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I. INTRODUCTION

During the past two years I have been engaged in research in an attempt to develop a laboratory test by which blood of homozygous Rh+ individuals (DD) can be regularly distinguished from that of heterozygotes (Dd). Hitherto, no such means have been known. Certain approaches by other workers have either since come to be doubted or reveal only statistical differences between groups of subjects and cannot be reliably applied to individuals.

II. THE PROBLEM

The reasons for the difficulty in developing such a test arise from the nature of the genes D (tending to cause Rh-positiveness in the individual) and d (tending to cause Rh-negativeness). To say that the genotypes DD and Dd are phenotypically indistinguishable is to say that D behaves as a gene completely dominant to gene d. Geneticists generally hold, however, that when two different genotypes appear phenotypically identical, it is merely because we have not sufficiently sensitive means of recognizing such qualitative or quantitative differences as do exist. The structural difference between two alleles at a given locus should logically

lead either to qualitatively different synthetic products or to failure to initiate synthesis of a particular product at one allele but not at the other.

The conventional genes in the Rh system are: C and c, D and d, and E and e. With one exception, each of these genes causes its carrier's erythrocytes to contain the pertinent antigen, as demonstrable by agglutination and/or lysis of erythrocytes when reacted with the appropriate antiserum. The one exception is d. No anti-d serum is known to be produced naturally or upon direct stimulation. One cannot induce production of an anti-d serum by challenging an Rh+ (DD or Dd) individual with the RBCs of an Rh- (dd) individual. There have appeared occasional reports[9, 10, 11] of apparent recovery of an anti-d serum, but these results were not reproducible and are largely discounted now[17, 18]. Thus it is quite possible that the d allele does not give rise to production of any antigen, or that production of its associated antigen by all erythrocyte-bearing animals precludes antigenic response on direct stimulation.

III. PREVIOUS APPROACH TO THE PROBLEM

The most promising approach hitherto developed was that of Masouredis[12, 13, 14]. He reasoned that under the "One gene : One enzyme" hypothesis, erythrocytes of

DD genotype might contain more Rh+ antigen than Dd erythrocytes and that this difference might be reflected in greater binding of antibodies by DD than by Dd erythrocytes.

His technique was to label with I^{131} the gamma-globulins derived from a repeatedly-sensitized Rh- mother. The gamma-globulins were reacted with measured amounts of pooled lyophilized Rh+ erythrocyte stroma. The sensitized stroma was washed free of excess globulin. Then the bound I^{131}-globulin was eluted from the RBCs and its activity counted. A curve was constructed showing the activities measured for 199 different individuals' erythrocytes, and a bimodal distribution was noted. One group consisted mainly of the individuals considered on the basis of either family Rh patterns or the individuals' own C-c and E-e genotypes to be Dd; similarly the other group seemed largely composed of DD individuals.

The mean I^{131}-globulin uptake by presumed DD individuals was 1.6 times that for Dd persons. There was, however, considerable overlap for the two populations, so that although the contrast between the means was found to be statistically significant, the procedure could not be relied on to provide an unequivocal test result for an individual of unknown "D" genotype.

IV. THE PEPPER RATIONALE

My approach to the problem was inspired by the findings of Dr. K. C. Atwood and myself in our work on the ABO system at the University of Chicago. The relevant historical data are presented here.

Ashby[1] (1919) showed that when type "A" or type "B" erythrocyte suspensions were reacted with corresponding antisera, 0.03% to 3.5% of the cells remained free. McKerns and Denstedt[15] (1950) reasoned that if Ashby's free cells were inagglutinable, then their number should increase upon successive agglutinations. Experimentally, however, they found no such increase, indicating that the non-agglutinating cells in a given stage are probably not inagglutinable. Meanwhile, Gray and Sterling[8] (1950) developed the technique of using radioactive chromate to tag RBCs for blood volume studies.

Atwood[2, 5, 6] (1958, 1959) tagged RBCs with Cr^{51} (radioactive) and reacted them with appropriate anti-A or anti-B serum. He separated the free cells from the agglutinate, and in the second stage he added to the free cells from Stage 1 fresh suspension of untagged cells of the same type (carrier cells) and again added appropriate antiserum. Successive stages were carried o out precisely as the second. Counting free cell activi-

ties and correcting for volume changes revealed that in successive stages, progressively fewer of the original cells were removed, so that after about the fifth stage, a constant number of free cells remained after each agglutination. This constant number was considered the truly inagglutinable fraction. This fraction, it was postulated, might be the result of somatic mutation of gene I^A or I^B to I^O in erythropoietic cells.

Should somatic mutation be the correct explanation, then three factors might be expected to increase the free-cell frequency: age, exposure to mutagenic agents, and zygosity. No age effect has been detected in comparing individuals of different ages, but a better study would follow the individual through several years of his lifetime.

Mutagenic agents were found to increase free-cell frequencies markedly. Scheinberg and Reckel[20] (1960) reported free-cell frequencies increased 1.5 to 4 times base values in pigeons subjected to total-body X-radiation and in human polycythemics exposed to P^{32}. Atwood and Megill[3] (1959) found that in polycythemic patients of blood type "A", there was a 10-fold increase in "A_2" cell frequencies and a 2-fold increase in "O"

cell frequencies within two weeks of treatment with 4 mc. of P^{32}.

My contribution was in connection with the zygosity factor. Studies thitherto had yielded inagglutinable cell frequency curves at two general levels, one substantially higher than the other. In order to determine whether zygosity could explain this difference, I obtained donors of known genotype. Since no laboratory means existed for distinguishing between BB and BO individuals of type "B", or between AA and AO individuals of type "A", I located families in which both parents were type "AB". Type "A" offspring thus had to be of AA genotype; "B" offspring were of genotype BB.

Our studies showed a significant difference between BB and A_1B subjects in frequencies of cells non-reactive to anti-B sera. Consistent, but more complicated findings were obtained for "A" reactivity. This was consistent with the expectation that if somatic mutation was producing the free cells, and if such mutation was a wholly random event, then phenotypic change from type "B" to type "O" would be less commnn from BB cells (requiring two independent mutations from gene I^B to gene I^O) than from BO cells (requiring only one such mutation). For reasons not yet sufficiently clear, we

found the fraction of "O" cells in BB blood to be higher than the (expected) square of the "O" cell fraction in BO blood, but nevertheless significantly lower than the unsquared fraction. Thus the differences found were in the predicted direction, though the homozygous free cell frequencies were only 1/5 to 1/20 those of the heterozygotes, some 50 to 200 times higher than the squares of the heterozygote frequencies as postulated.

The above approach seemed to me to be quite appropriate to the Rh zygosity problem. It was hoped that studies would reveal a higher frequency of non-"D" cells in heterozygous than in homozygous Rh+ persons.

A priori, one difference between the ABO and the Rh situations would make a difference. As a complete recessive, d's presence cannot be determined by any presently-known means, and therefore it is impossible to prove that a person is of genotype DD. In occasional instances, however, it is possible to make a presumption of zygosity on genetic grounds, on a high order of probability.

This has been accomplished by locating a family in which the father is Rh- (dd) and the mother and all nine children are Rh+. The children are thus all Dd, and

the mother's chance of being DD is 2^9 : 1.

V. TECHNIQUE AND RESULTS

The development of a technique by which my approach could be applied to the Rh zygosity problem has required long searches and is studded with many false starts. We now have one or two methods which sometimes work but which are far from completely satisfactory.

The main problem has been that of separating agglutinated from unagglutinated cells in each stage.

The initial attempt involved the use of millipore filters on the assumption that the 10-micron pores would retain aggregates of two or more cells and allow single, unagglutinated cells to pass through. However, the filter pores became clogged almost immediately, and there was no flow at all.

The next approach was to apply suction to the bottom of the millipore filter apparatus to force the cells through the pores. The result was total breaking-up of the cell aggregates so that all passed through the filter.

The remaining attempts all have involved passive sedimentation. At first, cell suspensions were treated with albumin-reacting anti-D serum. The albumin-react-

ing antibodies are considered to be the blocking or incomplete type of antibody, according to immunological "dogma" and require a protein substance for aggregation. The agglutinate formed in this reaction system proved quite weak and too unstable for separation.

Since then the saline-reacting anti-D serum has been used. It yields a more firm agglutinate. Eventually the following procedure was arrived at.

Five drops of a 2% suspension of RBCs from an Rh+ individual were tagged to maximum capacity with Abbot Rachromate. The cells were then washed repeatedly until subsequent washings showed negligible radioactivity. The 2% suspension was reconstituted byaaddition of 5 drops of normal saline. To this was added 5 drops of "saline anti-D" serum. The reaction mixture was incubated at 37° C. for 30 minutes, then centrifuged at 1000 r.p.m. The tube was tapped gently, causing the pellet to break up into smaller cell aggregates. The clumps of cells quickly settled to the bottom of the tube, and after 5 minutes the supernatant containing the suspended free cells was drawn off by means of a capillary pipette. The agglutinate was counted in a Picker well counter. The supernatant was counted, centrifuged,

and separated, and both the activities of the unagglutinated cells and the saline were determined. To the unagglutinated Cr^{51}-labeled cells from stage #1 (UAg-1) were added 5 drops of a 2% suspension of unlabeled cells from the same donor, plus 5 drops of "saline anti-D" serum to form the second reaction mixture (RM-2). Third and subsequent stages were executed as the second. The process was discontinued when two successive stages yielded unagglutinated cells of the same, or nearly the same, activity. An example of results obtained by this method is presented in Table I. The donor was unselected for genotype and, indeed, no presumption of genotype was attempted.

Table I.

Stage	RM	UAg	% of RM-1
1	12,071	10,610	
2	10,428	3,264	
3	3,170	319	
4	328	46	
5	37	47	0.3893%

Table I: Pilot test using genotypically unselected Rh+ erythrocytes. RM = reaction mixture; UAg = unagglutinated cells.

In this example, the final inagglutinable cell fraction was 47/12071, or about 4×10^{-3} of the original reaction mixture. This figure was in the same order of magnitude as frequencies obtained in the ABO studies.

The next step was to test the validity of the test by adding measured amounts of Cr^{51}-labeled, type "O", Rh- cells to suspensions of unlabeled type "O", Rh+ cells and proceed as above. The result was satisfactory; i.e., there was little loss of reactivity of the unagglutinated cell fraction from stage to stage.

Finally, the test was used on bloods of individuals of known or presumed Rh genotype. The subjects were from the family described above: the mother, presumably DD, and one son, known to be Dd (assuming true paternity of the husband, which is not doubted here).

The initial attempts to employ the test as described above were met with considerable difficulty, in that the degree to which cell pellets would break up varied over a wide range, from very little to almost completely. Nevertheless, by persistence it was possible to execute the test. The summary of results is presented in Table II.

Table II.

	Donor Type	
	Dd	DD
RM-1:	76,323	92,412
UAg-6:	31,644	3,492

	Dd	DD
% of RM-1	41.5	3.7

Table II: Comparison of known Dd and presumed DD bloods as to unagglutinated cell activities after several stages of agglutination.

These results were somewhat disappointing, because even though the difference in the free cell frequencies was in the predicted direction, it was felt that the technique was not yielding precise results. I strongly doubted that the true inagglutinable cell fraction was so very high, and I knew that such frequencies could not reflect true somatic mutation rates.

Next there was an attempt to use the method of Dacie and Mollison[7, 16] to agglutinate and separate the cells. This differed from the above by calling for a two-hour incubation period (rather than one-half hour) and three centrifugations per stage (rather than one), each centrifugation being followed by gentle tapping, to suspend the small clumps of cells before they are allowed to sediment. The results were quite disappointing, in that agitation sufficient to disturb the pellet at all caused total breaking-up of the agglutinate with all cells going into suspension, thus precluding separation.

The most recent and most successful variation thus

far employed has not required centrifugation of the reaction mixtures at all. Very fresh cells and very new, very potent "saline anti-D" serum are used. The reaction mixture is incubated at 37° C. for 15 minutes, during which time the formed cell aggregates settle out. The supernatant is then drawn off and we proceed as described above. This technique yielded the results given in Table III when last employed.

Table III.

DD donor	RM-1	UAg-1	UAg-2	UAg-3	UAg-4	UAg-5	UAg-6
1	114645	97282	4887	2198	1684	1308	1119
2	124452	91689	3719	1481	966	878	808
3	97318	92034	703	448	261	155	192
4	101807	86728	363	151	113	68	105
Dd donor							
5	106189	74170	52438	27273	20818	16426	14768
6	75343	70193	32335	21088	17337	13263	13073
7	129619	110464	22788	14632	13101	9027	8699
8	118001	92363	24913	18575	15943	13145	12780

	UAg-6/RM-1
DD donor	
1	9.78×10^{-3}
2	6.49
3	1.97
4	1.03
Dd donor	
5	139×10^{-3}
6	173
7	67
8	108

Table III: Results of simultaneous testing of four samples each of known Dd blood and presumed DD blood.

VI. DISCUSSION

A. The Laboratory Methods. I have made it clear in the above discussion that I feel much remains to be accomplished in the development of a reliable laboratory technique by which my approach to the basic problem of genotype determination may be utilized. The results are, indeed in the direction predicted by the assumption that phenotypic expression of somatic mutation would be more common in heterozygotes than in homozygotes. However, I feel that the end-results are still quite variable; i.e., that the free-cell frequencies obtained for a given individual will not always be the same in two successive tests. The wide variation between stages of the same test promotes this feeling. If individual results vary too widely, it will be impossible to genotype accurately a person whose genetic endowment is unknown.

The largest part of the difficulty stems from the weakness of the Rh agglutinating system. This is striking to one who has worked with the ABO system, in which agglutination is almost instantaneous and dramatic, and in which the agglutinate, once formed, remains formed.

Hitherto, it has not been necessary for clinical

clinical laboratories to develop accurate means of producing a firm agglutinate in Rh testing, because such laboratories are interested only from the qualitative standpoint. Therefore, it is by no means certain that the optimal conditions for promoting Rh agglutination are known. Further work will definitely be expended in this direction so that these tests may proceed more confidently.

One approach to the problem has not been tried at all yet; that is the use of a lytic system rather than an agglutinating system. Little is known of the possibilities of such an approach.

Still another approach not yet attempted would employ Coombs serum to further bind the agglutinated erythrocytes. The Coombs system is considered to be rather weak, however, and may not contribute substantially to our progress.

B. Importance of the Problem. Nevertheless, despite the technical difficulties, I have persisted in this project for two reasons: (1). The theoretical considerations upon which this project is based are genetically sound; and (2). Such excellent results were obtained in our work on the ABO system.

A great amount of information may be gleaned from this project if it is carried further and more reliable results are obtained. The most obvious practical application would be in the area of genetic counseling, the typical family in need of advice being an Rh-incompatible one in which all offspring thus far born are Rh+ and the couple wants to know the liklihood of producing an Rh- child that would be unaffectable by erythroblastosis fetalis.

Information as to mutation rates is another potential value from this study. I have indicated above that I do not consider the present results to be applicable to that area yet, inasmuch as the free-cell frequencies are several times higher than the true somatic mutation rates must be.

Still another large area on which these studies might shed some light is the nature of the D and d genes themselves. If it turns out that an Rh+ individual can produce Rh- cells but an Rh- person cannot produce Rh+ cells at all, it would suggest that d represents a deletion of a locus or at best a null at the D locus.

C. A note on laboratory "genotyping". It is

common practice to request from a clinical laboratory information as to the genotype of an individual, with reference to his Rh system. The requisition is met by a report that the patient's C, D, and E genetic configuration are thus-and-such, with the genes even arranged in linkages on chromosomes. Apparently both the clinician and the laboratory technician are under the impression that the actual D or d genotype is thus obtained. I wish to make it clear that this is not the case. The conclusion presented to the clinician is based upon Race and Sanger's[19] surveys of gene frequencies in England. The survey did not indicate the means by which D genotype may be determined on the basis of the subject's C and E genotypes. Thus as far as the D portion of the genotype is concerned, the result obtained is merely an estimate, and quite likely to be mistaken. For example, one couple screened for this study was Rh-incompatible. There had been seven pregnancies, with five deaths from jaundice and prematurity. Only the first and third offspring had survived, the latter with the aid of transfusions at birth. Both the living children were Rh+. The probability is very great indeed that the father in this case is DD and is incapable of producing an Rh- child, yet the couple had been advised, on the basis of "genotyping" that the father was a heterozygote. The implications for this family are obvious.

VII. SUMMARY AND CONCLUSIONS

This paper is a discussion of the problem of determining the genotype of Rh+ individuals and of my attempts to solve this problem in the laboratory. This is in the nature of a progress report on a project not yet completed, but through which we have already obtained some worthwhile information.

The approach described herein is based on the assumption that somatic mutation will lead to the production of a population of inagglutinable, Rh- cells, within the larger population of Rh+ cells in the blood of an Rh+ individual. It is postulated that if somatic mutation is a wholly random event, then homozygous cells will reveal mutation phenotypically much less commonly than heterozygous cells will. The results obtained thus far are consistent with this prediction.

Potentially fruitful areas of further investigation are duscussed.

VIII. ACKNOWLEDGMENTS

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BIBLIOGRAPHY

1. Ashby, Winifred, The Determination of the Length of Life of Transfused Blood Corpuscles in Man, J. Exptl. Med. 29:267-81, 1919.

2. Atwood, K. C., Examination of Human Erythrocyte Populations for Somatic Mutation at the ABO Locus, Proc. Natl. Acad. Sci. U. S. 44:1054, 1958.

3. _____, and Megill, Donald, Erythrocyte Automosaicism in Polycythemics Treated with Phosphorus-32, Science 130:1411-2, 1959.

4. _____ and Pepper, F. J., Erythrocyte Automosaicism in Some Persons of Known Genotype, Science 134:2100-2, 1961.

5. _____ and Scheinberg, S. L., Isotope Dilution Method for Assay of Inagglutinable Erythrocytes, Science 129:963-4, 1959.

6. _____ and _____, Somatic Variation in Human Erythrocyte Antigens, J. Cellular Comp. Physiol. 52, suppl. 1:97-123, 1958.

7. Dacie, J. V. and Mollison, P. L., Survival of Normal Erythrocytes from a Donor with Nocturnal Haemoglobinuria, Lancet i:390, 1949.

8. Gray, S. J. and Sterling, K., Determination of Circulating Red Cell Volume by Radioactive Chromium, Science 112:179-80, 1950.

9. Haberman, Sol, Hill, J. M., et al, The Demonstration and Characterization of the Anti-d Agglutinin and Antigen Predicted by Fisher and Race, Blood 3:682-8, 1948.

10. Hill, J. M. and Haberman, Sol, Two Examples of Sera Containing the Anti-d Agglutinin Predicted by Fisher and Race.

11. _____, _____, et al, Additional Evidence for the Anti-d: Statistical Studies, Blood,5:1049-58, 1950.

12. Masouredis, S. P., Quantitative Evaluation of Rh_o (D) Variants with I-131 Anti-D, Bibl. Haemat. 13:237-40, 1962.

13. _____, Reaction of I-131 Anti-D with Enzyme-Treated RBC, Transfusion 2:363-74, Nov.-Dec., 1962.

14. _____, Rh_o (D) Genotype and Red Cell Rh_o (D) Antigen Content, Science 131:1442, 1960.

15. McKerns, K. W. and Denstedt, O. F., The Free-Cell Phenomenon in Isohaemagglutination, Can. J. Research E28:152-68, 1950.

16. Mollison, P. L., Blood Transfusion in Clinical Medicine, Springfield, Charles C Thomas, 1961. 3rd ed., p. 136-8.

17. ibid., 1956. 2nd ed., p. 220-1.

18. Race, R. R. and Sanger, Ruth, Blood Groups in Man, Springfield, Charles C Thomas, 1959. 3rd ed., p. 162.

19. _____, et al, The Rh Chromosome Frequencies in England, Blood 3:689-95, 1948.

20. Scheinberg, S. L. and Reckel, R. P., Induced Somatic Mutations Affecting Erythrocyte Antigens, Science 131:1887-8, 1960.