Papers and Originals

Prevention of Rh-Haemolytic Disease: Results of the Clinical Trial

A Combined Study from Centres in England and Baltimore*†

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Finn (1960), in Liverpool, first put forward the idea that it might be possible to prevent immunization of Rh-negative mothers by giving them antibody to destroy Rh-positive foetal cells. The suggestion derived from our earlier work on ABO incompatibility in relation to Rh immunization (Clarke *et al.*, 1958), and the progress of the research since then has been described in four main papers. In the first two (Finn *et al.*, 1961 ; Clarke *et al.*, 1963) we described experiments which were successful in preventing Rh immunization in Rh-negative male volunteers. The basis of the procedure was to remove rapidly from the circulation previously injected chromium-tagged Rh-positive red cells by giving high-titre anti-D as an infusion of plasma. Later Gorman *et al.* (1963) (see also Freda and Gorman, 1962 ; Freda *et al.*, 1964) suggested the use of anti-D gamma-globulin instead of plasma, and since then we have used gamma-globulin.

In our third paper (Woodrow *et al.*, 1965) we showed that injected Rh-positive foetal cells could be cleared from the circulation of Rh-negative women volunteers as effectively as the Rh-positive adult cells were cleared from the Rh-negative men. We also produced evidence to show that the majority of cases of appreciable transplacental haemorrhage occurred during labour or very shortly before it, and, moreover, the greater the number of foetal cells found after delivery the greater was the likelihood of subsequent immunization. In this third paper we also stated that we had begun a clinical trial, in collaboration with colleagues working in Sheffield, Leeds, Bradford, and Baltimore, and the preliminary results of this were reported in August of last year (Clarke and Sheppard, 1965). Subsequently we described experiments which indicated that 1 ml. of gamma-globulin might be as effective in giving protection against the development of antibodies as the 5 ml. which we had used up to then (Clarke *et al.*, 1966).

In the present paper we describe the clinical trial, which has demonstrated that, anyhow up to six months after delivery, the technique which was successful in protecting against experimental Rh immunization has also protected women from Rh immunization by their Rh-positive foetuses.

Materials and Methods

Design of the Clinical Trial

The fact that we found a correlation between the number of foetal cells in the maternal circulation after delivery and subsequent immunization had an important bearing on the way we designed our trial.

We knew we were able to choose by the post-partum foetal-cell count a group of patients who were at considerable risk of developing Rh antibodies. By using only these women in our treated and control groups we thought we should be able to detect a significant difference (if one existed) between the two groups much sooner than if we took all Rh-negative mothers with Rh-positive babies regardless of the foetal-cell count. Furthermore, we decided to limit the trial to primiparae in order to avoid any confusion due to possible effects of previous pregnancies, and thus we have included only those women whom we considered to be "high-risk" primiparae. In this way we made the best use of our limited supplies of gamma-globulin and kept this initial trial reasonably small so that in the event of any adverse effects the minimum number of people would have been treated.

The organization has been similar in all five centres, and the precise details of the Liverpool trial have been as follows. Five maternity units were visited in the morning of each week-day and a sample of blood was obtained from all Rh-negative women just delivered of their first baby. Films of these samples were eluted and stained and counts of the number of foetal cells in 50 low-power fields made (Kleihauer *et al.*, 1957 ; Woodrow *et al.*, 1965 ; Woodrow and Finn, 1966). Where the foetal-cell score was five or more, and where the corresponding cord blood was Rh-positive and ABO compatible with the mother, the case was included in the trial.

The aim was to treat alternate cases with 5 ml. of gamma-globulin containing high-titre anti-D (usually between 1 in 1,280 and 1 in 4,096 in albumin with R_2r cells) by intramuscular injection. However, for various reasons there has been considerable upset in the strict alternation of the cases. For instance, as we decided that the gamma-globulin should be given not later than 36 hours after delivery, all cases where the baby was born between Friday afternoon and Sunday morning have been used as controls and additional week-day deliveries treated to make the numbers equal. When gamma-globulin supplies have been short we have had runs of controls, and then compensated by treating several consecutive patients. Other difficulties encountered were delay in finding out the ABO group of the baby and the impossibility on occasion of reaching the patient within the time limit. Four patients refused treatment and were used as controls.

Nevertheless, despite these difficulties it will be seen that no large blocks of controls were unduly concentrated at any particular period of the trial. Even though a considerable proportion of the controls had to be chosen from the week-end deliveries, we are confident that there are no differences between

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the treated and control cases with regard to obstetric management. In fact, there is no significant difference between the mean foetal-cell scores for the combined control and the combined treated groups in the U.K. centres. (Mean square roots: 4.06 for controls and 4.511 for treated: S.E. of difference = 0.387, $P>0.2$). Similarly for the Baltimore data, where the results are expressed as volumes of foetal blood, the mean square roots are 0.802 for controls and 0.659 for treated, the S.E. of difference = 0.112, $P>0.2$.

When the trial was started in May 1964 it was calculated on the basis of our previous work that 70 treated and 70 controls should be observed for six months after delivery, since this would be enough to give a statistically significant answer regarding the efficacy of the treatment (see Clarke and Sheppard, 1965). It was realized, however, that final proof of the complete prevention of immunization would only be obtained after the effects of second pregnancies had been observed.

Kleihauer–Betke Technique for Detecting Foetal Cells

This is an acid elution technique which enables foetal cells to be demonstrated and counted among a population of adult red cells (Kleihauer *et al.*, 1957). The precise details of how we have used the test and the difficulties encountered with it have been described elsewhere (Woodrow *et al.*, 1965 ; Woodrow and Finn, 1966). Thus in approximately 2% of post-delivery samples many intermediately staining cells are found due to an increase in maternal Hb F, and it is then impossible to obtain an accurate foetal-cell score. Before this was appreciated three such cases were treated, but the post-injection blood films showed no change. These cases, and subsequent similar ones, have been excluded from our trial. In general, however, the technique has proved satisfactory and we had little difficulty in counting the foetal cells and selecting those cases with a count of 5 (or more), which we calculated was the equivalent of about 0.25 ml. of foetal blood. In the treated cases a foetal-cell count was usually carried out about 24 hours after the injection of the gamma-globulin, and in some patients this was repeated later to test further for the clearance of the foetal cells.

Tests for Antibody

In Liverpool, tests for Rh antibodies were made on samples of serum taken immediately after delivery and three and six months later. The sera were tested in the first instance against four different types of Rh-positive cells, and where a positive

TABLE I.—*Prevention of Rh-haemolytic Disease : Overall Results of Clinical Trial at Six Months or Later After Delivery*

Centre	Controls			Treated			
	No.	Immunized	Not Immunized	No.	Immunized	Not Immunized	Doubtful
Liverpool	40	10*	30	40	0	40	0
Sheffield	15	5	10	14	0	12	2†
Leeds	5	0	5	6	0	5	1‡
Bradford	4	0	4	5	0	5	0
Baltimore	14	4	10	13	0	13	0
Total	78	19	59	78	0	75	3

* One of these had no antibody at delivery but had anti-D six months later, detectable only by papain technique (titre 1/4). Retested 18 months after delivery: findings similar.

† Three months after delivery reaction positive by papain technique, demonstrable only in neat serum. Negative Coombs test. Similar findings, though weaker, at six and eight months.

‡ Tests negative three months after delivery. Presence of anti-D queried at 10 months, but all tests negative 14 months after delivery (see Table IV) for details.

Statistical analysis: Comparison of the combined control and treated groups for antibody production gives $P = 1{\cdot}73 \times 10^{-4}$ even if the three doubtful cases are regarded as immunized (one-tailed test).

TABLE II.—*Liverpool Series*

Controls

Serial No.	Date of Delivery	Foetal-cell Counts at Delivery	Cell Survival Where Known	Immune Antibody Production with Titres	
				3 Months	6 Months
				After Delivery	
280	24/5/64	56, 78	—	Nil	Nil
292	29/5/64 r	13, 11; 5, 11; 8, 4; 5, 4	—	,,	,,
307	6/6/64 w	17, 19	—	,,	,,
362	2/7/64 r	7, 6; 5, 8	—	Anti-D present ICT 1/8 (ins.)	Anti-D present Alb. 1/16 Sal. nil ICT 1/16 Pap. 1/8
403	17/7/64 w	16, 18; 19, 17	—	Anti-D present Alb. 1/2 Sal. 1/2 ICT 1/2 Pap. 1/8	Anti-D present Alb. 1/16 Sal. 1/2 ICT 1/64 Pap. 1/4
446	8/8/64 w	74, 46; 81, 55	—	Anti-D present (ins.)	Anti-D present Alb. 1/2 Sal. 1/4 ICT nil Pap. 1/4
469	14/8/64 w	68, 71	5, 5 (20/10/64)	Nil	Nil
486	28/8/64 w	8, 7	4, 2 (20/10/64)	,,	,,
509	16/9/64	12, 14	7, 9 (20/10/64)	,,	,,
551	6/10/64 r	7, 7; 10, 6	8, 6 (22/10/64)	,,	,,
567	18/10/64 r	18, 8; 15		,,	,,
575	25/10/64 u	7, 8; 11, 8	5, 6 (11/11/64)	,,	,,
589	1/11/64 w	10, 8; 9, 7	12, 7 (10/11/64)	,,	,,
610	16/11/64 c	18, 14; 29, 30	—	,,	,,
617	21/11/64 w	17, 37; 36, 32	—	,,	,,
638	1/12/64 c	7, 7	—	,,	Anti-D present Alb. 1/2 Sal. 1/2 ICT 1/4 Pap. 1/4
657	9/12/64 u	10, 15	—	,,	Papain anti-D antibody Alb. nil Sal. nil ICT nil Pap. 1/4
659	10/12/64 u	10, 8	—	,,	Nil
674	21/12/64	6, 4	—	Anti-D present (ins.)	Anti-D present Alb. 1/2 Sal. 1/2 ICT nil Pap. 1/4
698	8/1/65 g	18, 12	—	Anti-D present Alb. 1/4 Sal. 1/2 ICT 1/4 Pap. 1/2	Anti-D present Alb. 1/4 Sal. 1/2 ICT 1/8 Pap. 1/4
785	9/3/65	26, 32; 50, 41	—	Anti-D present Alb. 1/4 Sal. 1/2 ICT 1/2 Pap. 1/4	Anti-D present Alb. 1/2 Sal. 1/2 ICT 1/2 Pap. 1/2
798	13/3/65 w	27, 27; 30	0, 1 (4/5/65)	Nil	Nil
822	8/4/65	10, 11	0, 0 (25/5/65)	,,	,,
850	13/5/65	17, 17	—	,,	,,
868	27/5/65	5, 8; 11	—	,,	,,
869	29/5/65 w	6, 5; 6, 7	—	,,	,,
895	19/6/65 w	6, 3; 7, 8	1, 0 (2/8/65)	,,	,,
913	29/6/65 u	11, 8	3, 4; 6, 5 (5/8/65)	,,	,,
997	7/8/65	18, 20; 29, 26	1, 0 (1/10/65)	,,	,,
1042	6/9/65	23, 27; 25, 27	2, 0 (25/10/65)	Anti-D present Alb. 1/4 Sal. 1/4 ICT 1/8 Pap. 1/8	Anti-D present Alb. 1/4 Sal. Nil ICT 1/8 Pap. 1/8
1050	11/9/65 w	3, 7; 6, 5	1,0 (25/10/65)	Nil	Nil
1055	14/9/65	36, 38	4, 5; 7, 6 (19/10/65)	,,	,,
1061	17/9/65 w	16, 7	—	,,	,,
1068	18/9/65	9, 9	—	,,	,,
1080	26/9/65	8, 7; 8	—	Anti-D present Alb. 1/8 Sal. 1/8 ICT 1/4 Pap. 1/8	Anti-D present Alb. 1/8 Sal. 1/2 ICT 1/8 Pap. 1/8
1097	2/10/65	5, 6	—	Nil	Nil
1116	9/10/65 w	9, 14	1, 1 (15/12/65)	,,	,,
1140	23/10/65 w	27, 27	5, 5 (17/12/65)	,,	,,
1162	1/11/65	83, 44; 61	—	Anti Le^b	Anti Le^b
1221	8/12/65	72, 71; 69	27, 28 (10/1/66)	Nil	Nil

c = Cord blood group not available; baby grouped later. g = Gamma-globulin not available. r = Refused treatment. w = Week-end control.
u = Unable to go to hospital to treat. ins. = Insufficient serum for further tests.

result was obtained the sera were tested against a further panel of cells (R_1R_1, R_2R_2, R′r, R″r, $R_1^{w}r$, and three different rr samples). This panel included all blood-group antigens apart from the extremely rare ones. Activity in saline and albumin, and by Coombs and papain techniques, was tested for routinely. Similar procedures were carried out in the other centres.

Results

Table I gives the overall findings together with the statistical analysis, and Tables II–VI the details for the five centres. Certain differences between the centres are dealt with below. Others, such as differences in the apparent length of survival of passive antibody in the treated cases and in the assessment of specificity of induced antibodies in the controls, probably reflect differences in technique in different laboratories.

Interpretation of Results of Clinical Trial

It is clear that the final results of this trial extend and confirm our preliminary report and there is no doubt that anti-D gamma-globulin given soon after delivery is effective in preventing the development of immune Rh antibody in the subsequent six months. Taking the most favourable view—that is, when we compare the presence or absence of definite Rh immunization in the treated and controls—protection is complete (0 as against 19). However, it is possible that the two treated Sheffield women (Nos. 305 and 308) who still had antibodies eight months after delivery are in fact immunized, and, furthermore, the doubtful Leeds antibody (No. 13724/64) may indicate some unusual form of immunization rather than an error or the remains of the injected gamma-globulin. If these three cases are in fact failures, protection would still be of the order of 80% (3 as against 19). Nevertheless, passive antibodies can last longer than six months, and it is very unusual for immune antibodies, once formed, to disappear (though their titres may fluctuate), and we therefore feel that the three cases mentioned are in fact unlikely to be failures. However, we shall follow them up with great care and also the control whose antibodies were demonstrable only by the papain technique (Liverpool series, No. 657).

It can be seen that there is considerable variation between the five centres as regards the proportion of women in the controls who have developed antibodies, but statistical analysis shows that there is no significant heterogeneity between the centres.

It is encouraging that other clinical trials carried out in the U.S.A. (Freda *et al.*, 1966 ; Gorman *et al.*, 1966) and Germany (Preisler and Schneider, 1966) have produced similar findings to our own. The results of these, which are all based on tests for antibodies at six months or more after delivery, are shown in Table VII.

It is interesting to compare the proportion of immune antibodies found in the controls in the series of Pollack *et al.* (1966) (about 11%) with our own (about 24%). The explanation of this is that in the New York and California series all Rh-negative women delivered of Rh-positive ABO-compatible babies entered the trial, and the Kleihauer technique was not used. In our series, on the other hand, we treated high-risk cases only (foetal-cell count more than 5) and the higher immunization rate in our controls supports the view that the quantity of foetal cells detected after delivery *is* a guide to the degree of risk of immunization.

In the Freiburg series all Rh-negative women free of antibodies but regardless of parity and ABO group of the foetus, in whom one or more foetal cells were found in post-delivery blood films, were admitted to the trial. The inclusion of many low-risk cases as well as those with ABO incompatibility may account for the reduced incidence of antibodies among the controls. However, there may be other factors, and it will be interesting, when the full German data are published, to see the distribution of the foetal-cell scores, as it may well be that varying obstetrical procedures in different countries have a bearing on the frequency of Rh immunization.

From our earlier experiments with male volunteers and from a consideration of all the evidence on the timing of immunization we had anticipated that we ought to be able to protect about 75% of Rh-negative women, but we thought that our method might be too late for the remaining 25%. However, in the event the protection rate is probably very much higher. One reason for this may be that until the last few weeks of pregnancy transplacental haemorrhages large enough to cause immunization are much rarer than we had thought. In addition, we know (*a*) that Rh antibodies sometimes take six months or more to develop, and (*b*) that it is very unusual for a first Rh-positive baby to be affected. Yet another reason may be that, in contrast to the increase in titre of pre-existing antibodies which may occur in pregnancy, the primary immune response in the pregnant woman may be temporarily depressed as it is in some animals (Heslop *et al.*, 1954 ; Medawar and Sparrow, 1956 ; Anderson and Benirschke, 1964 ; Anderson, 1965), and if this

TABLE II.—*Contd.*

Treated

Serial No.	Date of Delivery	Foetal-cell Counts at Delivery	Foetal-cell Counts after Treatment with 5 ml. Gamma-globulin		Antibody Production	
			Approx. 24 hr. Later	Subsequent Counts at Stated Times	3 Months After Delivery	6 Months After Delivery
273	20/5/64	6, 7; 4, 3; 9, 13	2, 2	1, 0 (4 d.)	P	Nil
294	31/5/64	23, 28; 35, 24	N.T.	5, 3 (42 hr.) 2, 1 (7 d.)	,,	,,
355	29/6/64	24, 26; 30, 33	0, 0	—	,,	,,
414	23/7/64	78, 86	18, 24	2, 2 (4 d.)	,,	,,
419	26/7/64	3, 6; 5, 6	6, 6	—	Nil	,,
436	3/8/64	24, 26; 21, 29	N.T.	1, 1 (46 hr.)	P	,,
453	10/8/64	16, 10; 8, 10	1, 0	—	,,	,,
504	13/9/64	5, 4; 6, 5	4, 4	1,0 (3 d.)	Nil	,,
571	21/10/64	87, 96	5, 5	—	P	,,
583	29/10/64	3,5 ; 8, 11	2, 1	—	Nil	,,
587	1/11/64	8, 5; 5, 4; 5, 5	0, 0	—	,,	,,
619	23/11/64	54, 58	4, 2	—	,,	,,
656	8/12/64	16, 9; 10, 24; 24	4, 6	0, 0 (6 d.)	P	,,
665	13/12/64	4, 5; 6, 5	1, 0	—	,,	,,
668	15/12/64	34, 28	12, 12	—	Nil	,,
696	5/1/65	28, 30	0, 0	—	P	,,
767	24/2/65	67, 74	N.T.	2, 1 (36 hr.)		,,
775	1/3/65	40, 38	3, 5	—	,,	,,
781	8/3/65	4,9; 5, 10	2, 2	—	Nil	,,
782	8/3/65	7, 7	1, 1	—	P	,,
817	28/3/65	33, 30; 33	4, 10	2, 1 (3 d.)	,,	,,
820	31/3/65	7, 9	1, 2	—	,,	,,
840	26/4/65	20, 20	0, 1	—	,,	,,
844	3/5/65	31, 35	1, 2	—	,,	,,
884	10/6/65	13, 10; 13, 14	8, 7	0, 0 (48 hr.)	,,	,,
902	22/6/65	24, 15	0, 0	—	,,	,,
924	5/7/65	5, 8; 3, 4; 6, 5; 9	0, 1	—	,,	,,
979	28/7/65	82, 86; 81	N.T.	0, 1 (44 hr.)	,,	,,
992	4/8/65	123, 134; 116, 120	15, 12	1, 0 (48 hr.)	,,	,,
994*	6/8/65	5, 5	2, 2	0, 0 (48 hr.)	,,	,,
1013	20/8/65	15, 16	4, 6	0, 0 (48 hr.)	Nil	,,
1039	7/9/65	15, 16	2, 1	—	,,	,,
1058	15/9/65	48, 55	2, 1	—	P	,,
1120	12/10/65	4, 6; 6, 3; 6, 7	N.T.	0, 0 (48 hr.)	,,	,,
1143	26/10/65	6, 8	3, 4	—	,,	,,
1077	28/9/65	9, 8	0 ,0	—	,,	,,
1166	4/11/65	52, 79	5, 6	—	,,	,,
1187	24/11/65	6, 8; 6, 10	0, 0	—	,,	,,
1191	26/11/65	6, 6	0, 0	—	,,	,,
1211	3/12/65	6, 4; 5, 3; 8, 3	0, 0	—	,,	,,
1224	9/12/65	29, 27	5, 5	—	,,	,,

N.T. = Not tested.

P = A weak reaction in albumin and by the Coombs and Papain techniques, demonstrable only with neat serum and presumably due to *passive* antibody.

* After submission of this paper it was discovered that this patient was not Rh-negative but D^u-positive ($R^{u}r$). She is therefore omitted from Table I and replaced by case 1224. It is of interest that patient 994 showed no adverse effects from the injection of anti-D and that the Rh-positive foetal cells were removed. However, since it has been shown that D^u red cells take up only 7 to 25% as much antibody as ordinary D-positive cells, the foetal cells would preferentially absorb the injected anti-D and therefore be rapidly cleared.

Prevention of RH-haemolytic Disease: Clinical Trial. Liverpool Series

Each of the treated women was given 5 ml. of anti-D gamma-globulin intramuscularly within 36 hours of delivery. In all but five of the treated women a sample of blood was obtained approximately 24 hours after treatment to determine clearance of foetal cells and in some cases samples taken later than this were also tested. In some of the controls samples of blood were obtained at varying times after delivery to determine cell survival.

To determine the foetal-cell score the routine practice was for one observer to count each of two slides. On occasion other observers repeated the counts, and these results are also included.

is so red cells crossing the placenta will not often evoke an antibody response until after delivery. If such suppression of antibody response really does take place the exact timing of a transplacental haemorrhage would be of academic importance only.

The above considerations have a bearing on the interesting work of Zipursky *et al.* (1965), who, because they thought it likely that about 25% of patients become immunized during pregnancy, set out to protect this group by giving anti-D to the mother during pregnancy. An initial dose of 1 ml. of anti-D gamma-globulin was given in the last trimester, followed at three-week intervals by 0.4 ml. This treatment was carried out on 45 women at the time of the report, and 30 of them had produced healthy Rh-positive infants, none of whom were anaemic though two had a weak-positive Coombs test on the red cells (*Medical World News,* 1965 ; Zipursky, 1965). We have as yet no information on how far the treatment prevents immunization, but it appears to be a safe procedure. However, it would not often be necessary if the initiation of Rh immunization is usually suppressed until after delivery.

Second Pregnancies

It has been suggested that by giving anti-D all we are doing is preventing immune-antibody production but that some of the women are in fact "sensibilized" (see Nevanlinna, 1953) and that in second pregnancies immune antibodies will become

TABLE IV.—*Leeds Series*

Controls

Serial No.	Date of Delivery	Foetal-cell Counts at Delivery	Immune Antibody 3 Months after Delivery	Immune Antibody 6 Months After Delivery or Later
12818/64	18/10/65	9, 6	Nil	Nil
13465/64	31/10/65	5, 8	,,	,,
36228/64	25/11/64	5, 8	N.T.	,,
26709/64	4/3/65	11, 12	,,	,,
37991/64	3/4/65	100, 68	,,	,,

Treated

Serial No.	Date of Delivery	Foetal-cell Counts at Delivery	Score 24–48 hr. after Treatment with 5 ml. Gamma-globulin	Immune Antibody 3 Months after Delivery	Immune Antibody Six Months after Delivery or Later
18894/64	13/10/64	12, 6	2	Nil	Nil
13724/64	2/12/64	18, 20	6,6	,,	Doubtful*
28681/64	17/1/65	110, 100	20, 15	,,	Nil
6155/65	18/3/65	11, 8	0, 0	Anti-D present Alb. nil Enzyme positive (ficin and papain)	,,
4684/64	19/8/65	173, 117	75	Nil	,,
24286/65	1/9/65	26, 17	21, 20	,,	,,

N.T. Not tested.

* Although tests by the indirect antiglobulin technique suggested that a weak anti-D was present in this serum, further tests by Low's papain technique were negative and tests using ficinized cells gave inconclusive results. Unfortunately there was insufficient serum remaining of the specimen to confirm these results. Further samples at 14 months after delivery were tested both in Leeds and in Liverpool and found to be negative.

TABLE III.—*Sheffield Series*

Serial No.	Date of Delivery	Foetal-cell Score				Antibody Production	
		Delivery	24 hr	48 hr	3 mth	3 Months After Delivery	6 Months After Delivery
				Controls			
27	13/1/65	17	19	—	0	Anti-D present Sal. 1/2 Alb. 1/4 ICT + Enzyme pp +	Anti-C + D present Sal. w Alb. + ICT + Enzyme pp +
51	15/2/65	254	234	161	0	Anti-D present Sal. 1/2 Alb. 1/4 ICT w Enzyme pp +	Anti-C + D present Sal. nil Alb. 1/2 ICT + Enzyme pp +
76	17/3/65	6	5	4	0	Nil	Nil
129	10/5/65	6	5	3	0	,,	,,
167	13/6/65	15	15	15	0	,,	,,
176	27/6/65	18	13	15	0	,,	,,
203	21/7/65	12	10	10	0	,,	,,
230	28/8/65	5	6	6	0	,,	,,
254	9/9/65	5	4	4	0	,,	,,
265	20/9/65	17	23	25	0	Anti-D present Sal. nil Alb. w ICT w Enzyme pp +	Anti-C + D present Sal. 1/1 Alb. 1/2 ICT + Enzyme pp +
266	19/9/65	18	29	20	0	Nil	Nil
303	9/10/65	20	18	16	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w	Anti-C + D present Sal. 1/1 Alb. 1/1 ICT + Enzyme pp +
307	18/10/65	12	8	7	0	Anti-C + D present Sal. w Alb. w ICT w Enzyme pp +	Anti-C + D present Sal. 1/1 Alb. 1/1 ICT + Enzyme pp w
309	17/10/65	7	5	5	0	Nil	Nil
331	9/11/65	9	13	12	0	,,	,,
				Treated			
47	11/2/65	5	3	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w	Nil
54	17/2/65	7	4	0	0	Nil	,,
58	24/2/65	40	15	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w	,,
118	22/4/65	5	2	2	0	Anti-D present Sal. nil Alb. nil ICT w Enzyme pp +	,,
				Treated continued			
143	21/5/65	9	2	1	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp +	,,
170	18/6/65	10	2	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w	,,
197	13/7/65	7	0	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp +	,,
220	12/8/65	7	4	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp +	,,
278	27/9/65	14	5	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w	Anti-D presen Sal. nil Alb. nil ICT neg. Enzyme pp w (absent at 8 months)
279	27/9/65	54	33	7	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w	Nil
301	10/10/65	8	10	1	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp +	,,
305	13/10/65	10	5	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp +	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w (still present at 8 months)
308	18/10/65	8	5	0	0	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp +	Anti-D present Sal. nil Alb. nil ICT neg. Enzyme pp w (still present at 8 months)
315	20/10/65	22	7	0	0	Anti-D present Sal. nil Alb. nil ICT w Enzyme pp w	Nil

pp = Papainized panel. + = Present when tested with neat serum. w = Weakly present when tested with neat serum.

overt. It is likely that this state of affairs sometimes occurs naturally, the mothers being immunized by the first Rh-positive pregnancy even though no antibody can be detected in the post-partum period. Antibody may then appear late in the next Rh-positive pregnancy, probably as the result of a few foetal cells crossing the placenta.

We have studied six (untreated) primiparae who had a foetal-cell score of 5 or more after their first delivery, who were free of antibodies six months later, and who have produced an Rh-positive ABO-compatible second baby. Two of these six women developed antibody during the second pregnancy. If this small series is representative, then for every woman showing antibodies by six months after the first delivery approximately one other woman will develop them by the end of the second Rh-positive ABO-compatible pregnancy. This means that, untreated, about 50% of women in this category can be expected to have antibodies by the end of the second Rh-positive pregnancy. It is therefore important to note that six of the *treated* women in our trial have been delivered of normal ABO-compatible Rh-positive second babies, and all are free of antibody. Furthermore, although it is likely that the risk of antibody developing during the second pregnancy is somewhat lower for the New York and the Freiburg series, the fact that six treated women in the former (Pollack, 1966) and six in the latter (Schneider, 1966) have reached the end of the second Rh-positive pregnancy without showing antibodies is very encouraging. (In the German series, the six women had had bleeds with the first baby of 0.16, 0.06, 0.12, 0.1, 0.17, and 0.05 ml. respectively—that is, " small " bleeds.)

There is also experimental evidence to support the view that we are not merely suppressing antibody formation. In a Baltimore study in 1963 (not previously reported) we gave 5 ml. of Rh-positive blood to 13 men who had earlier been protected by anti-D after four successive Rh-positive blood infusions. In 12 cases the injected blood survived normally, which makes it unlikely that the volunteers were in a state of " sensibilization." (The thirteenth case showed moderately reduced red-cell survival of both Rh-positive and Rh-negative cells.) Furthermore, Freda *et al.* (1966) gave 14 Rh-negative male volunteers three injections of Rh-positive blood, the first two stimuli being followed by injections of anti-D gamma-globulin and the third one not. This third injection of 1 ml. of blood was given 10 months after the second injection, and in no case was it followed by anti-D production. These workers were therefore mimicking a subsequent Rh-positive pregnancy, and they concluded that the protection afforded by the two gamma-globulin injections was complete. Taking all the available evidence into consideration, we feel that it argues strongly against the view that treatment with anti-D merely suppresses the appearance of immune antibody until the next pregnancy.

TABLE V.—*Bradford Series*

Serial No.	Date of Delivery	Foetal-cell Score at Delivery	Score 24 hr. after Treatment with 5 ml. Gamma-globulin	Immune Antibody 6 Months after Delivery
		Controls		
64/1627	1/12/64	10	—	Nil
65/124	1/3/65 (twins)*	14	—	
65/1406	7/8/65	8	—	,,
65/1402	20/9/65	19	—	,,
		Treated		
64/1780	26/12/65	17	8 (4 at 48 hr.)	Nil
65/143	8/3/65	10	2	,,
64/2318	26/3/65	6	2	,,
64/2254	21/4/65	16	6	
65/1230	16/9/65	65	16	,,

* One was Rh-positive; the other died soon after birth and was not grouped.

TABLE VI.—*Baltimore Series*

Date of Delivery	Estimated Volume of Foetal Bleed (ml.)	Immune Antibody 6 Months Post-delivery
	Controls	
7/1/65	1·4	Anti-D present
4/2/65	0·25	Nil
22/2/65	0·43	,,
7/3/65	3·0	,,
10/4/65	0·33	,,
20/4/65	0·25	Anti-D present
4/5/65	1·65	Nil
18/5/65	0·38	,,
22/5/65	1·0	Anti-D present
22/7/65	0·47	Nil
23/8/65	0·3	,,
13/10/65	0·26	,,
25/10/65	0·27	Anti-D present
7/12/65	0·84	Nil
	Treated	
25/1/65	0·58	Nil
23/2/65	1·65	,,
1/3/65	0·36	,,
7/4/65	0·36	,,
19/4/65	0·3	,,
19/4/65	0·48	,,
25/4/65	0·3	,,
25/4/65	0·25	,,
7/5/65	0·25	,,
22/5/65	0·26	,,
17/8/65	0·58	,,
20/8/65	0·5	,,
29/10/65	0·33	,,

TABLE VII

	Centre	Controls			Treated		
		No.	Immune Antibody		No.	Immune Antibody	
			Present	Absent		Present	Absent
Trial begun April, 1964	New York and California (Freda *et al.*, 1966; Pollack, *et al.*, 1966)	158	17	141	160	0	160
Trial begun October, 1963	Freiburg (Schneider and Preisler, 1965; Preisler and Schneider, 1966)	47	2	45	55	0	55

Mechanism of Protection by Anti-D

The precise way in which the passively administered anti-D prevents active immunization is unknown, and more than one mechanism may be responsible.

1. Importance of Site to which Rh-positive Cells are Removed

An explanation of the protection conferred by maternofoetal ABO incompatibility is that in the presence of naturally occurring anti-A or anti-B, Rh-positive foetal red cells are either haemolysed intravascularly or are rapidly removed to the liver. In the latter case the paucity of immunologically competent cells results in a failure of the Rh antigen to stimulate antibody production. However, there is the anomaly that when a predominantly saline-active anti-D was used experimentally (Clarke *et al.*, 1963) enhanced anti-D formation resulted. This may be because the red cells were not cleared from the circulation, or because of a difference in complement-binding, or because the lower titre of antibody may have resulted in the red cells being removed to the spleen (Mollison, 1961), where they would come into contact with numerous immunologically competent cells. On the other hand, we know that when incomplete anti-D is used removal is also to the spleen (Clarke *et al.*, 1963), and yet in this case protection results and immune antibody is not formed. Thus the mechanism of protection by incomplete anti-D cannot depend on the site to which cells are removed.

2. Specific Inhibition of the Immune Response

It has been postulated that as part of a physiological homoeostatic mechanism regulating the production of antibody there is a " feed-back " effect by which passive antibody inhibits the further production of antibody (Uhr and Baumann, 1961). The

great variety of antigenic systems in which suppression of primary immunization by the passive administration of antibody has been observed lends support to this view (see Neiders *et al.*, 1962). How the inhibition occurs is not known, but two possible modes of action have been suggested.

Blocking of Antigen Sites.—When the Rh-positive foetal cells encounter the injected incomplete anti-D coating of the cells occurs and the antigen sites are blocked by antibody. This blocking action may persist in the reticuloendothelial system of the spleen, and though there is no direct evidence to support this view the work of Stern *et al.* (1961) favours it. They demonstrated that if Rh-positive cells are coated with incomplete anti-D before injection into Rh-negative men the formation of antibodies is prevented. The cells were washed in saline before injection, and presumably there was no free antibody present. Finkelstein and Uhr (1964) suggest that the effect of such red-cell blocking might be that within the macrophage of the reticuloendothelial system there is a failure to "process" the antigen, and the lymphoid cells are thus not stimulated to produce antibody.

Inhibitory Effect on the Antibody-producing Cell.—Using sheep erythrocytes as antigen in rats, Rowley and Fitch (1964) showed in a series of experiments that passively administered antibody was apparently bound to potential antibody-forming lymphoid cells. This was associated with a failure of the cells to proliferate in response to administered antigen, and was interpreted as suggesting a direct inhibitory effect of the antibody on the potential antibody-producing cells.

It is uncertain how long the suppression of the immune response lasts after injection of the gamma-globulin, and Chown (1965) thinks that it may persist even after the passive antibody has disappeared. However, in our Baltimore study (see above), of the 13 men protected, four produced anti-D when challenged seven months later with 5 ml. of Rh-positive blood.

It is not known whether the giving of anti-D gamma-globulin will protect against the development of antibodies other than anti-D. This could be tested by giving a suitable dose of antibody directed against a red-cell antigen other than D and seeing whether or not anti-D was produced. An experiment based on this principle is in progress. Kell-negative Rh-negative volunteers have been injected with Kell-positive Rh-positive red cells, and then anti-Kell antibody administered. If protection is non-specific neither anti-Kell nor anti-D would be produced, but if the suppression of immunization is specific one would expect anti-D to be produced in some cases.

Design of our Further Clinical Trial

Earlier this year we showed that 0.5 ml. of gamma-globulin effectively clears 5 ml. of injected Rh-positive blood in 48 hours (Clarke *et al.*, 1966). Though, in a current experiment, we have not demonstrated protection with this dose—none of the controls and none of the treated has so far (nine weeks later) developed immune antibodies—yet we feel it is unlikely that we have caused enhancement, and therefore that we are justified in beginning a new clinical trial with a smaller dose of gamma-globulin than previously. This new trial began on 9 June 1966, and, as in the first clinical trial, only Rh-negative primiparae who have had Rh-positive ABO-compatible babies are being included. However, on this occasion we are treating, within 36 hours of delivery, alternate patients who have a Kleihauer score of from 0 to 4 inclusive, and these will be given 1 ml. of gamma-globulin. The original trial will be continued, and women with a foetal-cell score of five and over on the Kleihauer test will continue to be given 5 ml. of gamma-globulin.

ABO Incompatible Pregnancies

Although it is well recognized that ABO incompatibility between mother and foetus protects against Rh immunization, this is not invariably the case. In a six-month follow-up of 90 Rh-negative women who had Rh-positive ABO-incompatible first babies we found anti-D in one case. Since the incidence of anti-D in a similar follow-up of ABO-compatible pregnancies is approximately 8%, and as compatible pregnancies are about four times as common as incompatible ones, it follows that of 32 antibodies developing after a first pregnancy approximately one will be the result of an ABO-incompatible pregnancy. In order to prevent these occasional cases of immunization it would be necessary to treat a considerable number of "low-risk" mothers, and perhaps this should be done only when supplies of gamma-globulin become freely available.

Supplies of Gamma-globulin

If the new clinical trial gives satisfactory results the reduced dosage will greatly ease the difficulty of providing gamma-globulin for the whole country, and the position can be further helped by the use of plasmapheresis. If all women "at risk" with Kleihauer scores of from 0 to 4 were given 1 ml. of gamma-globulin and the remainder 5 ml., it has been estimated that about 72,000 ml. of gamma-globulin a year would be needed. There are three possible sources from which this could be obtained, and they are listed in order of desirability:

1. Rh-negative women immunized by recent pregnancy who have high-titre incomplete antibodies in their serum. This source has the advantage that no booster doses are required, and therefore there is no risk to the donor.

2. Post-menopausal Rh-negative women sensitized by pregnancy or transfusion, and Rh-negative men sensitized by transfusion who would be prepared to agree to being hyperimmunized.

3. Post-menopausal Rh-negative women and Rh-negative men who would agree to being deliberately immunized.

Sources 2 and 3 carry the slight risk of homologous serum jaundice due to injection of donor blood, but this can be minimized by very careful selection of the donor.

By one or other of these methods, panels of donors could be established so that adequate sources of antibody would be available, and then by the use of plasmapheresis, whereby each donor provided 500 ml. of plasma at each attendance, ample supplies of gamma-globulin would be ensured. In order that excessive demands are not made on donors' time we feel that they should not be asked to attend more often than once a month.

Value of the Kleihauer Technique

In 1965 we published some information on the relation of foetal-cell score at delivery to production of Rh antibody six months later (Woodrow *et al.*, 1965), and we now have further results on this problem (see Table VIII).

It will be seen that there is an increasing likelihood of the mother developing antibodies as the foetal-cell count rises, and the Kleihauer technique is clearly of great value in assessing the risk of subsequent immunization. Because of this, and because of the uncertainty of how much gamma-globulin is needed to protect against large bleeds, we feel that at present it is wise to employ the Kleihauer technique wherever possible.

TABLE VIII.—*Relation of Foetal-cell Count After Delivery to Subsequent Rh Immunization as Measured Six Months After Delivery. Cases are All Rh-negative Primiparae with Rh-positive ABO-compatible Babies*

	0	1–4	5–60	60+	Total
Antibody (%)	5 (2·59)	11 (8·33)	9 (16·98)	3 (50)	28 (7·87)
No antibody ..	188	121	44	3	356
Total ..	193	132	53	6	384

N.B.—The data do not represent a random sample of deliveries, as 37 women with a foetal-cell score of 5 or more (31 with a score of 5–60 and 6 with one of over 60) had been treated with anti-D gamma-globulin during this survey, and are not included.

It seems to us inadvisable simply to treat everyone with 1 ml., because this may not be enough to protect against a large transplacental haemorrhage—though this view may need revision in the light of further experience. Admittedly there are difficulties in learning the technique, and extra staff would be needed in the various regions if one were planning to use it on a national scale, the number depending on how often the ABO and Rh grouping of the baby is carried out at the hospital. Where it is, as few as two junior technicians (working under supervision) plus a full-time clerk might be able to do the work. In Liverpool two junior technicians carry out about 15 tests in one day, and this includes the time taken to prepare the buffer. If it were only a question of dividing big from small bleeds, very many more cases could be looked at. Where there was any doubt, the bleeds would be classed as "large" and those mothers with large bleeds and with "intermediate" or doubtful cells would be given 5 ml. of gamma-globulin, while 1 ml. should be sufficient to protect those with small or no bleeds.

Wider Applications of this Research

It is well known that in animals the giving of passive antibody has resulted in some prolongation of survival of grafts (Billingham *et al.*, 1956 ; Parkes, 1958 ; Brent and Medawar, 1961 ; Nelson, 1962), and it is possible that the immunological approach described here may have some practical application in the field of tissue transplantation in man. Furthermore, it might conceivably be of use in the prevention of some types of autoimmune disease. For example, it would be worth trying to protect NZB mice who are "at risk" for autoimmune nephritis (Russell *et al.*, 1966) by the injection of gamma-globulin from those animals which had already developed the disease.

Summary

A successful clinical trial of a method of preventing Rh immunization of Rh-negative mothers by their Rh-positive babies has been carried out. The technique has been developed over a period of six years, and was first shown to be successful in preventing Rh immunization of male volunteers.

Soon after the baby is delivered the woman is given an intramuscular injection of 5 ml. of gamma-globulin containing a very high titre of incomplete anti-D.

Included in this trial are 156 Rh-negative primiparae shown to be at considerable risk of Rh immunization because a number of foetal cells were detected in their blood after delivery of an Rh-positive baby. Half of the women were given 5 ml. of gamma-globulin and the other half served as controls.

Testing of the serum for antibodies at least six months after delivery has demonstrated no certain case of Rh immunization in the 78 treated women with 19 immunizations in the controls.

Three doubtful cases among the treated (two at Sheffield and one at Leeds) have occurred, and while reasons are given for thinking these women are not immunized the possibility remains that they may be ; but, even so, in these high-risk cases the order of protection would still be about 80%.

Workers in New York and California and Germany, using a similar type of approach, have also obtained very favourable results, though the design of their experiments differs slightly from our own.

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 second Rh-positive baby. Results for this so far are encouraging, as no antibodies have developed in any of the 18 women who have had the treatment and who have had a subsequent Rh-positive ABO-compatible baby—six in our trial, six in Freiburg, and six in New York—and we can be very optimistic that the technique is not merely postponing the development of immune antibodies. The evidence to date seems to indicate that nearly all cases of maternal Rh immunization can be prevented by the injection after delivery of high-titre incomplete anti-D.

Experiments have been carried out which suggest that 5 ml. of gamma-globulin may be an unnecessarily large dose and that 1 ml. may be a safe dose for most women. A second clinical trial has been started in which alternate Rh-negative women whose blood after delivery contains no, or very few, foetal cells are being given 1 ml. of anti-D gamma-globulin.

The mechanism by which the injection of anti-D prevents Rh immunization, the problem of the supply of anti-D gamma-globulin, and the wider implications of this method of influencing immunological reactions are discussed.

We are grateful first of all to those mothers at the five centres who volunteered to enter the trial, and to the blood donors whose plasma has been the source of the gamma-globulin prepared in this country. The investigation here would not have been possible without the help of Dr. W. d'A. Maycock, Mr. L. Vallet, and the staff of the Lister Institute of Preventive Medicine, who prepared the gamma-globulin for us. Similarly, we acknowledge with thanks the Ortho Research Foundation, who provided it for the Baltimore trial.

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The Adrenal Cortex in Internal Medicine*—Part II

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Aldosterone

The same interval elapsed between discovery of aldosterone in 1952 by Tait *et al.* and recognition of the first aldosterone-producing tumour by Conn in 1954, as between recognition of adrenaline and the first adrenaline-producing tumour. If the rate of clinical progress has not changed in 45 years, that of technical progress has been immensely greater, making possible the isolation of aldosterone from human urine, its crystallization, and the determination and proof by synthesis of its chemical structure, all within two to three years. Shortly afterwards isotope-labelled samples of the hormone were prepared, and with this the daily secretion rate was first estimated. This rapid progress was all the more remarkable because the daily production is only about one-hundredth of that of cortisol.

More recently plasma-aldosterone levels have been estimated, though the technique is exacting and the use of isotopes is essential: they average only about a thousandth of the normal plasma cortisol, or about a five-millionth of the normal plasma-glucose content. The now familiar estimations of similar low concentrations of vitamin B_{12} are made possible by the peculiar sensitivity of a living organism, but there is no equivalent specific organism or chemical reaction for aldosterone. Analysis still involves laborious separation by orthodox chromatographic methods, with assay by modern double isotope-derivative techniques, because the quantities are much too small even for classical microchemical methods. But in spite of the difficulties the general dynamics of aldosterone within the body have now been fairly completely worked out.

Aldosterone is secreted into the systemic blood by the zona glomerulosa of the adrenal cortex at a rate which averages 130 μg. a day, but varies from 50 to 200 μg. in normal circumstances. Circulating aldosterone, unlike cortisol, is only mildly bound to plasma protein, the mean being 65% compared with 95% for cortisol (Daughaday *et al.*, 1961). When blood passes through the liver its aldosterone is almost completely removed. The liver is probably the only major site of aldosterone destruction, for indirect isotopic estimates of the amount of blood cleared of aldosterone per minute coincide very closely with the estimated total liver blood-flow (Bougas *et al.*, 1964). This volume of blood has been called the metabolic clearance rate for aldosterone. The rate of removal from the circulation is such that half the blood content is destroyed in about 30 minutes, and under normal circumstances this will be replaced by freshly secreted aldosterone. The plasma concentration at any time reflects the balance between secretion and destruction, the two being in approximate equilibrium. If any two of these three variables can be measured the third can be calculated.

Estimates of total plasma aldosterone obtained either indirectly or directly, by the double isotope-derivative method, give a mean of 0.007 μg./100 ml. (Peterson, 1964). Some of this minute amount leaks into the urine; the renal clearance for free aldosterone is about 14% of the inulin clearance (Gfeller and Siegenthaler, 1965), and that for the 3-oxo-conjugate is about 250% of the inulin clearance (Siegenthaler *et al.*, 1964). If confirmed, this would indicate an active secretion by the renal tubules, but the loss in the urine rarely exceeds 10% of the daily production.

Although most of our knowledge of the behaviour of aldosterone in disease derives from changes in the urinary content, this is not the ideal criterion because of its uncertain relation to the plasma levels, especially in impaired states of renal function, which are frequent in just those conditions where the behaviour of aldosterone is of greatest interest. Direct measurement of plasma concentrations, after a great deal of labour, reflects the state of the internal environment at an instant in time. The aldosterone secretion rate, feasible as a research procedure, is more valuable, giving a mean value for 24 to 48 hours; but it requires the skilled use of isotopes and has the disadvantage of not necessarily reflecting the prevailing plasma levels, because in disease the rate at which aldosterone is destroyed in the liver varies (Luetscher *et al.*, 1965).

Most Useful Index

The most useful index of aldosterone behaviour in the body is likely to be an indirect estimate of the mean plasma-aldosterone concentration over 24 hours. Tait (1963) derives this estimate by dividing the aldosterone secretion rate by the rate of removal of aldosterone from the blood-stream, the latter being measured during constant infusion—the so-called metabolic clearance rate. This index is technically more feasible and probably more valid clinically than most of the others, but as yet few data have been published. In my laboratory Glaz and Pearson have studied the procedure, but it is not yet suitable for routine clinical investigation, calling for considerable patience and technical skill.

Aldosterone production in the body is as labile and variable as that of cortisol, but it rises and falls in response to quite

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