Immunology, 1975, **28**, 349.

Effect on Primary Rh Immunization of Delayed Administration of Anti-Rh

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(*Received* 21*st June* 1974; *accepted for publication* 5*th July* 1974)

Summary. An intramuscular injection of 100 μg of anti-Rh, given 13 days after an intravenous injection of 1 ml of Rh-positive red cells appeared to suppress primary Rh immunization: at 6 months, none of thirteen subjects so treated had detectable anti-Rh in their plasma, whereas anti-Rh was present in five out of twelve control subjects injected with Rh-positive cells alone ($P = 0{\cdot}015$, Fisher's exact test, one-tailed). Primary immunization was not suppressed in all treated subjects since, following a second injection of Rh-positive cells, 7-day survival was subnormal in three subjects, all of whom had anti-Rh in their plasma after a further 2 weeks. In three other treated subjects, primary Rh immunization appeared to be completely suppressed: survival was normal, or initially normal, following a second injection of Rh-positive cells and anti-Rh was detectable only after a third injection.

INTRODUCTION

In most experiments on the suppression of primary Rh immunization by passively administered anti-Rh, the antibody has been injected not later than 24 hours after the red cells, although in one series the interval was 3 days (Pollack, Singher, Gorman and Freda, 1967). It was felt to be of some practical importance as well as of theoretical interest to discover whether Rh immunization could be suppressed when antibody was given as late as 2 weeks after an injection of Rh-positive cells and the following experiments were therefore undertaken.

MATERIALS AND METHODS

Subjects

All subjects were previously untransfused Rh-negative male blood donors who volunteered to take part in the work after the various potential hazards had been explained to them. They were told that if they formed potent anti-Rh, they would be invited to become plasmapheresis donors for the production of anti-Rh immunoglobulin and several later did so. (Unless otherwise stated, Rh and D are used interchangeably in this paper.)

Donor

The same Rh-positive donor (Mrs Lim.) was used throughout. She had been a plasma-

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pheresis donor for over 2 years, during which time she had given over a hundred donations. Each donation, including those used for the present study, was found to be negative for Australia antigen. Earlier tests were done by immunoelectrophoresis and later ones by radioimmunoassay. The plasma donations had been used to prepare fibrinogen for clinical use and no hepatitis had been reported in any of the recipients. Weekly liver function tests of the donor had always been normal. Further details of the donor's red cell antigens were as follows: O, CcDEe, K-negative, S-positive, Fy^a-positive, Jk^a-positive.

Blood was collected into citrate–phosphate–dextrose (CPD) and after being stored for not more than 24 hours at 4° the red cells were washed once in the CPD solution described by Mollison, Robinson and Hunter (1958) and then labelled with ^{51}Cr. The final suspensions prepared for injection contained approximately 1 ml of red cells, labelled with about 40 μCi of ^{51}Cr in a volume of about 5 ml.

Plan of the experiments

The subjects were randomly divided into two groups, twelve to act as controls and thirteen to be 'treated'.

The control subjects received two injections of red cells at an interval of approximately 6 months (actually 190 days), each injection being of 1 ml of ^{51}Cr-labelled Rh-positive red cells. Survival was followed on each occasion for a period of 6 weeks. One subject (number 9) received a third injection of labelled red cells 5 months after the second injection.

The 'treated' subjects were given an initial injection of 1 ml of Rh-positive red cells, followed after an interval of 13 days by an intramuscular (i.m.) injection of 100 μg of anti-Rh. Blood samples were taken approximately 48 hours later to confirm that all the positive red cells had been removed from the circulation. Six months later, a second in-injection of 1 ml of ^{51}Cr-labelled Rh-positive red cells was given, this time without an injection of anti-Rh. Any subject who did not form anti-Rh after the second injection of Rh-positive red cells was, after a further period of approximately 5 months (actually 149 days), given a third injection of Rh-positive red cells, the survival of which was also followed.

In both groups, tests for anti-Rh were made if survival in any particular subject fell to 5 per cent or less during the period of study; if anti-Rh was not detected, further samples were taken at weekly intervals for 3 weeks and all subjects were tested 6 months after each injection.

Anti-Rh immunoglobulin

The preparation used was a standard batch (B170G) prepared by the Blood Products Laboratory of the Lister Institute for the routine prophylaxis of Rh immunization in recently delivered women. Three ampoules of this batch were assayed by Dr N. C. Hughes-Jones and estimated to contain 63, 50 and 62 μg of anti-Rh/ml respectively. One ampoule, containing 2 ml or approximately 110 μg of anti-Rh, was given by i.m. injection into the deltoid to each of the thirteen subjects in the treated group, as described above.

Blood samples

After each injection of red cells a sample was taken at 5–10 minutes and subsequent samples were taken at approximately weekly intervals for 6 weeks or until survival had fallen to 5 per cent or less (see Table 1). The haemoglobin concentration was determined

TABLE 1

Cr SURVIVAL AFTER SUCCESSIVE INJECTIONS OF 1 ml OF Rh-POSITIVE RED CELLS IN TWELVE SUBJECTS (CONTROL GROUP) WHO RECEIVED RED CELLS ALONE AND IN THIRTEEN SUBJECTS (TREATED GROUP) WHO ALSO RECEIVED AN i.m. INJECTION OF 100 μg OF ANTI-Rh 13 DAYS AFTER THE FIRST INJECTION OF RED CELLS; SECOND INJECTION OF RED CELLS GIVEN 6 MONTHS AFTER THE FIRST; THIRD INJECTION 5 MONTHS AFTER THE SECOND. MEAN NORMAL VALUES FROM GARBY AND MOLLISON (1971) (see text)

	Cr survival (per cent)																
	First injection				Second injection							Third injection					
Day:	7	14	28	42	0	7	14	21	28	35	42	7	14	21	28	42
Mean Normal						84·5	73·5	63·5	53·5	46·5	39·0					
Control group																
1	84	69	2	0	*	0										
2	88	70	6	0	*	0										
3	88	74	50	29	*	0										
4	83	73	53	38	*	0										
5	84	72	59	42	*	0										
6	85	44	0			20	2	*								
7	90	74	0			25	2*									
8	85	73	0			39	7	*								
9	§	(65)†	54	23		85	73	54	18	5*		87	76	66	52	31‡
10	84	(60)†	56	42		77	68	62	55	§	41					
11	84	74	59	45		86	75	67	61	51	47					
12	88	71	57	44		79	68	59	50	47	40					
Treated group																
13		69¶	0¶			32	2	*								
14		26	0			19	2	*								
15		69	0			71	30	5*								
16		68	0			83	20	3		*						
17		74	0			85	72	63	40	§	0	0*				
18		74	0			80	62	49	43	34	25	0*				
19		78	0			82	71	63	56	49	40	0*				
20		76	0			80	71	18	2			83	74	54	24	5
21		70	0			85	73	64	55	49	33	82	73	61	61	46
22		70	0			82	72	58	44	§	26	86	70	62	54	35
23		76	0			80	69	66	54	43	40	86	77	70	63	48‡
24		70	0			81	70	65	53	§	35	85	74	64	61	50
25		79	0			81	72	65	59	§	45	87	82	69	65	53

* Anti-Rh first detected.
† Samples on day 21 instead of day 14.
‡ Samples on day 46 instead of day 42.
§ No sample.
¶ In treated subjects samples were taken on days 13 and 15 after the first injection.

in a Coulter 'S' counter on the day of sampling. All samples were counted together at the end of each experiment and results were expressed as ct/g of haemoglobin. The value of the 5–10-minute sample was taken as 100 per cent and all subsequent values were expressed as a percentage of the value in this sample.

Tests for anti-Rh

All samples of serum were examined for Rh antibodies by the indirect antiglobulin test with R_1R_2 cells, using a serum:cells ratio in the incubation mixture of approximately 100 : 1. It was shown that the lowest concentration of anti-Rh(D) detectable by this method was approximately 0·01 μg/ml.

In addition, quantitative estimates of anti-D were made on all samples, using the AutoAnalyzer.

Samples were tested for anti-C and anti-E as well as for anti-D and were also 'screened' for the presence of antibodies outside the Rh system, using a panel containing all the common red cell antigens. The methods used were agglutination in saline at room temperature and 37°, agglutination with papain-treated cells and the indirect antiglobulin test.

RESULTS

Table 1 summarizes the results. The mean normal figures for Cr survival up to 28 days are taken from Garby and Mollison (1971); the figures for normal survival at 35 and 42 days are based on extrapolations from the same data.

CONTROL GROUP

Following the first injection of red cells, survival was definitely normal at 28 days in seven subjects, and was either normal or only slightly subnormal in all these subjects at 42 days. In the remaining five subjects (numbers 1, 2, 6, 7 and 8), survival fell to zero within 21 days in one case, within 28 days in two further cases and within 42 days in two more cases. All these five subjects proved to be 'responders' but, as Table 1 shows, there was no obvious correlation between rapid clearance of red cells following the first injection and the production of serologically detectable anti-Rh at 6 months.

Following a second injection of Rh-positive red cells, all five subjects (numbers 1–5) with detectable anti-Rh before the injection had cleared all the cells by the end of 7 days. In three more subjects (numbers 6, 7 and 8), survival was very subnormal at 7 days and these subjects all had detectable anti-Rh in their plasma within 21 days of the second injection. Of the remaining four subjects, three (numbers 10, 11 and 12) had a strictly normal survival and were classified as 'non-responders'. In the remaining subject (number 9), although survival was normal at 7 and 14 days, it was subnormal from 21 days onwards and anti-Rh was detected at 5 weeks. The presence of anti-Rh was confirmed in samples taken at 6 weeks and 7 weeks. Because of this rather unusual result, i.e. initially normal survival following a second injection of Rh-positive red cells in a subject who proved to be a responder, a third injection was given 6 months after the second injection and, as Table 1 shows, was followed by strictly normal survival. No anti-Rh could be detected at the time of the third injection or during the following 6 months.

TREATED GROUP

Thirteen days after the first injection and immediately before the injection of anti-Rh was given, survival was normal in twelve subjects but in the remaining subject (number 14) was greatly reduced.

Twelve of the subjects were tested 48 hours after the injection of anti-Rh and all had virtually no surviving cells (^{51}Cr survival less than 1 per cent); the remaining subject (number 18) could not be tested for 14 days after the injection of anti-D, when Cr survival was less than 1 per cent. At 6 months, none of the subjects had detectable anti-Rh in their plasma.

Following the second injections of Rh-positive cells, survival was subnormal at 7 days in three subjects (numbers 13, 14 and 15) and these three all produced serologically detectable anti-Rh within 3 weeks. One further subject (number 16), in whom survival was normal at 1 week, had very subnormal survival at 2 weeks and had serologically detectable anti-Rh in his plasma at 5 weeks.

The nine subjects without anti-Rh in their plasma at 5 months after the second injection were then given a third injection of red cells. Three subjects (numbers 17, 18 and 19) cleared the cells at 7 days, by which time they had detectable anti-Rh. Of the remaining six, five (numbers 21–25) had strictly normal survival and were classified as non-responders. Donor number 20 had normal survival up to 14 days after both his second and third injections and was thought to be in all probability a non-responder.

CHARACTERISTICS OF ANTIBODIES

In the control group, as Table 1 shows, in five subjects (numbers 1–5) anti-D was not detected at 6 weeks, but was detected at 6 months, after the first injection; in these five antibody levels were $<0{\cdot}1$–$1{\cdot}5$ μg/ml and the specificity was anti-D alone in four cases and anti-C plus anti-D in one case. Within 2–4 weeks of the second injection, antibody levels were between $1{\cdot}1$ and 92 μg/ml and in four cases the specificity was anti-C plus anti-E as well as anti-D and in the remaining case anti-C plus anti-D. In four more subjects (numbers 6–9), antibody was first detected 2–5 weeks after the second injection, when anti-D levels were between $<0{\cdot}1$ and $0{\cdot}3$ μg/ml; they remained at low levels during the following 2–3 weeks; all specificities were anti-D only.

In the treated group, antibody was first detected 3–5 weeks after the second injection in four subjects (numbers 13–16) and had the specificity anti-D only in all cases; antibody levels were $<0{\cdot}1$ μg and remained at low levels during the following 2–3 weeks. In three further subjects (numbers 17–19), anti-D was not detected 5 months after the second injection but 1 week later, after a third injection of red cells, had reached levels of 4·9–39 μg/ml; in these three cases, the specificity was anti-C plus anti-D plus anti-E in two and anti-C plus anti-D in the remaining case.

In five cases (three controls and two treated), samples agglutinated Rh-positive cells in saline as well as giving an indirect antiglobulin test.

In no cases were any antibodies outside the Rh system detected.

DISCUSSION

Analysis of results

In analysing the results, either all subjects can be considered or the analysis can be con-

fined to responders. By definition, subjects who produce serologically detectable anti-Rh are responders and donors numbers 1–9 in the control group and numbers 13–19 in the treated group thus fall into this category. It must be admitted that the results in donor 9 are very puzzling in that shortened survival of the second injection with the production of serologically detectable antibody within 5 weeks was followed by strictly normal survival of a third injection of Rh-positive red cells. In view of the fact that the sample taken 5 weeks after the second injection was tested repeatedly and the presence of anti-Rh confirmed, and that anti-Rh was also demonstrated in samples taken at 6 weeks and 7 weeks, this subject is considered as a responder from the point of view of analysing the present results.

Subjects numbers 10–12 in the control group and numbers 21–25 in the treated group had normal survival of their second injection of red cells and the latter subjects (numbers 21–25) also had strictly normal survival following their third injection of red cells, so that all these subjects seem definitely to be non-responders. Subject number 20 twice showed diminished survival (second and third injections) but, as on both occasions survival was normal at 14 days after the injection, this subject has been classified as a non-responder.

In summary, the proportion of responders was nine out of twelve in the control group and seven out of thirteen in the treated group. The over-all frequency of responders (sixteen out of twenty-five or 64 per cent) agrees well with that observed in the large series of Archer, Cooke, Mitchell and Parry (1971).

The prevalence of serologically detectable anti-Rh 6 months after the first injection of Rh-positive cells in the control and treated groups was five out of twelve vs zero out of thirteen or, considering the responders only, five out of nine vs zero out of seven. The probabilities of getting such differences by chance are 0·015 for all subjects or 0·029 considering only responders (Fisher's exact test, one-tailed).

Evidently, primary Rh immunization was not completely suppressed in all subjects in the treated group: in subjects numbers 13–15, following the second injection of Rh-positive cells, 7-day survival was subnormal and all these subjects had detectable anti-Rh in their plasma at 3 weeks. The findings in these subjects were very similar to those in subjects numbers 6–8 in the control group. On the other hand, in subjects numbers 17–19 in the treated group, survival was normal or almost normal for 4 weeks after the second injection and anti-Rh was detectable only after a third injection of red cells given 5 weeks later. In these subjects, then, primary immunization appeared to have been completely suppressed. In one further treated subject (number 16), results were intermediate: red cell survival following a second injection was normal at 1 week but thereafter rapidly became subnormal and anti-Rh was detectable 5 weeks after injection.

There was no obvious difference between the specificities of the antibodies found in the control and treated groups; in both series, specificity was almost always anti-D alone at the time when antibody was first detected and became broader (anti-C plus anti-D with or without anti-E) after boosting.

In a previous paper, it was suggested that in responders given a second injection of Rh-positive cells about 6 months after a first injection, survival was always subnormal 7 days after the second injection, suggesting that in responders enough antibody was always produced after a first injection to diminish the survival of 1 ml of red cells given 6 months later (Mollison, Frame and Ross, 1970). In the present series, one exception to this rule was encountered, namely in subject 9 in the control series; as already discussed, the response in this subject was highly unusual in that, although anti-Rh was detected

after a second injection, the antibody subsequently disappeared and Rh-positive cells survived normally after a third injection, suggesting the development of tolerance.

In the treated group, the interval between the injection of 100 μg of anti-Rh and the giving of the second injection of red cells was 190 days. Assuming that the antibody was being metabolized at a constant rate with a $T_{\frac{1}{2}}$ of 26 days (see Mollison, 1972), the total amount remaining after 190 days would be expected to be about 0·6 μg. Such an amount was estimated previously to be just capable of slightly reducing the survival of 0·3 ml of Rh-positive red cells (Mollison and Hughes-Jones, 1967). In the present study, following the second injection of 1 ml of red cells, survival was normal at 7 days in all except three subjects, all of whom formed serologically detectable anti-Rh within 3 weeks of the second injection. In one subject (number 18) in whom survival was normal at 7 days, it was slightly subnormal from 14 days onwards, and it is just possible that in this case there was persistence of enough anti-Rh to affect the survival.

The present results re-emphasize that the absence of serologically detectable anti-Rh following an initial injection of Rh-positive cells does not imply that Rh immunization has been suppressed completely. It may therefore be necessary to draw more cautious conclusions from some previously published data. For example, based on the failure to find serologically detectable anti-Rh, it was concluded that the injection of approximately 4000 μg of anti-Rh following the transfusion of a whole unit of Rh-positive blood suppressed Rh immunization (Pollack, Ascari, Crispen, O'Connor and Ho, 1971). Although the data decisively showed the suppression of overt antibody formation, it remains uncertain whether primary immunization was completely suppressed.

Are some Rh-positive cells more immunogenic than others?

In the present series of control subjects, five out of twelve made serologically detectable antibody after a single injection of red cells and three more made antibody within 3 weeks of a second injection. There was only one other responder in the series (subject number 9) so that, of nine responders, eight had made anti-Rh within 3 weeks of a second injection of Rh-positive cells.

By contrast, in a previous series of ten Rh-negative subjects, only one made antibody after a single injection of 1 ml of Rh-positive cells and no others made antibody within 4 weeks of a second injection, although three had anti-Rh in their plasma when tested 5 months after a second injection and one further subject formed anti-Rh after a third injection. In all, then, out of five responders in this other series, only one made anti-Rh within 3 weeks of a second injection of red cells (Series 'A' of Mollison, Hughes-Jones, Lindsay and Wessely, 1969). The difference between the two series, namely eight out of nine vs one out of five making anti-Rh within 3 weeks of a second injection, is just significant at the $P = 0{\cdot}05$ level. Since in both cases 1 ml of red cells was injected on each of the two occasions and the other details of the two series were similar, the question arises whether the difference could have been due to the use of different donors. In the previous series, the donor was of phenotype R_1r, whereas in the present series, as already mentioned, the phenotype of the donor was R_1R_2. If the difference is to be ascribed to the use of different donors, the question then arises whether the difference is due simply to the greater number of D sites on R_1R_2 red cells compared with R_1r red cells or whether it is due to some qualitative difference between the two phenotypes or to some other difference between the two donors.

Although there does not seem to be any direct evidence that the R_2 antigen is more

immunogenic than R_1 when injections of red cells are given to Rh-negative subjects, there is the evidence of Murray (1957) and Murray, Knox and Walker (1965) that R_2r infants are more likely to immunize their mothers than are R_1r infants.

Relation of 'collapse' curves to primary immunization

The significance of 'collapse' curves and their relation to primary immunization remains obscure. The term 'collapse' curve has been used (Mollison, 1961) to describe a red cell survival curve which is within normal limits for an initial period of about 2 weeks but then rapidly falls so that all the cells are cleared from the circulation by about 4 weeks after transfusion. Survival curves of this kind are found after about 30 per cent of all transfusions of a few millilitres of red cells exchanged between ABO-compatible, Rh-compatible subjects (Adner, Foconi and Sjölin, 1963; Mollison, 1972). When a second injection of a few millilitres of cells is given from the same donor to the same recipient, survival is normal or almost normal in about 50 per cent of cases, but is shorter than on the first occasion in the remaining 50 per cent, with complete clearance of all cells in about 10 days. In these latter cases, alloantibodies are occasionally found (Adner *et al.*, 1963).

In the present series of control subjects, following the first injection of Rh-positive cells, there was no close association between early clearance of the cells and a good primary response. Indeed, amongst the five subjects who made serologically detectable anti-Rh after a single injection of cells, survival at 6 weeks was strictly normal in two cases and almost normal in a third case. Good survival at about 6 weeks after a first injection in subjects who made serologically detectable antibodies without a further stimulus was described for anti-K by Adner *et al.* (1963) and for anti-Rh by Mollison *et al.* (1970).

Implication of present results

The fact that in some responders, at least, insufficient humoral antibody is formed in the first 6 weeks to affect the survival of a small dose of Rh-positive red cells is surprising, particularly in view of the fact that, as the present observations indicate, primary immunization is sometimes initiated within 2 weeks (13 days) of the first injection of Rh-positive red cells.

It is generally supposed that Rh-positive red cells become immunogenic only after they have been removed from the circulation by the reticuloendothelial system. Accordingly, when an injection of anti-Rh is given approximately 2 weeks after an injection of 1 ml of Rh-positive red cells, as in the present work, approximately 10 per cent, or 0·1 ml, of the cells is expected to have reached the RES due to normal senescence of cells. Such an amount of Rh-positive red cells is certainly capable of inducing primary Rh immunization (Jakobowicz, Williams and Silberman, 1972). The present observations are consistent with the idea that the suppression of Rh immunization by passively administered anti-Rh is mediated by some effect of antigen–antibody complex on antibody-forming cells. Evidence that such an effect is mediated by the Fc portion of the bound antibody has been discussed by Chan and Sinclair (1973).

ACKNOWLEDGMENTS

We are greatly indebted to the donors who acted as subjects in these experiments. We are also grateful to Dr T. E. Cleghorn, Director of the North London Blood Transfusion

Centre, for helpful advice and to Mr J. B. Harris for testing samples in an AutoAnalyzer to confirm the results obtained by manual methods and to provide quantitative estimates. Finally, we should like to thank Dr J. M. England for help in analysing the results.

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