because they seemed to recover in two or three days, had their symptoms attributed by parents to some dietary indiscretion. The fact that so many children in this home fell ill in so short a time, immediately suggested that this outbreak was of an infectious nature. During a period of 16 days 19 cases occurred, 13 of them in the space of six days, but an accurate incubation period cannot be deduced from these figures. So far as the children were concerned, the numbers were too small to detect whether age or sex was associated with susceptibility to the virus.

Roller towels were in use by the children, and it is possible that the infection was transmitted in this way. The adults, on the other hand, had their own toilet and washing facilities and so were less liable to this form of contamination. The two adults who were affected were mother and daughter, and the young son of this family was also a case. It is therefore a possibility that transmission could have taken place via a towel, W.C. seat, chain-pull, etc., in this house. On the other hand, the father and other son, who were also similarly exposed, were not affected.

Examination of stool specimens suggested that on the average the period of infectivity ceases a fortnight after the patient becomes afebrile, although the two adults affected remained positive for eight and nine weeks respectively after clinical defervescence. With such small numbers it is impossible to state categorically that adults remain positive excretors for longer than children.

Summary

An outbreak of an enterovirus infection in a children's home is described.

Of a total of 27 children, 17 showed clinical signs of infection; and of 10 adults connected with the home two were affected. The 19 cases occurred in a 16-day period, 13 of them within the space of six days.

The most common presenting symptom was abdominal pain, although frontal headache, alone or associated with abdominal pain, was fairly common. The average temperature was 100° F. (37.8° C.) and the duration of pyrexia ranged from two to six days.

E.C.H.O. type 9 virus was isolated from the faeces of 12 of the 19 subjects who developed clinical illness. Serum from 14 of the 19 cases was examined, and antibodies were present in all 14.

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EXPERIMENTAL STUDIES ON THE PREVENTION OF Rh HAEMOLYTIC DISEASE

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ABO Incompatibility

In the context of haemolytic disease of the newborn, ABO incompatibility means that the father's blood is unsuitable for transfusion into the mother, and our interest in this stems from the work of Levine (1943), who noted a deficiency of such mating types in the parents of affected children. He deduced, therefore, that ABO incompatibility affords a degree of protection against Rh haemolytic disease, and this has been confirmed by many workers. Of particular interest are the experiments of Stern *et al.* (1956), who showed that male volunteers could be much more easily sensitized to Rh if the injected blood were ABO compatible.

Nevanlinna and Vainio (1956) emphasized the importance of the sensitizing foetus and showed by means of a family study that once sensitization had occurred there was no evidence that ABO incompatibility between mother and foetus afforded any further protection. We carried out a somewhat similar survey in order to determine the ABO group of the Rh-sensitizing foetus, and in 14 ABO incompatible matings in which the sensitizing foetus could be determined with certainty its ABO group was always compatible with the mother (this can happen because of heterozygosity in the father—for example, a group O woman married to an AO man can produce a group O child). The probability of our results being found by chance alone was less than 1 in 500 (Clarke *et al.*, 1958). Nevertheless, the sensitizing foetus is not invariably ABO-compatible, and we have recently found one which is incompatible.

One of us (R.F.) has investigated a series of 85 families with Rh-negative mothers where four or more children have been born and yet no haemolytic disease or Rh sensitization occurred. In 22 the husband was also Rh negative, but in the remainder the percentage of ABO incompatible matings was 42.9% $\pm$ 6.23 compared with 33.6% in the general population (as judged by phenotype frequencies) and 22.0% $\pm$ 2.83 in the investigated families with haemolytic disease (Finn, 1961).

The most obvious explanation for the protection afforded by ABO incompatibility is that suggested by

Race and Sanger (1950). They point out that if Rh sensitization is due to foetal red cells entering the maternal circulation, it seems possible that they may be eliminated before they have time to act as Rh antigens—for example, foetal cells containing A antigen would be removed by a maternal serum containing anti-A.

Foetal Cells in Maternal Circulation

“Massive” transplacental haemorrhage from the foetal into the maternal circulation was first postulated by Wiener (1948), who described a probable case. Chown (1954) proved the point by means of differential agglutination and by tests for foetal haemoglobin, but neither of these techniques is sensitive enough to detect small foetal bleeds such as may be responsible for most cases of Rh sensitization. Kleihauer *et al.* (1957) described a method which is very suitable, the principle being the differential elution of adult and foetal haemoglobin. This is effected by means of a citric-phosphate buffer on an ordinary blood smear, the adult cells appearing as ghosts while the foetal cells stand out as dark refractile bodies (see Fig. 1). Zipursky

FIG. 1.—Large numbers of foetal red cells in the maternal circulation demonstrated by the phosphate elution technique.

et al. (1959) used this method to demonstrate foetal red cells in the blood of women after delivery. They studied 42 cases and detected foetal red cells in 11 (21%).

We decided to use a modification of this technique in a large series of Rh-negative women to find out: (a) the incidence of transplacental foetal bleeding, (b) the correlation between circulating foetal cells and the production of Rh antibodies, (c) the role of the placental barrier in protection against Rh sensitization, and (d) the time of foetal bleeding.

Technique

The Kleihauer technique has been used, the only modification being that the blood smear has not been stained. To establish its sensitivity, one of us (R.F.) examined blood from 50 men along with an equal number of specimens from post-partum women, the observer being unaware of the source of the material. Two smears from each of the subjects were made, and each was examined twice for three minutes, the number of refractile cells found being counted. Each individual

was given a quantitative figure (foetal score) by taking the average of the number of refractile cells counted in the four scans. No male score was found to exceed 0.5. To give a wide margin a figure of 2 or more has therefore been accepted as indicating definite transplacental foetal bleeding. In the application of the technique to the series of post-partum bloods from 206 Rh-negative women, only one smear was made and the foetal score is the average of two three-minute counts.

In order to arrive at a correlation between foetal scores and quantity of foetal blood in the maternal circulation, mixtures of adult and foetal blood were made at varying dilutions. Thus at 1 in 5,000 the score was 5 (SD ± 1.5). On the assumption of a maternal blood volume of about 5 litres, a score of 5 would therefore represent about 1 ml. of foetal blood in the maternal circulation.

Incidence of Transplacental Haemorrhage

Examination has been made of 256 specimens of post-partum blood, all the patients being unselected for ABO group and for parity. The first 50 were also unselected for Rh, but the remainder were all Rh-negative. The only other selection was that none of the women had a history of haemolytic disease nor any demonstrable Rh antibody. Table I shows the

TABLE I.—Foetal Scores in 256 Mothers After Delivery

Foetal Score	No. of Women
0	205
1	21
2	7
3	5
4	5
5-9	7
10-19	2
20 and over	4
Total	256

results of the foetal cell scores. It can be seen that foetal bleeding, as judged by our definition, has been detected in only 30 (11.7% ± 2.01) of the women. The blood groups of the baby were tested in 135 of the Rh-negative women. In none of the 21 cases in which foetal cells were detected in the maternal system was the foetus incompatible with the mother on the ABO system. In the 114 cases in which no foetal cells were detected in the mother the foetus was incompatible with the mother on the ABO system in 26 cases. These two groups are significantly distinct ($P=0.013$). Clearly, 11.7% is an underestimate of the frequency of foetal bleeding, since the 256 specimens examined must have contained a number of incompatible foetuses, and the presence of bleeding is almost certainly not detectable in such cases.

In most of the cases in which foetal cells were detected—that is, scores which were above 2—the quantity of foetal blood in the maternal circulation seems to be 1 ml. or less. Our data do not reveal any definite division into small and larger bleeds, but rather progressively smaller numbers of women with increasing quantities of foetal blood. Only four women had foetal scores indicating that they had received about 5 ml. or more of foetal blood. It would appear, therefore, that about 2% of women have what might be called “large” transplacental foetal haemorrhage (the bleeds of 50–100 ml., which cause anaemia of the infant, being called “massive”).

Correlation of Circulating Foetal Cells and Rh-antibody Production

We have followed up 85 of the Rh-negative women who had Rh-positive foetuses, and tested their serum for antibodies two or three months after delivery. The series is not random, in that it includes as many women as possible in whom foetal cells had been detected. The results are shown in Table II. It will be seen that of the

TABLE II.—*Transplacental Haemorrhage and the Development of Antibodies in the Puerperium*

Foetal Score	No. of Women	Antibody Present
Less than 2	75	1
2-19	7	—
20 and over	3	2
Totals	85	3

75 whom we had scored as having had no transplacental bleed, only one has developed an antibody. This, curiously enough, is an anti-C, even though the baby's Rh genotype was CDe/cde, and repeated testing has failed to reveal any anti-D. Of the women with "small" bleeds (foetal scores 2-19) none has developed an antibody. Of the three women scored as having had a "large" bleed, two have developed anti-D, active both in saline and in albumin, while the third has produced no antibody. There is a significant association between "large" foetal bleeds and antibody formation ($P=0.025$).

Role of Placental Barrier in Protecting Against Rh Sensitization

There are various factors which can play a part in preventing Rh-negative women mated to Rh-positive men from developing Rh antibodies. Our results suggest that the placental barrier is the most important, always provided that foetal bleeding is necessary for maternal sensitization. If this be so our data imply that about 85% of women are thus protected by the placental barrier. Two other known factors—ABO incompatibility in the mating and Rh heterozygosity in the husband—are much less important, though by no means negligible in a small-family society such as is found in this country. There is also the possibility that immunological tolerance plays a part in a small number of cases (see Race and Sanger, 1958).

Time of Foetal Bleeding

It seems probable that most though not all (see Discussion and Weiner *et al.*, 1958), foetal bleeds occur during labour. We are examining the blood of women at the 36th week of pregnancy to determine the precise incidence of ante-partum transplacental haemorrhage.

Experimental Protection Against Rh Sensitization

The weight of evidence given above strongly supports the hypothesis that circulating foetal cells are necessary to cause Rh sensitization. This raises the possibility that Rh sensitization might be prevented if such foetal cells could be destroyed or their antigen sites blocked. The mechanism by which ABO incompatibility protects, and the agglutination of foetal cells which we effected *in vitro* in a case of "massive" foetal bleeding (Finn *et al.*, 1950), made us consider methods which could be carried out *in vivo*. We decided to inject radioactive-chromium (Cr-51)-tagged Rh-positive cells into a number of Rh-negative male volunteers, and shortly afterwards to give half of them anti-D serum. Initially, only six men were used in order to give us information

on whether (a) the Rh-positive cells become coated with anti-D, and (b) the Rh-positive cell survival time was the same in the two groups. The results of this experiment are described here.

We are also studying whether or not Rh sensitization is similar in these six men and in a larger series injected later.

Method

The volunteers were male blood donors who were fully informed of the nature of the experiments. They were all of blood group A cde/cde. The donor of the Rh-positive blood injected into the men had previously given 8 pints (4.6 litres) of blood that was believed never to have caused jaundice in any of the recipients. His blood groups were A CDe/cde. Cross-matching tests between these cells and the volunteers' sera has not revealed any incompatibilities.

The donor was bled two days before the experiment, and this blood was tagged with Cr-51 on the day of the experiment. The amount of the Rh-positive blood injected intravenously was 5 ml., this volume being decided upon because it was known to be large enough to cause Rh sensitization, and yet not so large as to require a large volume of anti-D; it was also consistent with the amount concerned in the sensitization of the two women in our investigation who produced anti-D.

The anti-D serum had a titre with the donor's cells, both in saline and in albumin, of 1 in 64. We injected 10 ml. of it intravenously into three of the men chosen at random, half an hour after the Rh-positive blood injection.

Samples of blood were withdrawn from each of the six men, 2, 5, and 14 days later, and the following tests carried out.

1. The proportion of radioactive cells was estimated.

2. Samples were tested by the technique of Jones and Silver (1958) for the detection of minor cell populations. The method, as applied here, is to add incomplete anti-D to the blood so that any D-positive cells (but not D-negative cells) persisting will be coated with the antibody; the cells are then washed to remove excess antibody. Next, anti-human globulin (A.H.G.) is added, and this will attach itself to the antibody coating the D-positive cells. Because any D-positive cells will be sparsely distributed, agglutination (as in the indirect Coombs test) cannot occur. The cells are now washed to remove free A.H.G., and an equal volume of D-positive cells coated with incomplete anti-D are added. These will attach themselves to the anti-D plus A.H.G. coated cells to give "rosettes," each indicating the presence of a single original D-positive cell in the D-negative population. It is possible to count the number of rosettes per unit area and compare the figure with the number of unagglutinated cells (presumably the method could also be used for detecting an extremely weak anti-D).

3. Samples were tested as above, but omitting the initial treatment of the cells with incomplete anti-D. It will be appreciated from the method that no rosettes will be found unless the cells have been coated with anti-D *in vivo*.

4. Samples were tested for the presence of anti-D in the serum in saline, in albumin, and by the indirect Coombs test.

Results

Table III and Fig. 2 show the remaining percentage radioactivity at various times after the injection of the labelled cells. It will be seen that there is a great difference in the proportion of cells remaining in the circulation according to whether antibody had or had not been given. The administration of antibody seems at least to halve the number of circulating cells in the

first two days, but thereafter the differential decline is less pronounced. The difference between the two groups in the first sampling is highly significant ($P = < 0.02$).

Table IV gives the number of rosettes—that is, Rh-positive cells—counted at various times after the initial injection. Even though this test is qualitative and of unknown precision, it will be seen that the numbers are less in the volunteers who had received anti-D than in those untreated.

Table V gives the results of tests carried out as described in No. 3 above (in which the initial treatment with incomplete anti-D is omitted). It will be seen that rosettes were present only in those persons who had received the anti-D injection. This demonstrates conclusively that at any rate a good proportion of the Rh-positive red cells have been coated by the anti-D administered 30 minutes after the injection of the tagged red cells.

None of the volunteers' sera contained detectable anti-D, even though three of them had been injected

TABLE III.—Percentage of Cr-51-labelled Cells Remaining at Various Times After Their Injection

Volunteers	2 Days	5 Days	14 Days
Unprotected	106 94 98	88 85 87	69 67 77
Anti-D given	48 40 13	33 30 6	22 21 4.6

FIG. 2.—Remaining percentage radioactivity at various times after injection of 5 ml. of Rh-positive blood tagged with Cr-51 into 6 Rh-negative male volunteers.

TABLE IV.—Number of Rosettes—that is, Rh-positive Cells—Per Unit Volume of Recipient Blood at Various Times After Injection of 5 ml. Rh-positive Blood

Volunteers	2 Days	5 Days	14 Days
Unprotected	10 12 11	8 9 9	9 8 9
Anti-D given	8 9 9	5 6 4	5 5 4

TABLE V.—Number of Anti-D Coated Rh-positive Cells Per Unit Volume of Recipient Blood at Various Times After Injection of 5 ml. Rh-positive Blood

Volunteers	2 Days	5 Days	14 Days
Unprotected	0 0 0	0 0 0	0 0 0
Anti-D given	4 2 5	Not counted " "	4 4 4

with it two days previously. This was to be expected in view of the dilution of the anti-D by the recipient's serum.

Discussion

The results of our investigation into ABO incompatibility and foetal cells in the maternal circulation lend further weight to the hypothesis that sensitization against the D antigen is caused in most cases by circulating foetal cells in the maternal system. The results also suggest that if these cells were destroyed rapidly, as they are when the cells are incompatible with the mother on the ABO system, sensitization would be avoided. It therefore seems obvious that a way to prevent sensitization in an ABO-compatible mother would be to destroy the foetal cells rapidly by some other means. Since the cells are by definition D-positive, the injection of anti-D would be the first thing to try.

Our results show quite conclusively that the administration to Rh-negative male volunteers of 10 ml. of a particular anti-D has, firstly, coated at least a good proportion of previously injected Rh-positive cells, and, secondly, as judged by radioactive tagging, caused the elimination of at least 50% of them in two days. These results are very encouraging, particularly in view of two previous investigations. Thus, Jandl *et al.* (1957) showed that red cells fully coated with incomplete anti-D *in vitro* were rapidly removed from the circulation by the spleen, and that the proportion of cells destroyed depended on the completeness of the coating. Again, Stern and Berger (1960) have demonstrated that if Rh-positive cells are coated with anti-D before injection into Rh-negative men the formation of antibodies is prevented.

If rapid elimination of all foetal cells is necessary to prevent sensitization, our 50% destruction may mean that we did not give enough anti-D, and that a second dose was needed. On the other hand, most of the remaining Rh-positive cells have picked up antibody and have some blocking of their antigen sites, and this, together with the 50% destruction, may have been enough for complete protection. The matter is somewhat complicated because we used an antiserum which contained both complete and incomplete antibodies, and it is obviously necessary to try antisera of the incomplete type only before deciding on the most efficient type of anti-D. For protection with different antisera different doses may be needed.

The 5 ml. of Rh-positive blood which we injected into the volunteers introduces fewer red cells than the 5 ml. of foetal blood which we took as our dividing line between "small" and "large" foetal bleeds because of the higher number of cells per unit volume in foetal blood. If we are successful in reducing or eliminating sensitization in the three protected male volunteers, it would be important to give cells of different Rh-positive genotype because of the known differences in their ability to lead to haemolytic disease. Furthermore, the question of using foetal blood will have to be considered.

An important problem remains unsolved. We are uncertain what proportion of foetal bleeds occur before labour, and until we have more evidence on this point it is impossible to know how many women would be protected from sensitization by the giving of anti-D after delivery. The case in which an antibody was produced without our having detected any foetal cells in the mother's post-partum blood may possibly have

been associated with foetal bleeding many weeks before term. On the other hand, the case was anomalous in that anti-C without any anti-D was produced in spite of the foetus being of Rh genotype CDe/cde. We are unconvinced by the paper of Taylor and Kullman (1961) who, using the Kleihauer technique, found 18% of 40 ante-partum mothers and 17% of 58 post-partum mothers to be positive for foetal erythrocytes. Kleihauer *et al.* (1960), using a modification of their original technique, report that in 111 pregnancies, foetal cells were found before delivery in 37% and after in 71%. In the majority, however, they found only one foetal cell per 5–20 million maternal cells—that is, at dilutions which we would score as “no foetal bleed.”

Despite the unsolved problems, we feel that the signs are very hopeful, though the final proof of the feasibility of protection will have to await the results of tests for sensitization in these first six and many other volunteers. Waiting for antibodies to appear is inefficient, and a way of detecting sensitization may be to give, after about two months, a small provocative dose of Rh-positive cells to all the volunteers, whether they have received anti-D or not. Alternatively, repeated injections of Rh-positive blood could be given, the protected volunteers each time receiving further anti-D.

Should these experiments lead to an efficient method for preventing Rh sensitization, there ought to be no difficulty in detecting the women who need it, since the technique of screening for the presence of circulating foetal cells could be applied in any hospital laboratory. If it can be shown that incomplete anti-D is fully effective, the numbers of women requiring prophylaxis (about 2% of all pregnancies if all detectable foetal bleeds were treated) are not likely to be too large for available supplies of antiserum.

Summary

The various factors responsible for the occurrence of Rh haemolytic disease in only 1 in 20 of the families at risk are discussed. Of these we have investigated ABO incompatibility and the ability of the placenta to prevent the passage of foetal cells into the maternal circulation.

Our data give further support for the importance of ABO incompatibility and for the view that Rh sensitization is caused by the circulation of foetal red cells in the mother. Using a simple slide technique, we have found foetal cells in the blood of 11.7% of 256 post-partum women. It is estimated that most of the foetal bleeds had been of 1 ml. or less, but that in about 1.5% it had been “large”—that is, 5 ml. or more.

Rh antibodies developed in two of three women who had had “large” foetal bleeds and in 1 of 75 women in whom no foetal cells had been detected. This association of antibody formation with “large” foetal bleeds is statistically significant, and we conclude that the placental barrier is the most important of the factors preventing Rh sensitization.

Foetal cells were never found when the foetus was ABO incompatible with the mother, and ABO incompatibility probably protects because the foetal cells are rapidly eliminated by maternal anti-A or anti-B. It seemed possible that circulating Rh-positive foetal cells might be similarly eliminated by the administration of anti-D, thus preventing Rh sensitization of the mother.

To test this hypothesis we have injected Rh-positive blood tagged with Cr-51 into six Rh-negative male

volunteers, and given three of them 10 ml. of anti-D intravenously 30 minutes later. In the men not given anti-D the Rh-positive cells have survived normally, whereas in the three who received the antibody at least 50% of the injected cells had disappeared within two days. Using a modification of the technique of Jones and Silver (1958) for the detection of minor cell populations, we demonstrated that a proportion of the surviving foetal cells had been coated with the anti-D. This blocking of the antigen sites may protect from sensitization, and we are studying the men to see if the blocking and the 50% destruction prevent the production of Rh antibodies. On the other hand, a larger dose of anti-D will be needed if 100% elimination of foetal cells is necessary.

Our results suggest that it may be possible to prevent most cases of Rh sensitization, and thus in time eliminate Rh haemolytic disease. There remain, however, several problems, the chief being that of the women into whom the foetus bleeds before delivery.

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ADDENDUM.—A further 12 volunteers have been studied, and the radioactivity results confirm that about 60% of the injected Rh-positive cells are removed from the circulation by the administration of anti-D. It is now apparent that the major part of the fall occurs within a few hours.

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