# Papers and Originals

## Rh-immunization by Pregnancy: Results of a Survey and Their Relevance to Prophylactic Therapy

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**Summary: A series of Rh-negative primiparae has been studied in order to gain further insight into the process of immunization by pregnancy. The distribution of foetal cell counts in blood samples taken after delivery was determined for 2,029 mothers giving birth to ABO-compatible babies and for 417 mothers with ABO-incompatible babies.**

**A total of 760 mothers were tested for the development of Rh antibodies six months after the delivery of an ABO-compatible Rh-positive baby and 236 were further followed up through a second Rh-positive pregnancy. The incidence of anti-D six months after delivery is estimated to be 8.5%, and there is evidence of a direct relation between the count of foetal cells after delivery and the risk of developing antibodies. A further 8.5% of mothers were estimated to develop anti-D by the end of the second pregnancy, and it is postulated that these individuals had been primed by the first pregnancy. There is some evidence that the larger stimuli of Rh-positive blood in the first pregnancy are more likely to result in overt antibody formation, while the smaller stimuli are more likely to prime, antibodies not being detected until a second stimulus occurs during the second pregnancy.**

**These findings are relevant to the programme for preventing Rh-immunization by injecting anti-D gamma-globulin.**

### Introduction

Clinical trials conducted at several centres during the past few years to test the effectiveness of anti-D gammaglobulin in the prevention of Rh immunization by pregnancy have convincingly shown that such prevention is possible (Combined Study, 1966 ; Clarke, 1967 ; Freda *et al.*, 1967). This work has stimulated renewed interest in the details of the natural history of Rh immunization. The possibility that for some time to come supplies of gammaglobulin will be limited so that a degree of selection of mothers for treatment may be necessary and the fact that failures of prophylaxis have already occurred are just two reasons why a greater understanding of the process of immunization is needed.

A survey was started in Liverpool in 1963 with the aim of studying in some detail the development of Rh antibodies in a selected group of mothers, and some preliminary data have been published (Woodrow *et al.*, 1965). The latest results of this survey are presented, and their relevance to preventive therapy is examined.

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### Patients Included in the Study

The staffs of five maternity units in Liverpool co-operated in this survey. On the morning of each weekday the units were visited, and deliveries by Rh-negative primiparae were thus ascertained. Cord blood samples were tested for ABO and Rh groups and direct Coombs tests carried out. Blood samples were obtained from the mothers, usually within 24 hours of delivery, and always within three days. When the baby was Rh-positive the mother was visited at home six months later and a further sample of blood taken. The commonest reason for failure to obtain this sample was that the patient had left the district, co-operation otherwise being very good.

These mothers were further followed up by ascertaining as many second pregnancies as possible, four methods being used to this end : (1) the patient was given a card to post to the unit when a second pregnancy occurred ; (2) hospital case sheets were labelled, and patients in the survey who returned to the same antenatal clinic were thus ascertained ; (3) letters were sent after an interval to patients from whom nothing had been heard ; and (4) where the letter produced no response the mother was visited at home.

The ABO and Rh groups of the second baby were ascertained either from the results of hospital laboratory tests on the cord blood or by later testing of the infant where the delivery took place at home. A maternal sample of blood at the time of the second delivery was obtained in the majority of cases, but where this was missed the Regional Blood Transfusion records were consulted to obtain the results of all antibody tests carried out during the pregnancy.

### Methods

*Tests for Antibodies.*—These were carried out on the immediate post-delivery samples, and on any subsequent samples obtained. The samples of serum were stored at −20° C. and tested by saline, albumin, indirect Coombs test, and papain techniques (see Combined Study, 1966) against appropriate panels of cells.

*Tests for Foetal Cells.*—A modification of the Kleihauer–Betke technique (Kleihauer *et al.*, 1957) was used to detect and count the foetal cells present in the post-delivery maternal samples of blood (see Appendix). A quasi-quantitative method of scoring was used (see Woodrow *et al.*, 1965) giving a "foetal cell score." It is thought that a score of 5 is produced by the presence in the mother of approximately 0.2 ml. of foetal blood. Some of the problems involved in this method have been discussed previously (Woodrow and Finn, 1966) ; there it was pointed out that where the number of cells was low the reliability of the detection and scoring of foetal cells was limited.

However, it is believed that the score of cells does give a reasonable estimate of the volume of foetal blood present.

*Implications of Treatment of Some Mothers.*—When the survey had been in progress for a year a clinical trial of anti-D gammaglobulin was started. Alternate mothers showing a foetal cell score of 5 or more, the babies being ABO-compatible and Rh-positive, were treated with anti-D gammaglobulin after delivery. Though the foetal cell scores of these patients could be used in the survey, the results of later testing for antibodies could not. This meant that the mothers followed up were no longer as a whole representative of the population, and this fact has to be taken into account in analysing the results.

*Abortions.*—The occurrence of abortions between the first and second completed pregnancies was ascertained, as they might have played a part in the immunizing process. Where the second baby had been delivered in hospital the case notes were examined, and where the delivery had been at home the mother was visited and a history obtained.

## Transplacental Haemorrhage

In seven instances anti-D was already present at the end of the first pregnancy, and these are omitted from the results and analysis.

Post-delivery samples were obtained from 2,029 Rh-negative primiparous mothers just delivered of ABO-*compatible* babies. Twenty-nine samples could not be accurately scored (see below); the distribution of scores for the remaining 2,000 cases being shown in Table I. Altogether, 754 of the babies were Rh-negative, but there was no difference in the distribution of scores from that seen with Rh-positive babies, and the results were combined. It is seen that foetal cells were found in 56% of samples. Some idea of the upper range of transplacental haemorrhage observed can be obtained from the fact that there were four samples with scores of over 1,000, the estimated volumes of foetal blood being 40, 120, 140, and 170 ml. respectively.

TABLE I.—*Distribution of Foetal Cell Scores After Delivery; ABO-Compatible and ABO-Incompatible First Pregnancies*

| Pregnancy | | Foetal Cell Score | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3–4 | 5–10 | 11–39 | 40+ | |
| ABO-compatible | No. | 880 | 411 | 187 | 153 | 150 | 144 | 75 | 2,000 |
| | % | 44·0 | 20·5 | 9·4 | 7·6 | 7·5 | 7·2 | 3·8 | 100 |
| ABO-incompatible | No. | 314 | 65 | 24 | 6 | 5 | 3 | 0 | 417 |
| | % | 75·3 | 15·6 | 5·8 | 1·4 | 1·2 | 0·7 | 0 | 100 |

Table I also gives the distribution of foetal cell scores for 417 primiparous mothers just delivered of an ABO-*incompatible* baby. There is a marked difference in the two distributions. Of the compatible group 44% showed no foetal cells compared with 75.3% of the incompatible group. For every category of foetal cell score the incidence is higher for the compatible group, and the difference becomes more marked the higher the score.

There is no reason to suppose that the actual incidence of transplacental haemorrhage is different in the two groups, and the reason for the relative paucity of detected foetal cells with ABO-incompatible pregnancies is that by the time the sample had been taken most, if not all, the ABO-incompatible cells have been removed from the circulation.

*Samples Impossible to Score.*—In a previous report a description was given of certain blood samples which could not be scored for foetal cells because of the presence in the stained smear of many intermediately stained cells, rendering the counting of foetal cells impossible (Woodrow and Finn, 1966). This appearance is due to the presence in the maternal red cells of an increased amount of Hb F. This appearance was found in 29 of the ABO-compatible samples and in nine of the ABO-incompatible cases, the overall incidence being 38 (1.5%) out of 2,455. This is lower than the incidence previously found during pregnancy (6.3%), and these findings perhaps lend some support to those of Rucknagel and Chernoff (1955) and Kristoffersen (1964), who found that a rise in the level of Hb F tends to occur in the first trimester with a fall later in pregnancy. These 38 cases are excluded from the general analysis.

## Rh Antibody Developing in the Post-delivery Period

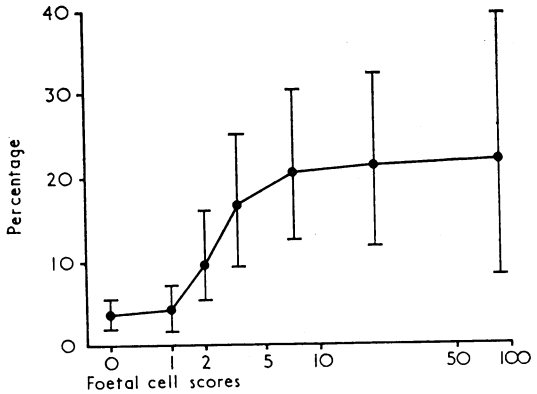
### ABO-Compatible Rh-Positive Pregnancies

Table II shows that samples were obtained six months after delivery from 760 mothers. The relation of anti-D formation to the foetal cell scores is also shown here and in the Chart. There appears to be in general an increase in the risk of anti-D developing as the foetal cell score rises. The data have been analysed by a ranking method (the Mann-Whitney U-test). The distribution of foetal cell scores is significantly different in those mothers developing anti-D from those who did not do so ($P<10^{-7}$).

TABLE II.—*Development of Anti-D Six Months After Delivery*

| | Foetal Cell Score | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3–4 | 5–10 | 11–39 | 40+ | Total |
| Anti-D present .. | 13 (4) | 6 (2) | 8 (2) | 10 (3) | 11 (2) | 9 (1) | 4 | 61 (4) |
| Anti-D absent .. | 346 | 141 | 73 | 50 | 42 | 33 | 14 | 699 |
| Total .. | 359 | 147 | 81 | 60 | 53 | 42 | 18 | 760 |
| % with anti-D .. | 3·6 | 4·1 | 9·9 | 16·7 | 20·8 | 21·4 | 22·2 | 8·0 |

Pregnancies all Rh-positive and ABO-compatible.
Figures in parentheses refer to patients in whom papain anti-D only was found.




Relation between the foetal cell score after delivery of an ABO-compatible Rh-positive baby and the percentage of mothers who developed anti-D during the subsequent six months. The 90% confidence limits on the percentages are given (calculated as symmetrical limits in terms of the angular transformation).

Table II suggests that up to foetal cell scores of about 10—that is, 0.4 ml.—there appears to be a close relation between the foetal cell score and anti-D production. It is rather surprising that, given the nature of the technique, such an apparently close relation is shown. Little increase in the incidence of anti-D formation was observed as the foetal cell score rose above 10.

These figures thus give some measure of the "risk" of developing anti-D for any particular mother. In order, however, to determine the overall risk, the fact that certain mothers had been treated must be taken into account. Table III sets out a method of doing this. The incidence of anti-D for each category of foetal cell score in Table II is taken as an estimate of the true incidence for that category in the population. The distribution of foetal cell scores for the 2,000 ABO-compatible

pregnancies, shown in Table I, is then used with these incidences to give an estimate of the true overall incidence of anti-D and the contribution to this of each category of foetal cell score. The estimated incidence of anti-D is thus seen to be 8.5%. A sizable contribution is made to this total by each category of patient, the large number of patients with the lower scores compensating for the lower risk of immunization.

TABLE III.—*Estimate of True Incidence of Anti-D Six Months After First Delivery*

| | Foetal Cell Score | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3–4 | 5–10 | 11–39 | 40+ | Total |
| No. .. .. | 880 | 411 | 187 | 153 | 150 | 144 | 75 | 2,000 |
| % developing anti-D | 3·6 | 4·1 | 9·9 | 16·7 | 20·8 | 21·4 | 22·2 | — |
| Expected No. with anti-D .. | 31·7 | 16·9 | 18·5 | 25·6 | 31·2 | 30·8 | 16·7 | 171·4 |

Pregnancies assumed to be Rh-positive and ABO-compatible. Thus of 2,000 mothers in the population 171 would be expected to have anti-D by six months after delivery—that is, 8·5%.

Of interest is the development of anti-D in patients whose post-delivery blood sample was scored as showing no foetal cells. Three possible factors might be operating here: (*a*) where only very small amounts of foetal blood are present, foetal cells may not be found in the areas of the slide scanned; (*b*) cells may have been present during the antenatal period, and may then have disappeared from the circulation; and (*c*) cells may be absent at the time of sampling but present shortly afterwards. Some evidence that such a delay in the appearance of foetal cells in the maternal circulation can occur is given by Hindemann (1966).

One further point of interest is that of the 54 antibodies found in this group 13 were detected by papain technique only, the other tests all being negative.

## ABO-Incompatible Rh-Positive Pregnancies

Samples were obtained from 208 mothers six months after delivery, and in two instances anti-D was found. The first mother was group O; she delivered a group B infant, no foetal cells being found after delivery, and six months later was discovered to have anti-D. The second was group O, and delivered a group A infant; a foetal cell score of 2 was obtained, and six months later anti-D, detected by papain technique only, was present. One further patient is of interest. A group O mother delivered a group B infant, and neither foetal cells nor antibody were found at delivery. Six months later anti-E with the behaviour characteristics of a "natural" antibody was found.

The incidence of anti-D for this group was thus shown to be approximately 1%. It appears that anti-D is 8.5 times more likely to appear in the antenatal period after first ABO-compatible pregnancies than after incompatible ones. One-fifth of all pregnancies are ABO-incompatible, and the figures therefore imply that of 35 instances of anti-D formation after a first Rh-positive pregnancy, one pregnancy is likely to be an ABO-incompatible one.

## Antibodies Developing During Second Pregnancy

Data are available on tests for antibody in 396 second pregnancies. Of these, 88 proved to be Rh-negative, and it is of interest that in no instance was antibody found during such a pregnancy where none had been found six months after the first pregnancy. This suggests that it must be very uncommon for anti-D to appear, as the result of the first pregnancy, *later* than six months post-delivery. Thus in the few instances where a second Rh-positive pregnancy had started when the six-months sample was taken, any subsequent appearance of antibodies during the pregnancy was judged to have been in part due to this second pregnancy.

In regard to abortions only two definite abortions and one possible abortion were found to have occurred between the first and second Rh-positive pregnancies, and in none of the mothers involved did anti-D appear during the second pregnancy. Thus there is no evidence that abortion determined the development of anti-D. The incidence of abortion appears to be unexpectedly low, but it must be remembered that the mothers in the survey are a very selected group who have had two full-term children within a fairly short period of time.

## First Pregnancy ABO-Compatible Rh-Positive

Of 246 such mothers followed through a second Rh-positive pregnancy, 10 had second Rh-positive pregnancies, antibodies having already been found after the first pregnancy. The outcome of the remaining 236 cases is presented in Table IV, where the development of antibodies is shown in relation to the foetal cell score after the first delivery. The class intervals used here are different from those used in Table II because of the smaller numbers. Both ABO-compatible and ABO-incompatible second pregnancies are included. It can be seen that there is considerable heterogeneity in the Table. Where no foetal cells were present after the first delivery 3.2% of mothers developed anti-D in the second pregnancy. Where the score was 5 or more 9.5% developed antibody. Very striking is the incidence of 16.5% antibodies where the foetal cell score (1–4) suggested a very small transplacental haemorrhage in the first pregnancy.

TABLE IV.—*Development of Anti-D During the Second Rh-positive Pregnancies*

| | Foetal Cell Score After First Delivery | | | |
|---|---|---|---|---|
| | 0 | 1–4 | 5+ | Total |
| Anti-D present .. | 4 | 15 | 2 | 21 |
| Anti-D absent .. .. | 120 | 76 | 19 | 215 |
| Total .. .. .. | 124 | 91 | 21 | 236 |
| Percentage .. .. | 3·2 | 16·5 | 9·5 | 8·9 |

The first pregnancies were ABO-compatible in all instances. $\chi^2$, 2 d.f. = 11·4, $P < 0.01$.

One element of possible bias must be mentioned here. Samples for antibody testing were obtained between the 32nd week and delivery, and for the past two years every attempt has been made to obtain a sample at the second delivery. There was some bias towards obtaining delivery samples in those cases with foetal cell counts of 5 or more who acted as controls in the clinical trial. It is possible that in a few instances anti-D might have developed after the last sample of blood was taken during pregnancy, but this applies only to foetal cell scores of 0–4. The incidence of antibodies for these scores in Table IV may thus be slightly underestimated.

Once again the fact that this follow-up excludes mothers treated after the first pregnancy means that the overall results in Table IV are not directly referable to the general population. However, the "risk" for each category of foetal cell score can be used in conjunction with the known distribution of scores in the population (Table I) to estimate what is probably occurring in the population. Table I shows that of 100 patients 44 would be expected to show no foetal cells, 38 a score of 1–4, and 18 a score of 5 or more. During the second Rh-positive pregnancy 1.4, 6.3, and 1.7 "patients" respectively would be expected to develop anti-D, giving a total incidence of 9.4%.

*ABO-incompatible Second Pregnancies.*—Table IV includes 29 instances where the second baby was ABO-incompatible with the mother. In one of these anti-D developed during the

pregnancy, but the numbers are insufficient to judge the effect of ABO incompatibility during second pregnancies.

*Estimate of Incidence of Anti-D Resulting from Two Rh-positive Pregnancies.*—The above results are combined in Table V. An estimate has been made of the incidence of anti-D in 100 Rh-negative mothers, (*a*) six months after delivery of the first ABO-compatible infant, and (*b*) during the subsequent Rh-positive pregnancy. The data suggest that 17% can be expected to have anti-D by the end of the second pregnancy. About equal numbers appear after the first pregnancy and during the second. The greatest contribution to total antibody production appears to come from mothers showing a few foetal cells after the first delivery, and this seems to result from (*a*) the frequency with which such small haemorrhages occur, and (*b*) the higher rate of priming. In contrast, mothers showing larger foetal cell scores are less numerous and are most likely to show anti-D production after the first pregnancy.

TABLE V.—*Estimate of Incidence of Anti-D After First and During Second Rh-positive Pregnancies*

| Foetal Cell Score | No. | Anti-D Expected after First Pregnancy | No. Starting Second Pregnancy Without Anti-D | Anti-D Expected During Second Pregnancy | Total with Anti-D |
|---|---|---|---|---|---|
| 0 | 44 | 1·6 | 42·4 | 1·4 | 3·0 |
| 1–4 | 38 | 3·1 | 34·9 | 5·8 | 8·9 |
| 5+ | 18 | 3·8 | 14·2 | 1·3 | 5·1 |
| Total | 100 | 8·5 | 91·5 | 8·5 | 17·0 |

The first pregnancies are assumed to be ABO-compatible in all cases and the numbers in the columns are an estimate of the outcome in 100 Rh-negative women.

## First Pregnancy ABO Incompatible

Seventy-eight mothers have been followed through a second pregnancy. In 16 instances the second baby was Rh-negative, and no antibodies developed. Two mothers developed anti-D after the first pregnancy (see above). Of the remaining 60 mothers two developed anti-D during the second Rh-positive pregnancy, an incidence of 3.3%. In both instances the mother was group A, the first baby was group AB, and the second baby was group A. One of the infants required transfusion. It can be estimated that for every 17 mothers who have an ABO compatible first baby and who develop anti-D by the end of the second Rh-positive pregnancy, there is one mother who does so after an ABO-incompatible baby.

## Rh Groups of Pregnancies after Immunization

The data above suggest that 8.5% of Rh-negative mothers develop anti-D after the first ABO-compatible pregnancy. One would thus expect that of the 246 mothers followed through the second Rh-positive pregnancy 20 would have already developed anti-D after the first pregnancy. In fact, only 10 such cases were found. A further 10 mothers with anti-D after the first pregnancy had Rh-negative second pregnancies. This apparent deficit of Rh-positive second pregnancies in mothers with anti-D is not significant and may disappear as the study continues. There is no evidence that the general incidence of abortion is higher in women with anti-D, and no bias in terms of follow-up or planning of pregnancy by these mothers is likely (the mothers were not told they had developed anti-D).

## Papain Antibodies

In some cases specific anti-D was found with a papain technique when saline, albumin, and indirect Coombs tests were negative. In 14 instances this type of antibody appeared after the first pregnancy, and in one instance it was first detected during a second Rh-positive pregnancy. It is generally agreed that this type of Rh antibody does not itself result in haemolytic disease. However, there is presumably a risk that a disease-producing antibody will appear during a subsequent pregnancy. It is of interest that in the two instances in which a second Rh-positive pregnancy occurred in women with these antibodies the anti-D became detectable by albumin and indirect Coombs tests by the end of the pregnancies and the babies were clinically affected. Conversely, there were three instances where the second pregnancy was Rh-negative, and in two of these the anti-D remained unchanged. One of these two patients was followed through two Rh-negative pregnancies over a period of three years, and the anti-D remained quite unchanged in its behaviour. In the third patient a papain anti-D was first found three months after the first delivery. It was unchanged three months later, but during a second Rh-negative pregnancy, two years later, no anti-D could be found.

## Sex of Foetus and Rh Immunization

Because of the finding of Renkonen and Timonen (1967), which suggested that Rh-negative mothers were more likely to be immunized by a male than by a female foetus, an analysis was carried out of the sex ratio of the firstborn infants in the present series in relation to anti-D production. Though the sex ratio was 1.5 : 1 (37 males to 25 females) in those first pregnancies which resulted in antibody production in subsequent months, the difference is not statistically significant. The sex ratios of immunizing foetuses in the two series of Renkonen and Timonen were 1.44 and 1.74 respectively, and it is of interest that the present survey is showing a similar trend in this respect.

The above authors suggested that the excess of males among immunizing foetuses might be because more foetal cells crossed into the maternal circulation in the case of male pregnancies. The distribution of foetal cells scores after delivery was looked at in relation to the sex of the corresponding first baby, and no difference was found.

## Discussion

The current concept of the natural history of Rh immunization by pregnancy is based on a modification of that put forward by Nevanlinna (1953). He pointed out that the pregnancy during which Rh antibodies are first detected is not as a rule the pregnancy providing the primary stimulus. Where antibody is present at the beginning of a second Rh-positive pregnancy or appears during it, the primary stimulus must have come from the first pregnancy. However, Nevanlinna greatly underestimated the frequency with which antibodies appear after a single pregnancy. It is now apparent that as the result of the first Rh-positive pregnancy Rh antibodies may appear in the postpartum period or priming may occur without antibody being detected. This latter state of immunity is recognized in the general field of immunology (Barr and Llewellyn-Jones, 1951 ; Uhr and Baumann, 1961), but there is no agreed simple terminology to describe it. It is thought to be associated with replication of the corresponding clone of lymphocytes but with no differentiation and antibody release (Leduc, Coons, and Connolly, 1955 ; Siskind, Dunn, and Walker, 1968). The proliferated cells act as " memory cells," and with a very small secondary antigenic stimulus differentiate and rapidly produce antibody. This state can be demonstrated in Rh-negative volunteers by the fact that in some cases, following the injection of Rh-positive blood, anti-D may not be detectable, but a small volume of injected Rh-positive cells is very rapidly eliminated (Woodrow, Finn, and Krevans, unpublished observations). The serological complexities which may be involved are illustrated by the following case.

A volunteer aged 70 years was tested for the presence of Rh antibodies; three different Rh-positive cells were used and the results were negative. Five millilitres of Rh-positive cord blood was given intravenously, and a foetal cell count two days later showed that all the injected cells had disappeared. The pre-injection serum was retested and reacted by a papain technique with the injected cord cells and with 2 out of 30 further samples of Rh-positive blood. The 48-hour sample of serum reacted with 15 of the 30 Rh-positive blood samples. Five days after the injection a strong anti-D reacting in saline and by indirect Coombs tests was found. The man, then requestioned, described having had a small blood transfusion for a severe shoulder injury in France 50 years previously.

It is presumed that priming may occur as a result of the first pregnancy, and that a very few cells passing over during a second or latter Rh-positive pregnancy are sufficient to cause the rapid appearance of anti-D. This concept is crucial to the prevention of Rh-haemolytic disease, for if priming by the first pregnancy can be stopped then most, if not all, antibodies due to appear before the end of the second pregnancy should be prevented. The present study shows that of the 17% of Rh-negative mothers who are immunized by the first ABO-compatible pregnancy half will show anti-D during the subsequent months and the remaining half not until the second Rh-positive pregnancy.

## Immunization and Foetal Cell Score

There is clearly a relation between the count of foetal cells after the delivery of the first Rh-positive ABO-compatible baby and the appearance of anti-D in subsequent months. It is realized that a count of foetal cells at one point in time does not necessarily indicate the actual volume of stimulus the mother may have received during the pregnancy. Evidence has been previously presented showing that foetal cells may pass into the maternal circulation during the last trimester (Woodrow and Finn, 1966), and in these instances the foetal cell score after delivery is likely to be lower than a count taken some weeks before. This is particularly so if the process of immunization has already started so that a rapid disappearance of foetal cells will occur. However, the previous studies make it likely that in many cases the foetal cell score after delivery does indicate the actual volume of foetal blood providing the antigenic stimulus. It is remarkable how small this score has been in mothers who have developed anti-D. It seems that anti-D may appear after a stimulus of less than 0.1 ml. of Rh-positive blood. This supports the experimental findings of Zipursky *et al.* (1965), who were able to immunize Rh-negative volunteers with repeated injections of 0.1 ml. of Rh-positive blood.

Knowing the technical limitations of the method used and the relative crudity of the method of obtaining the foetal cell score, we find it interesting to note how the percentage of mothers showing anti-D six months after delivery rises as the estimated volume of blood rises in stages from 0.04 to 0.4 ml. It could be that there is a group of women who are very sensitive to Rh-positive blood, and that the dosage in this group is critical. The curve in the Chart appears to flatten out with stimuli above 0.4 ml., and this suggests a more resistant group of Rh-negative women requiring increasing volumes of Rh-positive blood to immunize them. On the other hand, it may be that it is largely a matter of chance which mothers are immunized by the smaller volume of Rh-positive cells. Development of anti-D may depend on a certain number of contacts being made by D antigen sites with potential antibody-producing cells, and where the number of antigenic molecules is small chance could play a considerable part in the outcome.

The results relating to the development of antibodies in the second pregnancy are in contrast with the above findings. Anti-D appeared at this time most often when the foetal cell score after the first delivery was low (1–4). Because mothers showing only a few foetal cells are relatively numerous the end-result is that approximately 70% of the instances of antibodies present at the end of the second pregnancy are associated with small stimuli in the first pregnancy as judged by a foetal cell score after delivery of 0–4. This finding agrees in some degree with that of Zipursky and Israels (1967), who found that most of the women who produced antibody after delivery had less than an estimated 0.1 ml. of foetal blood circulating at delivery. Their interpretation was that immunization often resulted from small amounts of foetal blood entering the maternal circulation during pregnancy. There is little direct evidence that this is a common way in which primary immunization occurs, and it is likely that small haemorrhages late on in pregnancy or during labour are a more common cause of immunization or priming. This is supported by the excellent results of giving anti-D *after* delivery. Moreover, the rarity with which antibody appears during a first pregnancy speaks against transplacental haemorrhage in the antenatal period being a common cause of *primary* immunization. In the present study only seven instances of anti-D present at the end of the first pregnancy were encountered.

The above results are interesting from a theoretical point of view. Sterzl (1966) and Siskind *et al.* (1968) have shown that the dose of an antigen has an important role in controlling the character of the immune response. They produced evidence that large antigenic doses may result in good antibody production but in poor preparation for a second response, in contrast with small antigenic doses which can produce poor or undetectable primary antibody response and yet induce good preparation for a secondary response. The present findings fit with this reasonably well.

## Relevance of Results to Prevention of Rh Immunization

The current practice in clinical trials aimed at testing the effectiveness of anti-D gammaglobulin in preventing Rh immunization is to give the gammaglobulin immediately after delivery of an Rh-positive infant. In most cases only ABO-compatible pregnancies are included. In some centres multiparous as well as primiparous mothers have been treated. The aim is to prevent the appearance of anti-D both in the subsequent months and during the next Rh-positive pregnancy. The failure rate so far is extremely low, and this is perhaps a remarkable result taking into account the facts that multiparous mothers have been included in some centres, and that the dosage and strength of the anti-D preparation used have varied from centre to centre. The data presented here give an idea of the risk of immunization for the different groups of patients. Thus if, because of limited supplies of gammaglobulin, it were decided initially to treat mothers with 0.2 ml. or more of circulating blood after the first delivery, then it can be anticipated that about one-third of the instances of anti-D expected to occur by the end of the next Rh-positive pregnancy will be prevented.

ABO-incompatible pregnancies are shown to be responsible for one in 35 instances of primary immunizations resulting from first pregnancies. It is likely that these can also be prevented by giving anti-D, but this has not yet been proved. The low risk for such mothers would suggest that prophylactic therapy for them should be offered when supplies of anti-D are more liberal.

What constitutes a proper dosage of anti-D gammaglobulin is a question not yet completely solved. The evidence here presented suggests that Rh-negative mothers are receiving volumes of blood ranging from less than 0.1 ml. to more than 150 ml. of Rh-positive, and transplacental haemorrhage of 350 ml. has been recorded (de Wit and Borst-Eilers, 1968). If one were to use an anti-D of a constant affinity then presumably

the preventive dose of anti-D would vary with the volume of foetal blood in the mother. If a standard dose were used it might not be sufficient for all cases, and too small a dose might lead to enhancement of anti-D production (Clarke *et al.*, 1963 ; Pearlman, 1967 ; Pollack *et al.*, 1968). On the other hand, a dose which was sufficient for the largest transplacental haemorrhages would entail considerable wastage.

## Situation in Multiparae

It is likely that following the delivery of the second Rh-positive baby the natural history of Rh immunization is similar to that documented here, though it might differ in detail. Three factors are worth considering here: (*a*) There might be a different incidence of transplacental haemorrhages in later pregnancies. Our general experience is against this, and Sullivan and Jennings (1966) found no evidence of it. (*b*) The more sensitive mothers might have been already immunized, and thus mothers reaching the end of the second and later Rh-positive pregnancies without antibodies might include an undue proportion of resistant cases. (*c*) Priming may have resulted from a previous pregnancy, and it might be more difficult to prevent immunization in such mothers.

What is uncertain is whether a mother primed by a first pregnancy can experience a subsequent Rh-positive pregnancy and yet reach the end of it without developing antibodies. It may be that virtually all such mothers develop antibodies during the second pregnancy, and that mothers who reach the end of a second or later Rh-positive pregnancy and have no antibodies cannot have been primed and can therefore be protected to the same degree as primiparous patients. Certainly the very low rate of failure in multiparous mothers treated at various centres suggests that this may be true. The answer to this question will become apparent with further follow up of pregnancies subsequent to the administration of anti-D.

This work has been carried out in parallel with the other Liverpool studies relating to the prevention of Rh haemolytic disease, and we would like to thank Professor C. A. Clarke for his advice and encouragement.

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## Appendix. Technique Used (In Liverpool) for Obtaining Foetal Cell Scores

Venous blood is collected in Sequestrene and diluted with two volumes of saline. Two thin blood films are then made, and, after drying, the smears are fixed in 80% ethanol for five minutes and then eluted at 37° C. in a sodium phosphate/citric acid buffer at *p*H 3.3. After washing and drying, the films are stained in Ehrlich's haematoxylin for three minutes and counterstained in 2.5% watery eosin for two minutes.

On each of the two slides thus prepared 50 low-power fields (diameter 940 $\mu$) are scanned for foetal cells, and the average for the two slides is the foetal cell score. The mean number of maternal cells included within the 50 fields is approximately 150,000.

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