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PRELIMINARY REPORT

Efficiency of Human Monozygotic Twins in Studies of Blood Lipids

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The cost of human experimentation often severely limits the size of experimental groups in studies of human metabolism. In animal studies workers have found that monozygotic (MZ) twins are efficient experimental subjects, because small, within twin-pair differences allow experiments to be done with fewer animals. This communication presents a method of using uniformity trials (all experimental subjects treated alike) to estimate the efficiency of MZ twins relative to unrelated experimental subjects. Fasting blood lipids were measured in 44 sets of MZ twins. The within twin-pair variation was used to estimate the experimental error for studies using twins, while the between twin-pair variation estimated experimental error for unrelated subjects. A total of 18 blood lipid parameters were measured, and there was evidence that 17 could be studied more efficiently with MZ twins than with unrelated subjects. For example, it was estimated that an experiment requiring 24 individuals to test the effects of two treatments on plasma cholesterol could be done with three sets of MZ twins.

EXPERIMENTS to measure the effects of environmental factors, such as diets or drugs, on human subjects are complicated by the large amount of variation between individuals. If not controlled, this variation becomes a part of experimental error and makes it difficult to separate treatment effects from variation among experimental subjects. Researchers working with mice and

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other small animals use inbred (isogeneic) strains raised in large numbers and under standard environments to control among subject variation. Monozygotic (MZ) human twins are the product of a single egg and sperm and, as such, are "mini" isogeneic lines of which both members share a common environment before birth and often long after birth. Human twin studies have been used almost exclusively to partition variation into genetic vs. environmental factors (nature vs. nurture), but, in comparison to animals, relatively little use has been made of MZ twins in studies of human metabolism.

This communication discusses estimation of the relative efficiency of experimental designs using MZ twins vs. unrelated individuals and presents estimates of the efficiency of MZ twins in the study of human blood lipids, with the purpose of stimulating interest in using MZ twins as subjects in studies of human metabolism.

MATERIALS AND METHODS

Table 1 shows the analysis of variance models for testing the effects of two treatments using MZ twins vs. unrelated experimental subjects. In statistical terminology, the design for unrelated individuals is a completely random design and the twin design is a randomized complete-block design[1] (7.2, 8.3). (Statistical concepts presented in this paper are keyed to a standard statistical text[1] with the appropriate sections in parentheses.) These models were also constructed to allow estimation of laboratory error that may be controlled by replicate analyses and is not necessarily influenced by the choice of an experimental design.

The twin design excludes among twin-pair variance (estimated by s_{AP}^2) from the experimental error and increases the relative efficiency of this design when s_{AP}^2 is larger than within twin-pair variance (estimated by s_{WP}^2). However, when the number of twin-pairs equals the number of unrelated individuals in each treatment group, the twin design has only one-half the error degrees of freedom that conversely tends to make the twin design less efficient.

Table 1. Analysis of Variance Models for Testing Two Treatments Using Two Groups of Unrelated Subjects vs. Paired Comparisons of Monozygotic Twins

Source of Variation	df	Mean Square Variance Estimates
A. Unrelated model		
Treatments	1	$s_{LE}^2 + rs_{AI}^2 + rns_{AT}^2$
Among individuals within treatments = experimental error	2(n—1)	$s_{LE}^2 + rs_{AI}^2$
Among replicates within individuals = laboratory error	2n(r—1)	s_{LE}^2
B. Paired comparisons of MZ twins		
Among twin pairs	n—1	$s_{LE}^2 + rs_{WP}^2 + 2r_{AP}^2$
Between treatments	1	$s_{LE}^2 + rs_{WP}^2 + rns_{AT}^2$
Within twin pairs within treatments = experimental error	n—1	$s_{LE}^2 + rs_{WP}^2$
Among replicates within subjects = laboratory error	2n(r—1)	s_{LE}^2

n, number of subjects per treatment group; r, number of replicate laboratory determinations done on each sample; df, degrees of freedom; s^2, variance estimate; LE, laboratory error; AI, among unrelated individuals; AT, among treatments; WP, within twin pairs; and AP, among twin pairs.

To use the completely random design, (Table 1A) unrelated individuals are assigned at random to two groups, and the treatment effects on these two groups are tested by the F ratio, i.e., between treatments mean square over within treatments mean square[1] (7.14). In contrast, the twin or randomized complete-block design (Table 1B) requires randomly assigning one of each twin pair to one of the treatment groups, assigning the co-twin to the other group, and then testing the treatment effects with the F ratio, i.e., treatment mean square over within twin-pair mean square[1] (8.7). In both designs the numerator of this F ratio theoretically contains rn times the treatment variance. Therefore, if treatment effects and laboratory error are equal in both designs, the relative efficiency of the two designs is a function of the relative sizes of s_{AI}^2 and s_{WP}^2. If the within twin-pair variance (s_{WP}^2) is enough smaller than the among unrelated individuals variance (s_{AI}^2) to more than compensate for the loss of efficiency due to loss of error degrees of freedom, then monozygotic twins are a more efficient means of testing treatment effects.

Fisher[2] (6.8) defined the efficiency of an experimental design in terms of how much information the observed differences between treatment means may be expected to give about the true differences between treatment means. Fisher quantitated this efficiency (E) as:

$$E = (df + 1) / (df + 3)s_{EE}^2$$

where df = degrees of freedom for the experimental error and s_{EE}^2 = an estimate of the experimental error variance.

The relative efficiency (RE) of two designs may, therefore, be expressed using the error df and estimated experimental errors for designs 1 and 2, respectively.

$$RE = \frac{(df_1 + 1) / (df_1 + 3)s_{EE1}^2}{(df_2 + 1) / (df_2 + 3)s_{EE2}^2}$$

For ease of calculation this equation may be simplified algebraically to:

$$RE = \frac{(df_1 + 1)(df_2 + 3)s_{EE2}^2}{(df_2 + 1)(df_1 + 3)s_{EE1}^2}$$

If this ratio (RE) is larger than one, then design 1 (the twin design) is more efficient than design 2. For example, the relative efficiency of a twin design (design 1) using six sets of MZ twins (5 error df) and a random design (design 2) using two groups of six unrelated individuals (10 error df) would be:

$$RE = \frac{(6)(13)s_{EE2}^2}{(11)(8)s_{EE1}^2} = \frac{0.89s_{EE2}^2}{s_{EE1}^2}$$

Translated, this means that if the twin-design experimental error is more than 89% of the random-design error, then twins are less efficient because of the loss of error degrees of freedom. If, however, the twin-design experimental error (s_{EE1}^2) is 89% of the unrelated design experimental error, their efficiency is theoretically equal; if the twin-design experimental error is less than 89% of the random-design experimental error, then the twin design is judged to be more efficient.

To compare fully the efficiency of two designs using Fisher's method, the researcher needs estimates of the experimental error variances for both designs. In practice, complete pilot studies of both designs are seldom feasible. However, experimental error can also be measured by uniformity trials,[3] where all experimental subjects are treated alike, and the time and expenses of observing treatment effects are eliminated. When conducted with a panel of MZ twins, uniformity trials permit the simultaneous estimation of the error associated with both experimental designs. The within twin-pair variance is an estimate of the twin-design experimental error, while the variance among the unrelated twin-pairs provides an estimate of the variance among unrelated individuals in the population, which in turn estimates the experimental error for an experiment using groups of unrelated individuals (Table 1).

To relieve the investigator of time-consuming calculations, an "N" nomogram[4] (Fig. 1)

was developed to determine the number of MZ twin pairs needed to replace, with equal precision, a certain number of unrelated individuals. To use this nomogram, determine the number of unrelated individuals needed for an experiment based on an estimate of the treatment-effect magnitude, experimental error, and a desired confidence level[1] (5.11, 5.12). Then using the s_{WP}^2/s_{AP}^2 ratio for the variable to be studied, the relative efficiency of MZ twins and unrelated individuals may be calculated.

Blood samples were taken after a 12–15-hr fast, and the lipids and lipoproteins were analyzed in duplicate using MZ human, twin pairs living together and aged 5–20 yr. Phospholipids were quantitated following thin-layer chromatography by methods reported previously.[5] Free cholesterol and cholesteryl esters were separated by thin-layer chromatography on silica gel G (Brinkmann Instruments, Westbury, N.Y.), and a solvent was made of equal parts chloroform and cyclohexane. The cholesterol and cholesteryl esters were then quantitated by the method of Abell et al.[6] Quantitative plasma lipoprotein electrophoresis was done using the method and equipment of Gelman Instrument Co. (Ann Arbor, Mich.) modified as previously reported[7] and quantitated by scanning on a densitometer at 560 μ. Triglycerides were measured by the method of VanHandel and Zilversmit.[8]

The β-lipoprotein was quantitated by immunodiffusion on commercially available agar plates (Partigen plates, CBDS, Woodbury, N. Y.); α-lipoprotein was quantitated by immunodiffusion following separation from β-lipoprotein on agar gel electrophoresis.[7] All of the lipid and lipoprotein fractions measured were compared to a standard plasma collected over 1 mg/ml EDTA, sealed in ampules, frozen, and stored in liquid nitrogen.[7]

Twin zygosity was determined by multiple serum and erythrocyte factors[9] and by the probability method described by Gaines and Elston.[10] The systems employed included: blood types A_1, A_2, B, O; M, N, S; C, D, E, c, and e of the Rh series; Fy^a, Fy^b, K, k; P, p; JK^a, JK^b; haptoglobin, and phosphoglyceromutase markers by starch gel electrophoresis. Using these markers in like-sexed twins, the probability of misclassification is less than 1%.

RESULTS AND DISCUSSION

Table 2 shows the analysis of variance obtained for plasma cholesterol. From this analysis the estimates of within twin-pair variance (s_{WP}^2) and among twin-pair variance (s_{AP}^2) were calculated by the formulae (derived from the mean square variance estimates in Table 2):

$$s_{WP}^2 = \frac{\text{within twin-pairs mean square} - \text{laboratory error mean square}}{2}$$

$$s_{AP}^2 = \frac{\text{among twin-pairs mean-square} - \text{within twin-pairs mean square}}{4}$$

Table 3 is a tabulation of the within (s_{WP}^2) and among (s_{AP}^2) twin-pair variance estimates, laboratory error variance estimates (s_{LE}^2) and s_{WP}^2/s_{AP}^2 ratios

Table 2. Plasma Cholesterol Analysis of Variance in a Uniformity Trial Using 41 Pairs of Human Monozygotic Twins

Source of Variation	df	Mean Square*	Mean Square Variance Estimates
Among twin pairs	40	0.871	$s_{LE}^2 + 2s_{WP}^2 + 4s_{AP}^2$
Within twin pairs	41	0.074	$s_{LE}^2 + 2s_{WP}^2$
Between duplicate analyses	82	0.007	s_{LE}^2

For abbreviations see Table 1.

* Mean squares expressed as $(\mu moles/ml)^2$

obtained for plasma cholesterol, as well as the other blood lipids and lipoproteins studied. The calculated ratios s_{WP}^2/s_{AP}^2 ranged from 1.01 down to 0.05. For α-lipoprotein quantitated by electrophoresis, whose s_{WP}^2/s_{AP}^2 ratio was 1.01, there was no evidence that twins would be more efficient experimental subjects than unrelated individuals. However, for the lipid components whose s_{WP}^2/s_{AP}^2 ratios ranged from 0.69 down to 0.05, twins should be considered as a way of decreasing the number of experimental subjects and, therefore, hopefully decreasing experimental costs. For example, using plasma cholesterol ($s_{WP}^2/s_{AP}^2 = 0.17$) and testing two treatments estimated to require 12 unrelated individuals per treatment group, the same information about true treatment effects could be obtained by using only three sets of MZ twins (Fig. 1).

To make the calculated values of s_{WP}^2 and s_{AP}^2 more meaningful, a measure of their variability was needed. Therefore, 95% confidence limits of s_{AP}^2 and s_{WP}^2 were estimated by the method of Scheffe as reported by Sokal and Rohlf (Table 4).[11] The 95% confidence limits mean that with 95% certainty true population variances fall within these limits of the variance

Table 3. Estimates of the Among and Within Monozygotic Twin Sets and Laboratory Error Variances* for Human Fasting Blood Lipids and Lipoproteins

	No. of Twin Pairs	s_{AP}^2	s_{WP}^2	s_{LE}^2	s_{WP}^2/s_{AP}^2
Plasma					
Cholesterol	41	0.199	0.034	0.007	0.17
Cholesteryl esters	41	0.642	0.058	0.035	0.09
Triglycerides	22	0.262	0.175	0.175	0.67
Phospholipids					
Sphingomyelin	41	0.0032	0.0007	0.0002	0.22
Lysolecithin	41	0.0152	0.0026	0.0011	0.17
Lecithin	41	0.1058	0.0123	0.0051	0.12
Phosphatidylinositol	41	0.0019	0.0001	0.0004	0.05
Phosphatidylethanolamine	41	0.0017	0.0001	0.0005	0.06
Lipoproteins (quantitative electrophoresis)					
α	26	23.32	23.56	2.51	1.01
Pre-β	26	136.37	54.19	1.88	0.39
β	26	82.37	31.56	8.09	0.38
Immunodiffusion					
α	26	43.2	6.2	3.0	0.14
β	25	79.6	11.6	44.1	0.15
Erythrocyte					
Cholesterol	44	0.483	0.069	0.015	0.14
Phospholipids					
Sphingomyelin	44	0.0055	0.0021	0.0012	0.38
Lecithin	44	0.0052	0.0020	0.0021	0.38
Phosphatidylserine	44	0.0037	0.0000	0.0034	—
Phosphatidylethanolamine	44	0.0077	0.0053	0.0022	0.69

*Variance estimates for lipid components are expressed as (μmoles/ml)², electrophoretic lipoprotein determinations as (per cent of total lipoprotein)² and the immunoquantitation of lipoproteins as (mm of diffusion).²

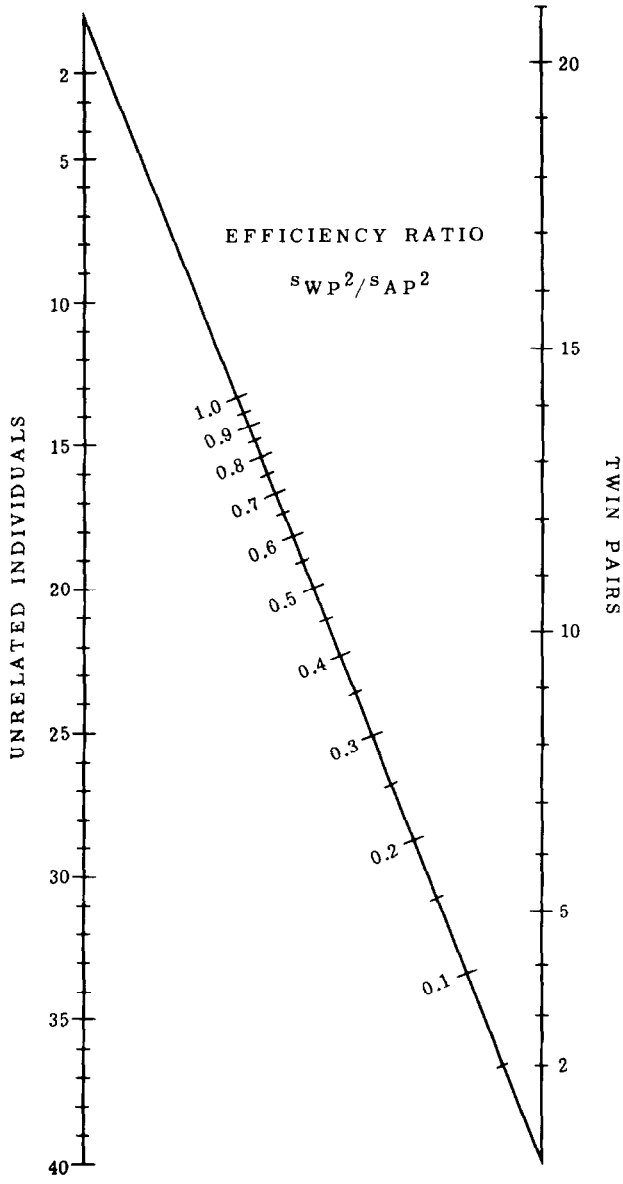

Fig. 1. "N" nomogram for calculation of number of MZ twin-pairs needed to replace an estimated number of unrelated individuals in each of two groups so that two treatments can be tested with equal precision. Instructions for use: (1) Estimate number of unrelated individuals needed in each of two groups to evaluate adequately two treatments and mark this point on left vertical line of the N (point 1). (2) Calculate s_{WP}^2/s_{AP}^2 ratio and mark it on diagonal line of the N (point 2). (3) Connect points 1 and 2 with a straight line and extend this line through right vertical line of N. Where this line intersects right vertical line of N is the equivalent number of twin pairs.

component estimate. Four of the confidence limits for s_{WP}^2 include zero (plasma phosphatidylinositol, phosphatidylethanolamine; immunodiffusion quantitation of β-lipoprotein and erythrocyte phosphatidylserine. This occurred because the within twin-pairs mean square was not significantly larger than the laboratory error mean squares for any of these components. In fact, the s_{WP}^2 calculated for erythrocyte phosphatidylserine was a negative number but is expressed here as zero because it is impossible for a variance to be less than zero. For those components with small s_{WP}^2 values that have relatively large confidence limits, it must be realized that the s_{WP}^2/s_{AP}^2 ratio may be

quite variable because the laboratory methodology is not refined enough to obtain an accurate estimate of s_{WP}^2.

Usually experiments will require testing more than two treatments. Dick and Whittle[12] discuss the efficiency of monozygotic twins in the "Incomplete Randomized-Block Design" that allows testing more than two treatments. These authors further computed the relative efficiency of MZ twins compared to unrelated individuals using more than two treatments as:

$$RE > \text{two treatments} = t\ RE/2\ (t—1)$$

where t = number of treatments and RE = the relative efficiency of twins comparing two-treatment designs.

Using plasma cholesterol again as an example, for two treatments three sets of twins were estimated to be as efficient as 12 unrelated individuals per treatment group, or a relative efficiency of 4.0 (12/3). The relative efficiency of MZ twins for studying the effects of four treatments on plasma cholesterol would be: $4 \times 3/2(4—1) = 2/1$. Therefore, six sets of twins (12 individuals) would be required to replace four groups of six unrelated individuals (24 individuals). Another factor to be considered in the incomplete block design is the minimum number of twin pairs per replication or "minimum replication unit." This number may be expressed as: minimum replication unit = $t(t—1)/2$, where t = number of treatments. Therefore, using four treatments,

Table 4. Estimated 95% Confidence Limits for s_{WP}^2 and s_{AP}^2 for Blood Lipids Studied

	Lower Limit	s_{AP}^2	Upper Limit	Lower Limit	s_{WP}^2	Upper Limit
Plasma						
Cholesterol	0.128	0.199	0.340	0.022	0.034	0.058
Cholesteryl esters	0.420	0.642	1.079	0.033	0.058	0.108
Triglycerides	0.093	0.262	0.653	0.099	0.175	0.375
Phospholipids						
Lysolecithin	0.0020	0.0032	0.0056	0.0004	0.0007	0.0013
Sphingomyelin	0.0087	0.0152	0.0237	0.0016	0.0026	0.0046
Lectithin	0.0688	0.1058	0.1788	0.0074	0.0123	0.0219
Phosphatidylinositol	0.0012	0.0019	0.0032	0.0000	0.0001	0.0003
Phosphatidylethanolamine	0.0011	0.0017	0.0029	0.0000	0.0001	0.0002
Lipoproteins (quantitative electrophoresis)						
α	6.11	23.32	56.37	14.11	23.56	45.61
Pre-β	68.7	136.37	287.12	32.87	54.19	103.91
β	41.4	82.37	173.49	18.63	31.56	61.72
Immunodiffusion						
α	24.8	43.2	85.7	3.6	6.2	12.3
β	36.8	79.6	185.6	0.0	11.6	48.9
Erythrocyte						
Cholesterol	0.313	0.483	0.820	0.044	0.069	0.118
Phospholipids						
Sphingomyelin	0.0032	0.0055	0.0101	0.0012	0.0021	0.0039
Lecithin	0.0029	0.0052	0.0097	0.0010	0.0020	0.0041
Phosphatidylserine	0.0022	0.0037	0.0065	0.0000	0.0000	0.0005
Phosphatidylethanolamine	0.0037	0.0077	0.0149	0.0031	0.0053	0.0094

a replication unit is six pairs of twins. This minimum replication unit is necessary, because to complete the analysis each treatment must occur once with every other treatment within a twin pair. If, for any reason, data are not collected on any member of this minimum replication unit, the whole unit must be eliminated from the analysis. Theoretically, the efficiency of MZ quadruplets in testing four treatments could be calculated the same way as for two treatments using twins, but the rarity of human quadruplets precludes their use.

One factor not considered in the nomogram or calculations is the expense of acquiring MZ twins as experimental subjects. Each investigator must evaluate this variable personally; however, MZ twins are not extremely rare and occur approximately once in each 300 births.[13] An example of a large, active twin panel is a roster of all twins born between 1917 and 1927 who have served in the U.S. Armed Forces.[14] Members of this panel live throughout the United States and at the present time are being studied by the National Heart and Lung Institute to determine hereditary influences on serum cholesterol, triglycerides, and specific lipoprotein concentrations.[15] Help in establishing twin panels may be obtained from centers where twin studies are in progress. The National Foundation-March of Dimes in September 1971 reviewed world-wide genetic services and published a "Directory of Genetic Units" that contains the address and director of 55 U.S. centers plus 101 world-wide centers doing twin studies.[16] In addition, there are national and local Mothers of Twins Clubs who are dedicated to participating in medical research. The National Organization of Mothers of Twins Clubs, Inc.[17] is made up of 8554 women in 44 states, and its major purpose is to cooperate with and actively participate in research.[18] Any investigator serious about using twins as experimental subjects should, therefore, be able to gather a twin panel. Twins, once ascertained, may be used repetitively and could become "semiprofessional" experimental subjects. They could learn how to record intake and output, as well as measuring other body functions. Perhaps the extreme example of efficiency would be a metabolic unit staffed entirely with twins.

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