Papers and Originals

Prevention of Rh-Haemolytic Disease*

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[WITH SPECIAL COLOUR PLATE FACING PAGE 8]

Brit. med. J., 1967, **4**, 7–12

The story of the Liverpool research to prevent Rh isoimmunization is a simple one, and its origins were unusual in that they stemmed from breeding Lepidoptera.

Single Gene Differences and the Origin of a Supergene in Papilio Butterflies

Plate 1 shows the Swallowtail butterfly *Papilio machaon*, which in this country flies in the Norfolk Broads. All that needs to be noted is that it is yellow. Plate 2 shows a related species, *Papilio asterias*, common in North America, and it will be seen that here the ground colour is black. In 1953, having earlier learnt how to hand-pair swallowtails (Clarke, 1952), hybridization between *P. machaon* and *P. asterias* was carried out (Clarke and Knudsen, 1953). The cross proved fertile, and in Plate 3 it will be seen that the F_1 hybrid was black and remarkably similar to the American parent. Though the hybrids were sterile *inter se*, yet it was possible to obtain back-crosses, and in the one to *P. machaon* there was clear-cut segregation (Plate 4), indicating a single gene difference as regards ground colour between the two species, black being dominant to yellow. During this study I met P. M. Sheppard, and it was decided to use the hand-pairing technique to investigate the phenomenon of mimicry, first in an African species, *P. dardanus* (Clarke and Sheppard, 1956, 1958, 1959, 1960), and later in a South-east Asian species, *P. memnon* (Clarke *et al.*, in press).

In mimicry certain butterflies, particularly tropical ones, resemble others, the models (often belonging to quite different families), which are distasteful to predators, particularly birds. Models and mimics usually fly together, and birds which have sampled the distasteful models tend to leave the mimics alone even though these are highly palatable. Plate 5*a* shows a mimetic form (*achates*) of *P. memnon* together with the distasteful model, *Parides coon* (Plate 5*b*). In both model and mimic it should be noted that (1) tails are present, (2) the white "window" of the hindwing is small, and (3) the body is yellow. Plate 5*c* is a non-mimetic form (*agenor*) of *P. memnon*, and breeding data appear to show that *achates* is dominant to *agenor* and that there is a single gene difference between the two forms. However, the matter is not as simple as this, for occasionally rearrangements take place, and Plate 5*d* shows a bred *memnon* insect where, though tails are present and the "window" is small, yet the body is black. This suggests that crossing-over has occurred and that the single gene is in fact a supergene composed of several closely linked loci (Fig. A).

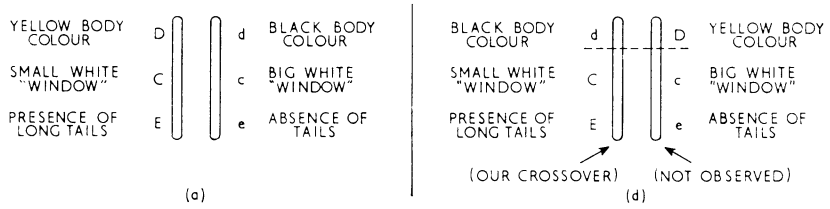

FIG. A.—Chromosomal arrangement of insects (*a*) and (*d*) in Plate 5.

It was this type of finding (originally in *P. dardanus*) and the many examples of genic interaction in the *Papilio* species which directed our attention to the rhesus blood group system, where crossovers probably account for the rarer genotypes and where there is a marked interaction between the Rh and ABO systems.

Rh and ABO Interaction

Levine (1943) first pointed out that there is a deficiency of ABO incompatible matings—that is, those where the wife cannot safely be transfused with her husband's blood—in families where children with Rh-haemolytic disease have occurred. This is because ABO incompatibility as between mother and foetus markedly protects against Rh immunization. The original observation has since been amply confirmed, and the most reasonable explanation is that ABO incompatible foetal red cells are destroyed in the maternal circulation by the naturally occurring anti-A or anti-B before they can sensitize the mother against the D antigen (the elimination probably taking place in the liver). This theory has support from the experimental work of Stern *et al.* (1956). They injected Rh-positive blood into Rh-negative male volunteers and found much higher titres of anti-D when compatible ABO blood was injected than when incompatible blood was used. Another observation made by them (Stern *et al.*, 1961) which turned out later to be very relevant to our research was that when Rh-negative male

* Lumleian Lecture delivered at the Royal College of Physicians of London on 3 May 1967.

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volunteers were injected with Rh-positive red cells coated with incomplete anti-D the men did not form immune anti-D.

To test further Levine's original observation, in 1958 we carried out in Liverpool a family study in order to determine the ABO group of the sensitizing foetus in families where there was Rh-haemolytic disease (Clarke *et al.*, 1958). The number of families investigated was 91, and of these the parents of 23 were incompatible on the ABO system. In 14 of these 23 families we were able to determine the sensitizing foetus with certainty and all were compatible with the mother on the ABO system. The probability of this occurring by chance alone is less than 1 in 500. Additional support for protection by ABO incompatibility, derived from this same series, was the marked deficiency of matings where the mother was O and the father A (16 as against 33 where the mother was A and the father O), whereas a 1:1 ratio would be expected if no protection existed. Nevertheless protection is not invariable—in a different study we did find one ABO incompatible sensitizing foetus, and it has also been described by other authors (and see Table II).

Shortly after this 1958 paper it occurred to us that it might be possible to mimic the protection afforded by ABO incompatibility by giving the mother anti-D after delivery, thereby destroying any Rh-incompatible foetal cells (which we assumed had at some time crossed the placental barrier) before they had time to sensitize her. This idea was put forward at a meeting of the Liverpool Medical Institution by Finn (1960), and by this time we had already begun work to test the hypothesis. This involved injecting Rh-negative male volunteers with chromium-tagged Rh-positive cells and later giving high titre anti-D as an infusion of plasma (Finn *et al.*, 1961). It was found that when complete (saline) antibody was given *enhancement* of immune antibody was produced in the "treated" compared with the controls, but that when incomplete (albumin) anti-D was used then significant protection was obtained (Clarke *et al.*, 1963). Though protection in men therefore seemed possible it was still uncertain whether the technique would work in mothers, but this problem was made much easier when it became possible to detect foetal cells in the maternal circulation, and this is next discussed.

Foetal Cells in the Maternal Circulation

Kleihauer *et al.* (1957) showed by means of an acid elution slide technique that it was possible to demonstrate and count foetal cells among a population of adult red cells, the latter appearing as ghosts and the former as dark refractile bodies. Zipursky *et al.* (1959) first used this method to detect foetal cells in the blood of women after delivery, and it was their paper which decided us to use the technique in our work. Our investigations showed that, while foetal cells can be demonstrated occasionally in the maternal circulation throughout pregnancy, they occur much more often and in greater numbers at or near delivery. Again, and most important from the protection point of view, the number of foetal cells found in the maternal circulation near or just after delivery correlated well with the frequency of the formation of immune anti-D in the mother six months later (Woodrow *et al.*, 1965 ; Woodrow and Finn, 1966). It was these findings together with our results in the volunteers which strengthened our view that giving anti-D to mothers after delivery might be effective. We therefore decided to carry out clinical trials, but before discussing these it is necessary to mention some important points relating not only to our work but also to that of others.

(1) Counting of Foetal Cells and Accuracy of the Acid Elution Technique

In most of our work the foetal cell score has been the number of foetal cells found in 50 low-power fields, and very roughly we assess that a score of 1–4 implies less than 0.25 ml. of foetal blood in the maternal circulation, a score of 5–60 means approximately 0.25–3 ml., and a score of over 60 means more than 3 ml. of foetal blood (Clarke *et al.*, 1963 ; Woodrow *et al.*, 1965). In this paper the term "significant bleed" means either of the last two.

A more accurate method of scoring is to relate the number of foetal to the number of adult cells, and Table I shows the result in our laboratory of a test carried out "blind" from material sent to us by Professor P. L. Mollison in which varying proportions of adult and foetal cells (samples A–E) were mixed. It will be seen that though our results (Mollison, personal communication) are not particularly impressive, yet the findings are in the right order of correctness though we failed to detect any cells in the most highly dilute mixture (sample D).

TABLE I.—*Accuracy of the Acid Elution Technique in Our Laboratory (See Text)*

	A	B	C	D	E
No. of foetal cells observed (two observers)	3+4	0+0	60+58	0+0	0+0
Ratio of total foetal cells to total number of adult cells scanned (our estimate) ..	1/50,000	—	1/3,000	—	—
Correct result	1/17,000	0	1/1,070	1/37,000	0

(2) Difficulties in Scoring

There are several inherent difficulties in the interpretation of the slides, because the acid elution technique does not distinguish between maternal and foetal Hb F. Maternal Hb F levels are increased in thalassaemia and in some other haematological disorders, and there may also be an increase in maternal Hb F during pregnancy. False positive results may also occur in association with a marked reticulocytosis. With experience it is usually, though not invariably, possible to identify those conditions with a high maternal Hb F, because the latter is usually unevenly distributed among many cells rather than concentrated in a few, as it is in a true transplacental haemorrhage (Woodrow and Finn, 1966). In a few cases this distinction is, however, not possible (see W.H.O. Report, in press).

(3) Foetal Cell Count and ABO Incompatibility

Table II shows well the effect of ABO incompatibility in reducing the foetal cell count in 1,000 Rh-negative primigravidae, the information kindly being supplied by Dr. C. C. Bowley, of Sheffield, who has been working with us in the group project (see *Brit. med. J.*, 1966). The reason why foetal cells survive in some of the ABO incompatible cases is because destruction is not always complete, and also because in certain cases the blood has been examined so quickly after delivery that there has not been time for the cells to be eliminated.

TABLE II.—*Comparison of Foetal Cell Counts in Foeto-maternal ABO Compatibility and Incompatibility (See Text)*

Foetal Blood	Significant Bleeds		Insignificant Bleeds		No Bleed		Total
	No.	%	No.	%	No.	%	
ABO compatible: D-positive	85	18·6	184	40·2	189	41·2	458
,, ,, D-negative	70	20·2	147	42·2	131	37·6	348
ABO incompatible: D-positive	5	4·2	28	23·5	86	72·3	119
,, ,, D-negative	3	4·0	20	26·7	52	69·3	75
Total ..	163		379		458		1,000

(4) Similar Research in the U.S.A.

Gorman, Freda, and Pollack, who started experimental prevention of Rh-haemolytic disease independently of the Liverpool group, were not seeking ways and means of eliminating foetal

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Plates 1–5 and Fig. A show the genetic investigations in *Papilio* butterflies which led to the Rh research.

PLATE 1
Papilio machaon.

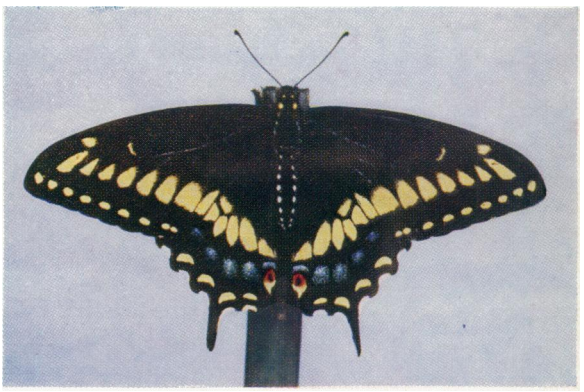

PLATE 2
Papilio asterias.

PLATE 3
F^1 hybrid.

PLATE 4
Back-cross to *P. machaon.*

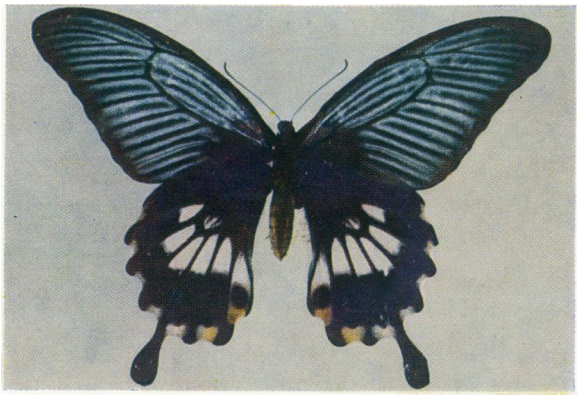

PLATE 5*a*
P. memnon f. *achates.*

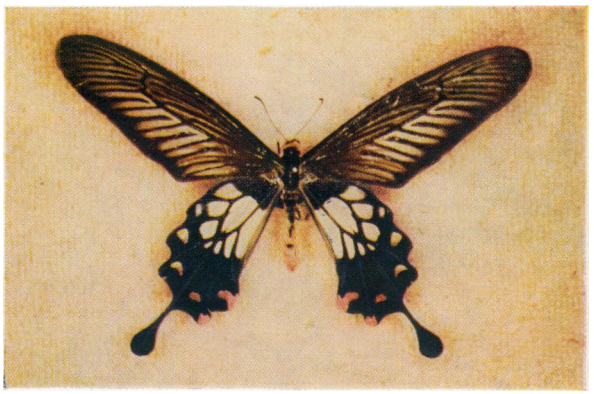

PLATE 5*b*
P. coon.

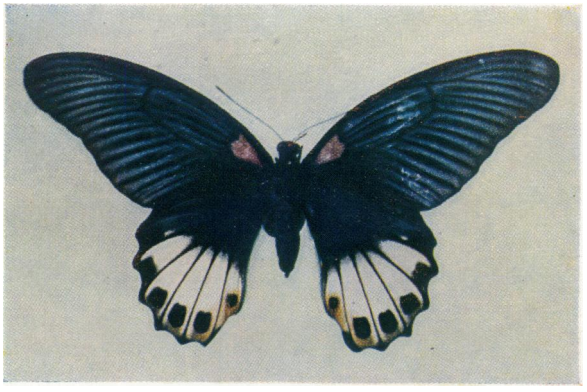

PLATE 5*c*
P. memnon f. *agenor.*

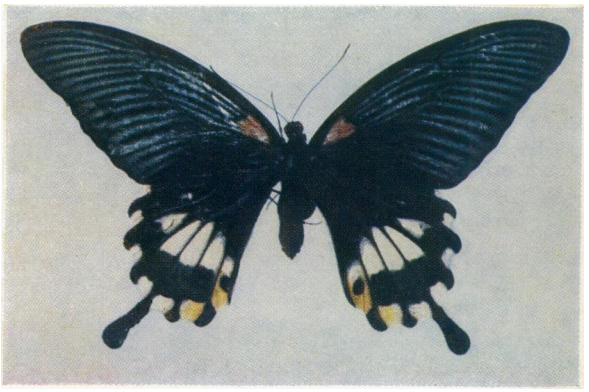

PLATE 5*d*
Bred insect showing cross-over of gene controlling body colour (see Fig. A).

cells (Freda and Gorman, 1962 ; Freda *et al.*, 1964). Rather they tried to put to practical use the finding of Smith (1909) that mixtures of diphtheria toxin and antitoxin, with antibody in excess, did not immunize when injected. Later it had been shown that if specific antibody is given passively to an individual subsequent injections of antigen completely fail to immunize, provided that there is a state of antibody excess at all times. Because of this, in the early New York experiments with male Rh-negative volunteers, the antibody injection was given before the Rh-positive cells were injected (Gorman *et al.*, 1963 ; Freda *et al.*, 1964) (see McConnell, 1966, and also Clarke, 1966).

There may, in fact, be no very great difference between our rationale and theirs, but the important contribution which the U.S.A. workers made was the introduction of anti-D gammaglobulin rather than plasma. Anti-D gammaglobulin carries no risk of jaundice, and being given intramuscularly is a much more convenient preparation to administer, and as soon as we knew of the U.S.A. advance we switched over to gammaglobulin, which has been made for us from the plasma of our hyperimmunized male volunteers by Dr. W. d'A. Maycock, Mr. L. Vallet, and their colleagues at the Lister Institute.

Clinical Trials

In May 1964 we decided to give, within 48 hours of delivery, 5 ml. of gammaglobulin containing incomplete anti-D (usually with a titre of between 1 in 1,280 and 1 in 4,096 in albumin with R_2r cells). This was given intramuscularly to alternate primiparae in whom the foetal cell score was 5 or more, and we decided to analyse this trial when about 70 controls and 70 treated had been followed up for at least six months. The results were highly satisfactory and were reported last year (*Brit. med. J.*, 1966). Of the 78 treated none had for certain produced immune antibody whereas 19 of the 78 controls had done so.

Similar trials, though not restricted always to primiparae and to "high risk" cases, have been in progress in U.S.A. and Germany, and Table III gives the overall results, so far as we know them, up to July 1967. It will be seen that at six months only one woman has become immunized in the treated group, a much better result than in our male volunteers. However, the controls pose some problems. It will be seen that in the various centres there is considerable heterogeneity among them and we do not know for certain the cause of this. While the figure in the "Liverpool group" is explicable because high-risk cases only were treated, yet this was not so elsewhere ; but it may be that different obstetrical practices are responsible for greater or less foeto-maternal bleeding. In this connexion it is of interest that Worlledge (1966) has shown in Nigeria that the Rh-immunization rate is only about one-third of what it is in this country, and this is probably not to be explained by the lack of antigenicity of the common R° antigen, since the immunization rate after transfusion with Rh-positive blood is about the same as it is here.

TABLE III.—*Combined Results from Various Centres. Immune Antibody Results Six Months and Later in Controls and After Injection of Anti-D Gammaglobulin*

	Control				Treated			
	Total	Imm.	Not Imm.	% Imm.	Total	Imm.	Not Imm.	Total in Study
Liverpool group (first trial)	136	29	107	21	131	1*	130	267
Germany[1]	76	3	73	4	76	0	76	152
Columbia, New York[2]	113	14	99	12	163	0	163	276
Long Beach, California[3]	176	21	155	12	176	0	176	352
Cornell, New York[4] ..	58	5	53	9	41	0	41	99
Edinburgh[5]	40	3	37	8	41	0	41	81
Totals ..	559	75	524	13	628	1	627	1,227

[1] Schneider and Preisler (1966) and Schneider (personal communication).
[2 3 4 5] Robertson (personal communication).
* Though there is no doubt about the complete failure in this case, it may be instructive. The patient developed a sharp local reaction to the anti-D gammaglobulin, and we then learnt that she had received early in pregnancy a prophylactic dose of anti-rubella gammaglobulin. Whether this has any bearing on the failure is being investigated. (See Wilson and Steinberg, 1965.)

Persistence of Injected Anti-D Gammaglobulin

The U.S.A. group have carried out tests to see how long the passive antibody can be detected and Table IV shows their results. We also seldom find passive antibody after six months, but occasionally traces have been detected for longer by an enzyme technique. For example, at the time of the original analysis of our Liverpool group clinical trial (*Brit. med. J.*, 1966) there were three cases among the 78 treated where it was uncertain whether it represented only the remains of the injected anti-D. This in fact turned out to be the case since in all three mothers the antibody eventually disappeared, though in one case it was thought to have lasted for as long as 13 months.

TABLE IV.—*Post-delivery Passive Rh-antibody Titres. New York Series (Pollack et al., 1967)*

Antibody Titre	Weeks Post-injection and Number of Patients in Each Category		
	5–7 Weeks	12–16 Weeks	24–26 Weeks
0	0	24	77
1	1	15	1
2	3	29	
4	15	18	
8	48	11	
16	58	2	
32	17		
64	3		
Totals ..	145	99	78

Subsequent Pregnancies (all Centres) up to June 1967

Though the six months' results were highly satisfactory there was always the possibility that the anti-D was only masking immunization and that in the next pregnancy the treated women would produce overt antibodies and "catch up" with the controls. The data in Table V strongly suggest that this is not the case, because none of the women who received the anti-D gammaglobulin after their previous pregnancy has, in fact, produced immune rhesus antibodies after having had a subsequent Rh-positive ABO compatible baby, whereas 15 further cases in the controls have done so. Nevertheless, larger numbers of second pregnancies are needed before concluding that the prophylaxis is really of the very high order it appears to be at present.

TABLE V.—*Combined Results. Subsequent Pregnancies (All Centres) Up to June 1967. Rh-positive ABO Compatible Infants*

	Control				Treated		
	Total	Imm.	Not Imm.	% Imm.	Total	Imm.	Not Imm.
Liverpool group (first trial) ..	26	4	22	15	21	0	21
Germany[1]	7	3	4	43	9	0	9
Columbia, New York[2] ..	14	4	10	29	21	0	21
Long Beach, California[3] ..	14	3	11	21	13	0	13
Cornell, New York[4] ..	1	1	0	100	2	0	2
Edinburgh[5]	1	0	1	0	0	0	0
Totals ..	63	15	48	24	66	0	66

[1] Schneider and Preisler (1966) and Schneider (personal communication).
[2 3 4 5] Robertson (personal communication).

Second Liverpool Trial

Having determined that 5 ml. of anti-D gammaglobulin protected the high risk cases, we decided in June 1966 to start a second trial, using only 1 ml. of anti-D, this again being given to alternate primiparae, but now only to those who had a foetal cell score of 0–4 cells, inclusive. Using these criteria, we knew

we should get many more cases, and it seemed highly likely that the immunization rate would be much lower. The indications are that this is so, since at the time of writing (July 1967) 3 out of the 54 controls have become immunized compared with one out of 51 treated, these all having been followed up for six months or more. The treated woman who produced the antibody did so between the fourth and seventh month of a second pregnancy. Whether this means that 1 ml. of the Liverpool anti-D gammaglobulin is too small a dose (see below), or whether the mother is one of the rare cases where antibody is produced in the first "at risk" pregnancy (to which this pregnancy approximates), remains to be seen. If the former is the case more antibodies will develop in the treated in this trial.

Because of the good results from the five Liverpool maternity units in the first trial, *all* primiparae in these hospitals where the cell score is 5 or more are now being given 5 ml. of anti-D gammaglobulin.

Indications for Prophylaxis in Rh-haemolytic Disease

The feasibility of a procedure is no indication for carrying it out, and, though there seems little doubt that the giving of anti-D is prophylactic, yet it has been suggested that the treatment is not without risk, and that the immunization of large numbers of volunteers and the mass inoculation of Rh-negative women is not justified. The critics feel that this is particularly so because only a small proportion of women at risk become immunized, and, even if they do, modern methods of treatment mean that the majority of children are saved. This clearly needs consideration and the risks must first be assessed.

Risks to the Mothers

In our experience the initial injection is safe. We have observed very occasionally local reactions, sometimes associated with fever, for 24 hours, but these are most exceptional and usually there is no upset of any kind. With anti-D gammaglobulin there appears to be no risk of causing homologous serum jaundice, since if the virus is present it is eliminated in gammaglobulin prepared by the alcohol or ether fractionation procedure (Mollison, 1967). We have twice given it in error to women who were later found to be weakly Rh-positive (D^u) and no reaction of any kind was observed, and probably there would be no serious trouble if the injection were given to women who were of the more usual Rh-positive types. There remains the question of the possibility of the mother becoming immunized to the gammaglobulin itself. We have investigated this with the help of Dr. Sylvia Lawler, who has tested the Gm groups in 20 of our treated women and in 20 controls, both matched approximately for the size of foeto-maternal "bleed." No anti-Gm was found in the controls, but it was in one treated case and the details of this are as follows:

Anti-Gm Study.—Mrs. N., a treated mother, has anti-Gm antibody, of Gm^{a+} specificity, in her serum not present before delivery. She herself is Gm^{a-} and so is her husband. Her mother, however, is Gm^{a+}, and Mrs. N. may have been originally sensitized from her mother, our gammaglobulin acting as a secondary stimulus.

It seems certain that here the gammaglobulin was responsible for the development of the overt anti-Gm, though it is possible that the primary stimulus was derived from the mother of the treated woman. However, the risks of a serious anaphylactic reaction if gammaglobulin needed to be given again in a subsequent pregnancy appear to be small, severe anaphylaxis being likely to occur only if the patient is hypogammaglobulinaemic (Grubb *et al.*, 1965).

When considering risks it seems right to take into account the views of patients. They in general are in no doubt about wanting prophylaxis, and it is in fact our controls who sometimes complain of being guinea-pigs.

Risks to the Volunteers

Deliberate immunization of Rh-negative volunteers carries the slight risk of homologous serum jaundice, but this can be minimized by injecting washed Rh-positive cells and always deriving these from a donor who has been known to have given blood to at least 10 recipients all of whom have been kept under supervision for a period of six months and not developed jaundice (see W.H.O. Report, in press). Nevertheless, the hazard remains and there is a genuine difference of opinion about the advisability of immunizing men on a large scale at this stage. The view of many is that supplies of anti-D from women who have been naturally immunized, or from men and women who have developed antibodies as the result of transfusion, should be used first. This is reasonable, but there are often difficulties in tapping these supplies, and they are in any case a dwindling source. The contrary view is that if protection can be obtained by the use of small quantities of anti-D when this is hyperimmune (donors repeatedly restimulated) then it would be preferable to have small numbers of Rh-negative volunteers in each region and the necessary supplies obtained by plasmapheresis. The extent of the problem if this were the procedure adopted is as follows. If it is assumed that for every million of a Caucasian population 1,600 Rh-negative women will give birth each year to an Rh-positive ABO compatible infant and that the suppressive dose of anti-D gammaglobulin is 1 ml. (but see below), then about 90 litres of anti-D antibody-containing plasma will be needed annually. Using plasmapheresis and assuming that each donor gives half a litre of plasma 12 times a year, 15 donors with acceptable anti-D antibody titre would be required per million of the population.

We think that the risks outlined above are very small and should be accepted. In all prophylactic inoculations large numbers of people are injected unnecessarily, but one of the chief functions of a clinician is to allay anxiety, and the evidence strongly suggests that Rh-negative women need no longer be anxious about their blood group. Also, though many babies are saved by medical care, yet the stillbirths remain a problem, and in general there is a great deal of distress in a not inconsiderable number of families and it seems likely that this can be avoided.

Lastly, the fact that exchange transfusion is feasible (though expensive, time-consuming, and not always successful) is no indication for preferring it to stopping the trouble at the source.

Problems Remaining to be Solved

(1) Latest Time at Which Gammaglobulin can Effectively be Given

In our trials the anti-D has been given within 48 hours of delivery and in the U.S.A. up to 72 hours. Since, for a variety of reasons, "at risk" patients might not be identified quickly, it is important to find out how long after delivery an injection of gammaglobulin is effective, and we are at present planning a trial in volunteer men to see if it is still protective when given a week after the injection of Rh-positive cells.

(2) Prophylaxis During Pregnancy

The results of the clinical trials to date giving the injection after delivery have been better than anticipated. It may, however, be that on occasion an effective immunizing dose of Rh-positive cells crosses the placenta during pregnancy. Zipursky *et al.* (1965) have shown that it is a safe procedure to give anti-D gammaglobulin during pregnancy, and while

we do not know whether this is protective yet it does not cause anaemia in the baby, though the Coombs test is occasionally positive. If failures with the conventional treatment after delivery do occur, their work would have great practical importance.

(3) Duration of Protection

The length of time during which injected anti-D affords protection is uncertain, but Chown (1966, personal communication) gives details of an interesting experiment which is summarized here. Three groups of six men in a local penitentiary were injected with 2 ml. of foetal Rh-positive blood. Then, 48 hours later: group (*a*) received 5 ml. of anti-D, group (*b*) received 1 ml. of anti-D, and group (*c*) received 1 ml. of commercial gammaglobulin.

Immune antibody production six months later was: group (*a*) 0/5 (1 man released), group (*b*) 0/6, and group (*c*) 3/6. At this time a further 2 ml. of Rh-positive foetal cells was given to each man.

Immune antibody production 10 months later was: group (*a*) 0/1, group (*b*) 0/3, and group (*c*) 5/5 (9 men relased).

Chown and his colleagues interpreted these results as supporting the concept of a suppressive effect on the potential specific antibody-forming cellular system, and as being in line with the experimental work of Finkelstein and Uhr (1964) and Rowley and Fitch (1964). The Canadian workers go further and think that the protection may last even after the antibody is no longer detectable.

On the other hand, Freda *et al.* (1966) showed that two out of eight of their previously protected male volunteers were still (10 months after their last injection of anti-D) capable of producing immune antibody when given a fourth and fifth antigenic stimulus *without* the anti-D gammaglobulin cover. The matter therefore remains open, but is of great current interest not only in this field but also in others such as in the prevention of autoimmune disease and in tissue transplantation.

(4) Optimal Dose of Anti-D Gammaglobulin

This is the most important current problem. Clearly it is meaningless to talk of 5 or 1 ml. of gammaglobulin and its strength must be assessed in some way. Titre has generally been used in the past, but this for technical reasons varies greatly in different laboratories, and, moreover, it only measures the amount of antibody which is combined with antigen on the red cell. Since the reaction between antibody and antigen is reversible, there is always some free antibody present in the plasma, of which titre takes no account. Nevertheless, this fraction may be important if central inhibition of the antibody-forming cells is of more account in preventing immunization than the blocking of the antigen sites on the red cells (see above). Therefore the total amount of anti-D in μg. per ml. is probably the most reliable measure, and it can be estimated by various labelling procedures (Hughes-Jones, 1967; Goldsmith *et al.*, in press).

However, for the present, since the precise method of protection is unknown, it seems best to estimate where possible both the titre *and* the total amount of anti-D present.

It is uncertain how the dose of anti-D in μg. per ml. has been related to its effectiveness in suppressing immunization, but some information is available. In the first clinical trial carried out by the Liverpool group, 5 ml. of gammaglobulin containing incomplete anti-D with a titre of usually between 1 in 1,280 and 1 in 4,096 in albumin with R_2r cells were used. Though the concentration of this is not known, it is believed to have been similar to later ones that have been estimated, and if so the 5 ml. contained about 1,000 μg. of anti-D, and this dose appears to be nearly always protective. (For this and other assessments of anti-D in μg. per ml. I am indebted to Dr. N. C. Hughes-Jones.)

However, both the New York and Winnipeg groups (W.H.O. Report, in press) have experimental evidence that a smaller amount is also effective. The former found that 0.1 ml. of their anti-D preparation protected four volunteers when given after 10 ml. of blood on six occasions, and the latter report that 1.5 ml. of another anti-D preparation protected 12 volunteers when injected with 2 ml. of blood. The anti-D concentrations of these two gammaglobulins, though also unknown, are thought to be of similar potency to later ones from the same centres, and if this is so the amount injected was of the order of 150 μg. of anti-D in the New York experiments and 300 μg. in those of Zipursky and his colleagues in Winnipeg. On the other hand, we have to remember that in her second pregnancy an immune antibody appeared in a woman in our second trial who had been given 1 ml.—that is, about 200 μg.—of anti-D.

At lower doses it would seem from work carried out in Germany and New York (see also W.H.O. Report, in press) that a dose of the order of 10 μg. of anti-D antibody is insufficient to prevent immunization. While these are only interim results they may serve as a basis for further experiments, but one fact is quite clear—namely, that the U.S.A. gammaglobulin is considerably stronger than that which we are using. Additional evidence that this is so is found when testing for passive antibody about 48 hours after a 1-ml. injection. Robertson (personal communication), using this dose of U.S.A. gammaglobulin, often finds a titre of 1 in 8, while, on the other hand, our 48-hour antiglobulin test with 1 ml. of the Lister product is always negative, the passive antibody being detected only by an enzyme technique. On using 5 ml. of the Lister preparation, as in the first Liverpool trial, our 48-hour antiglobulin titre is usually about 1 in 4. A particular case met with recently also gives some preliminary information about dosage (Gilliver, 1967).

A 21-year-old primigravida, blood group A rhesus (D) negative with no antibodies detectable at 34 weeks, was admitted to hospital on 12 September 1966.

After 54½ hours, an 8 lb. 5 oz. (3,770 g.) male infant was born by assisted breech delivery. His condition was satisfactory at birth but later the respirations became shallow and grunting and convulsions began, first right-sided and later generalized. About 24 hours after birth the infant's haemoglobin was 9.8 g./100 ml. and a blood film showed intense erythroblastosis. The boby's blood group was A Rh-positive and the direct Coombs test was negative.

Maternal blood was found to contain vast numbers of foetal cells, and this was confirmed by mixed-field agglutination with a saline anti-D serum. In a blood sample sent to Liverpool it was estimated that there were roughly 3,400 foetal cells per 50 low-power fields, and this was probably equivalent to about 170 ml. of foetal blood.

Seventy-two hours after delivery 5 ml. of gammaglobulin obtained from Liverpool was given, and 36 hours later the volume of foetal blood had fallen to about 80 ml., and at this time the Coombs test was positive. Free antibody at a titre of 1 in 4 was still present nine days after the injection, but the foetal cell score was 3 (representing less than 0.25 ml. of foetal blood) and the direct Coombs reaction had become negative.

Three and seven months later the follow-up revealed no antibodies detectable by the saline, albumin, Coombs, or enzyme techniques.

The baby improved satisfactorily after a transfusion of 85 ml. packed cells.

It will be interesting to see what happens in a subsequent pregnancy, but 5 ml. of the Liverpool gammaglobulin is evidently effective in clearing a very large T.P.H., and if this is, as we think, important in protection then it may well be that 5 ml. is an unnecessarily large dose in the majority of cases (see also Mollison and Hughes-Jones, 1967). But it must be remembered that in mismatched transfusions only about 50% of Rh-negative individuals are immunized by giving 1 pint (570 ml.) of Rh-positive blood (Mollison, 1967).

Until the mechanism of suppression of primary immunization is more precisely known, factors other than anti-D concentration and titre may be important. Thus it is possible that there may be variation in the biological activity of anti-D antibody obtained from different donors, and anti-D preparations are now made from pools of at least 10 donors. It will be interesting to see the results of clinical trials where the relative effectiveness of anti-D antibody derived from mothers who have had severely affected babies but who have not been deliberately restimulated is compared with that from donors who have been.

Summary

Genetic studies in *Papilio* butterflies, particularly those concerned with the evolution of mimicry, led to our interest in the Rh blood groups. Here, as in the mimetic butterflies, the various phenotypes are controlled by a series of closely linked genes (a supergene) and, again as in the butterflies, there are marked genetic interactions. The most important of these, described by Levine in 1943, is that ABO incompatibility as between mother and baby is nearly always protective against rhesus immunization of the mother. The mechanism of this is uncertain, but the most reasonable explanation is that any rhesus-positive foetal cells which have crossed into the maternal circulation are destroyed by the mother's naturally occurring anti-A or anti-B before they have had time to stimulate the production of Rh antibodies.

It occurred to us that it might be possible to imitate this by giving anti-D to Rh-negative mothers in the far more usual situation where mother and baby were compatible on the ABO system, and the idea was first put forward in 1960 at a symposium on medical genetics at the Liverpool Medical Institution.

Experiments on rhesus-negative blood-donor volunteers showed that protection could be achieved provided incomplete anti-D was given, and in these early experiments plasma was used from artificially immunized males. Concurrently with this research, the acid elution technique for the detection of foetal cells in an adult red cell population was used to show that transplacental haemorrhage was commoner at or near delivery, and, furthermore, that the greater the number of foetal cells in the maternal circulation just after delivery the more likely the mother was to produce immune Rh antibodies.

Similar work in the U.S.A., using anti-D in the form of gammaglobulin, which was much safer and more convenient, was first published in 1962, and after this we too used only anti-D gammaglobulin.

Details are given of several successful clinical trials carried out in this country, the U.S.A., and Germany, in which soon after delivery the mother is given an intramuscular injection of gammaglobulin containing a high titre of incomplete anti-D.

Definite proof that Rh immunization can be prevented by this technique will rest on the immunological state of the treated women just after they have had a subsequent Rh-positive ABO compatible baby. To date the results in these subsequent pregnancies are very encouraging, and the evidence indicates that nearly all cases of maternal Rh immunization can be prevented.

The risks to mothers and to the male volunteers are discussed and mention is made of the problem of supplies of anti-D gammaglobulin if the prophylaxis is to be made generally available. Other problems which remain to be solved, particularly the optimal dose of gammaglobulin, are outlined.

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