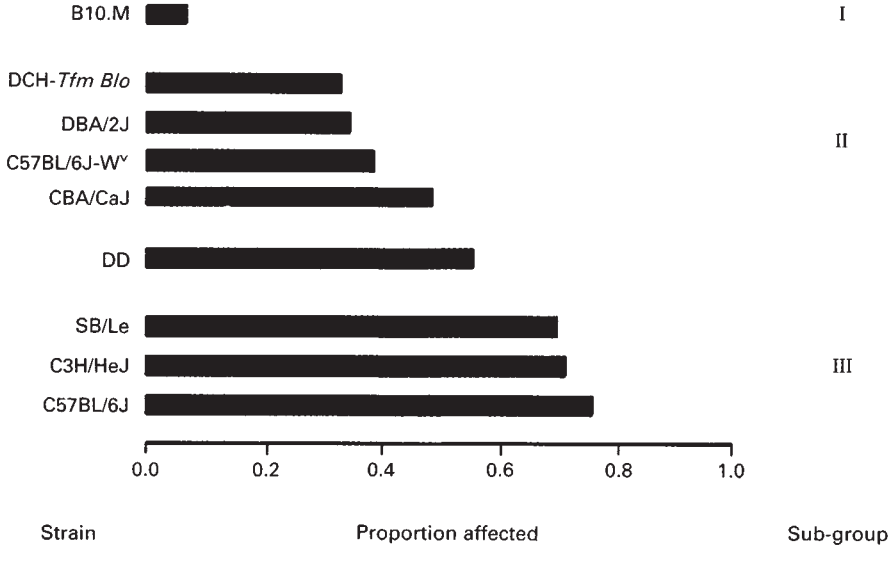

Fig. 2. *Proportions of mice, from the UPC study group, affected with polycystic kidney disease.* Data from 9 different strains were classified and then tested for within subgroup homogeneity [9] into Subgroups I, II, III [10]. The DD strain was not classifiable but was homogeneous with Subgroup II in the MSD study group.

affected) were homogeneous with respect to proportion. In the three most sensitive strains comprising Subgroup III, 70 to 76% of animals were affected. Inclusion of the DD strain (56% affected) in either subgroup II or III resulted in significant non-homogenitity (D = 13.164, $P = 0.001$ 4df. and D = 3.515, $P = 0.172$) 2df, respectively).

For the five strains given both drug preparations, the proportions of affected animals were also homogeneous within both Subgroups II and III but were significantly less than the corresponding proportions in the animals receiving the UPC preparation ($P < 10^{-5}$ for both subgroups). In the MSD group, homogeneity of subgroups II is preserved with inclusion of the DD strain (D = 20.001, $P = 0.0$, 3 d.f.). After separation according to drug source, examination for possible influence of

Table 2. Threshold analysis of the UPC study group

	B10M		DBA/2J		DCH-*TfmBlo*		C57BL/6J-W^v	
	OB	EX	OB	EX	OB	EX	OB	EX
N	46		58		63		34	
n_0	38	38.021	38	38.329	42	42.368	18	18.589
n_1	7	6.989	13	11.165	16	15.646	10	8.712
n_2	1	0.946	4	5.850	5	4.433	4	4.787
n_3	0	0.043	2	2.071	0	0.527	2	1.568
n_4	0	0.001	1	0.585	0	0.026	0	0.344
m		~0.8688		~0.6520		~0.4635		~0.1598
s		0.9237		1.5720		1.0373		1.3607
D		0.0001		0.9902		0.0112		0.3415
P		*		0.3197		*		0.5590
t	0.9388	0.9406	0.3993	0.4148	0.4307	0.4468	0.0738	0.1174
CI		±0.4717		±0.2731		±0.3035		±0.3443

Statistics and results from 9 strains of mice injected with hydrocortisone acetate (Upjohn). There are observed (OB) and expected (EX) columns for each strain. Numbers under OB except for the value of t (see below), are raw data. Numbers under EX are derived from the hypothesized normal distributions characterized by maximum likelihood estimates of the mean and variance.

For each strain:

N, total number of mice injected.

n_0, number with no evidence of PKD.

$n_1 \ldots n_4$, numbers with PKD in score categories 1+ to 4+.

m, maximum likelihood estimate of the mean.

s, maximum likelihood estimate of the standard diviation.

D, deviance. The likelihood ratio statistic for goodness-of-fit. $D = 2\sum_{i=o}^{4} n_{i\{OB\}} \ln(n_{i\{OB\}}/n_{i\{EX\}})$. Adjacent categories were combined when $n_{i\{EX\}}<1$. D is approximately distributed as chi square when the null hypothesis is true.

P, *P*-value associated with D. The values are not corrected for simultaneous inference. '*' indicates insufficient degrees of freedom for estimation.

t, threshold. Under OB, it is the value of z (the standard normal variate) such that the area to the left of z equals n_0/N. Under EX, $t = -m/s$.

CI, the number added to and subtracted from t to obtain approximate 95% confidence limits. It is based on a transformation of the diagonal elements of the Fisher information matrix of the likelihood function.

time, season, number of pregnancies, and generation revealed relatively wide fluctuations without regular pattern or significantly periodicity.

The intraclass correlation estimate (r_I) was 0.140, suggesting that the cystic trait has a low heritability. The interclass correlations within Subgroups II ($r_I = 0.027$) and III ($r_I = 0.0$) suggest no intra-subgroup genetic variation with respect to the quantal trait.

Threshold and liability distribution analysis

Summary data for the UPC group (Table 2) shows good agreement between observed and expected values in each score category. Likelihood ratio statistics (D) for the goodness-of-fit showed no significant deviation from normality ($P > 0.05$) for six of the seven strains in the UPC group that could be analyzed when considered as independent tests. However, when a Bonferroni correction for simultaneous inference [19] is applied, the DD strain distribution (uncorrected $P = 0.0364$) is also shown to be consistent with normality. This goodness-of-fit with the expected probability density function curves can be readily appreciated from visual inspection of Figure 3.

Although testing of the pooled data (generating a single probability density function for the entire UPC group) was consistent with normality ($D = 1.3266$; $P = 0.52$, 2 d.f.), the nine strains of the UPC group were not statistically homogeneous ($D = 177.85$, $P < 0.001$, 16 d.f.).

The distributions of Subgroups II and III, defined by differences of proportion, were different ($D = 103.84$, $P < 0.001$, 12 d.f.), but both Subgroup II (moderate resistance) ($D = 8.8169$, $P = 0.1841$, 6 d.f.) and Subgroup III (sensitive) ($D = 7.7469$, $P = 0.1013$, 4 d.f.) were internally homogeneous. The DD and the B10.M strains could not be fitted to either subgroup.

Summary data for the five strains receiving the MSD preparation are shown in Table 3. Goodness-of-fit testing for normality was the same as for the UPC group except that the DD strain was normally distributed and fitted quite well with Subgroup II. The agreement between observed and expected values remains close, but the distribution mean and calculated threshold for any given strain are different from those for the UPC preparations. Examples of these differences are shown in Figure 4. The variances within subgroups were homogeneous, even across study groups, when tested using likelihood ratios or the Bartlett statistic ($P > 0.5$ for both tests).

Discussion

In 1950, Baxter and Fraser first described the teratogenic induction of cleft palate by cortisone injections into pregnant mice [20]. Subsequent studies have amply confirmed that this steroid-induced malformation has a genetic basis and conforms closely to the model for multifactorial inheritance [21–25].

Our findings indicate that glucocorticoid-induced PKD also fits the multifactorial threshold model. First, different proportions develop PKD after injection with HCA in each of 18 strains of mice examined. Second, there are differences in liability related to the presence of single gene mutations on a constant background (C57B1/6J). Third, the severity of the disease, among the nine strains studied in detail, has a frequency distribution with significant goodness-of-fit to a normal

Table 2. *Continued*

CBA/CaJ		DD		C3H/HeJ		SB/Le		C57BL/6J	
OB	EX	OB	EX	OB	EX	OB	EX	OB	EX
132		153		316		23		297	
68	68.930	68	70.112	90	91.347	7	6.598	71	71.898
46	42.290	58	50.391	122	121.400	8	8.287	115	111.765
14	17.438	18	25.694	81	80.189	6	5.959	82	84.697
3	3.099	8	6.098	23	20.862	1	1.881	25	25.481
1	0.243	1	0.705	0	2.202	1	0.275	4	3.159
	~0.0586		0.1161		0.5533		0.5983		0.6990
	1.0532		1.1074		0.9950		1.0635		0.9991
	1.1779		4.3794		4.6470		0.0460		0.4060
	0.2778		0.0364		0.0979		0.8302		0.8163
0.0380	0.0557	~0.1397	~0.1048	~0.5686	~0.5561	~0.5119	~0.5625	~0.7093	~0.6996
	±0.1982		±0.1645		±0.1182		±0.3950		±0.1217

Table 3. Threshold analysis: MSD study group

	DBA/2J		DD		CBA/CaJ		C3H/HeJ		C57BL/6J	
	OB	EX	OB	EX	OB	EX	OB	EX	OB	EX
N	41		189		59		238		89	
n_0	35	35.141	137	137.561	46	46.090	148	147.679	52	51.956
n_1	4	3.989	34	33.851	12	11.808	55	55.092	28	28.006
n_2	2	1.442	15	13.662	1	1.081	26	26.356	8	8.166
n_3	0	0.358	3	3.369	0	0.021	7	7.482	1	0.841
n_4	0	0.069	0	0.556	0	0.000	2	1.391	0	0.030
m		~1.7168		~0.8469		~0.5945		~0.4156		~0.1994
s		1.6084		1.3969		0.7660		1.3547		0.9423
D		0.0095		0.3679		0.0131		0.2716		0.0002
P		*		0.5442		*		0.8730		*
t	1.0522	1.0674	0.5974	0.6063	0.7710	0.7762	0.3103	0.3068	0.2128	0.2116
CI		±0.4609		±0.1755		±0.3486		±0.1512		±0.2425

Statistics and results from 5 strains of mice injected with hydrocortisone acetate (Merck Sharp & Dohme). The index of symbols is the same as that of Table 2.

probability density function. And fourth, a change in the HCA preparation consistently produced a change in the proportion of mice affected, and a shift in the mean of the underlying liability distribution.

Classically, in an at-risk population for a single threshold trait, the multifactorial model assumes a set of genes affecting expression of the trait that interact with a set of environmental circumstances. The pool of genetic and environmental factors is collectively called liability [13]. It is considered that in any affected individual, a critical degree of liability—a threshold—has been exceeded, resulting in full expression of the trait. In this case, the underlying frequency distribution of the combinations of genes and environment is assumed to be normal or transformable to normal by appropriate scaling of measurement [13]. It is not possible to test the assumption of normality for any single liability distribution for a discrete phenotype.

This analysis assumes that the phenotype expression is continuous. We consider that each strain receiving the UPC or MSD product (Tables 2 and 3) constitutes a separate experiment. Each experiment represents a separate sampling of an underlying "pooled" liability distribution applicable to the subgroup of which the strain is a member. Furthermore, the strains of each subgroup can be considered samples of a theoretically larger number of strains to which the pooled subgroup liability applies. Goodness-of-fit tests indicate that the underlying distributions are not significantly different from normal. Tests for homogeneity indicate that the strains within subgroups do not differ from one another with respect to distribution and threshold. The apparent non-classifiability of the DD strain in the UPC study group is most likely an accident of sample since the MSD study group clearly places DD in Subgroup II.

Our analysis also allows some considerations of the relative contributions of the three basic elements contributing to a multifactorial threshold trait: genes, environment, and chance [26]. A normally distributed liability may follow from a simple "polygenic" model, if alleles of many genes in the pool have small, additive effects. By the second generation of random mating within an at-risk population, the Hardy-Weinberg equilibrium obtains and polygenic effects are binomially distributed. Thus, a critical number of genes might alone be sufficient to produce a threshold phenomenon [13]. At the other extreme is a model in which many small environmental perturbations predominate, also generating a normal distribution of liability but with a threshold. In this case, gene effects are minimal. Finally, computer modelling of random or "chaotic" effects suggest that threshold traits with continuous liability distributions of severity can arise out of stochastic events during organogenesis, even with single-gene traits [26].

In our model, insertion or deletion of a single genetic factor

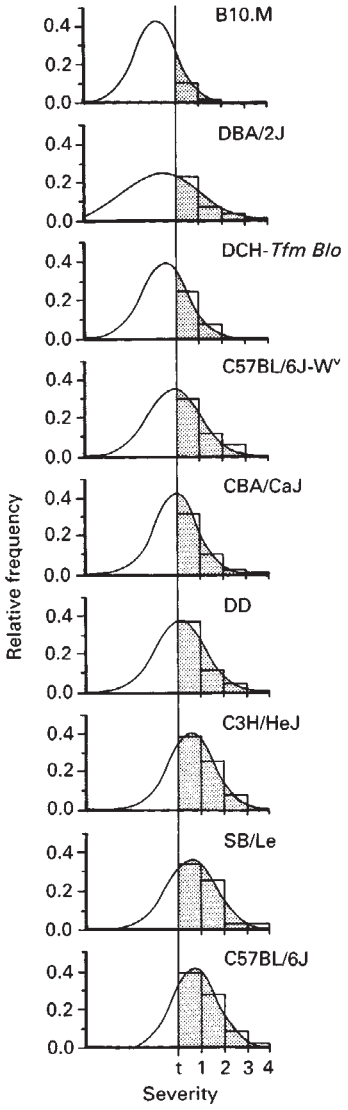

Fig. 3. *Comparison of liability distributions and observed proportions of affected mice on the UPC study group, showing a good fit to normal.* The scale points (1 to 4) on the abcissae were determined from the mean and variance of each normal distribution, taking the threshold (t) as 0. For each strain, the areas to the right of the threshold (shaded) and between successive scale points equal the expected proportions of affected mice in the respective score categories. The areas of the rectangles above each pair of successive points equal the observed proportions in the score categories. Because the base of each rectangle is 1, these proportions can be read from the ordinate heights. The curves and scale points were generated with an HP7475A plotter, Hewlett-Packard Company, interfaced with a Vax 8800 computer, Digital Corporation.

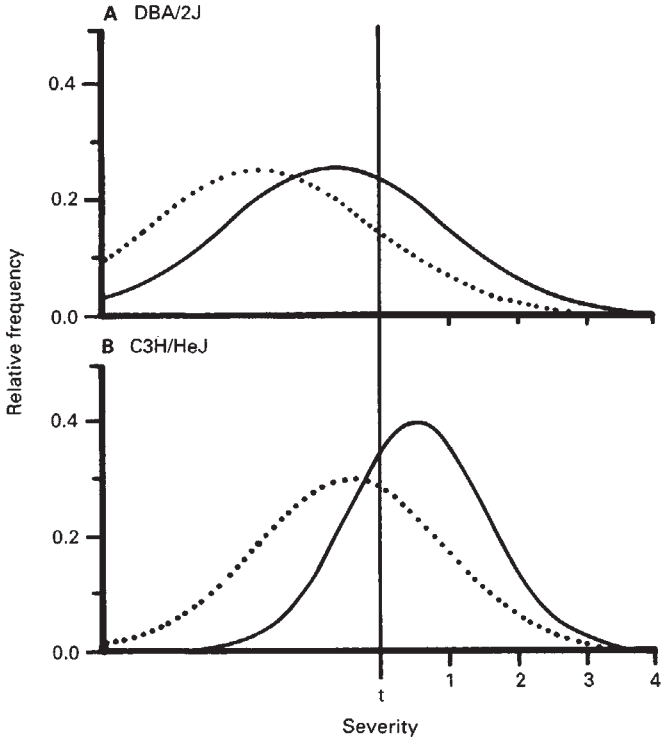

Fig. 4. *Effect of drug preparation on liability.* Symbols are: (—) Upjohn; (. . . .) Merck Sharp & Dohme. Two representative strains are shown. Note that both strains show a shift in the mean and therefore the liability when administered with the MSD preparation.

had the potential of changing the mean or variance of liability and threshold. Indeed, our results demonstrate a change in both mean and variance with the W^v mutation on the C57Bl/6J strain. Further genetic analysis, is now in progress to identify possible loci modulating liability, such as the histocompatability loci as suggested by the change in liability of the BIO.M strain.

Distribution parameters of liability were helpful in identifying an environmental effect. The relatively sudden decrease in proportions of affected animals at half-way through the experiments was initially postulated to be the result of some biologic element, such as generation, litter number or number of pregnancies. Effect of season was also considered, since Kalter [27] had observed such an influence on steroid-induced cleft lip and palate. However, time series analyses of thresholds (based on maximum likelihood estimates of the liability parameters) eliminated these variables, and showed that the decrease was directly related to a change in drug preparation. A major confounding environmental effect was thus eliminated and a more specific dissection of the remaining genetic and random elements in the expression of PKD was possible. Such considerations of changing liability and threshold may also apply to studies by Gardner, Evan and Reed [28] and Werder et al [29], who found similar changes in penetrance of PKD with shifts to and from a germ-free environment. Furthermore, our experiments demonstrate that in the presence of a well-controlled ambient environment and in the absence of any possible genetic variation, there is substantial variation in expressivity of PKD within strains.

A plausible explanation may be derived from the molecular biology of steroid action. With a uniform exposure of steroid receptors to circulating steroid molecules, the formation of activated steroid-receptor complexes will follow a Poisson distribution which, in the limiting case for large numbers of molecules, is indistinguishable from the normal distribution [30]. It follows that there may be a critical number of receptors that must be activated, likely at an inappropriate time in organogenesis in order to produce a change in cell phenotype. Assuming that kidneys will not show microscopic changes if

insufficient numbers of cells express the steroid-activated phenotype, it further follows that an increasing number of altered cells will generate increasing cystic changes that conform to a normal distribution of severity. It is in this sense that chance plays a role in morphogenesis, both in the development of and extent of the trait.

This analysis of steroid-induced PKD in mice has important implications for our understanding of PKD in man. Infantile and adult forms of the disease are considered to be Mendelian disorders arising from single gene defects. This interpretation has recently received substantial support from studies showing linkage of adult PKD to the alpha globin locus on chromosome 16 [31–32]. However, incomplete penetrance, extremely variable expressivity and discordance in twins [33–35] are clinical observations that defy simple genetic interpretation. Moreover, reports from several different centers of linkage heterogeneity for several kindreds suggest that more than one locus must be involved [36–39].

Some of these discrepancies can be resolved if PKD is considered a threshold trait in which chance is a major component of liability. In threshold traits, even a subtle change in the environment has the potential of changing the incidence of a trait and, under our hypothesis, its severity. For example, steroid exposure in utero [39] could also influence the expression of cystic change in the developing kidney. Moreover, other loci may also play a major role in modulating liability to PKD. Further study of the genetic basis underlying steroid induction of PKD in mice may eventually offer a more comprehensive explanation for the complex expression of this disease in humans.

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