an unjust criticism. All great advances in medicine must nowadays start with a great capital investment of time and manpower and energy. . .If we thwart or discourage research on transplantation today we are deliberately willing away part of the medical heritage of the future."

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Primary Immunization of Rh-negative Volunteers

H. H. GUNSON,* M.D., M.R.C.PATH. ; F. STRATTON,† M.D., D.SC., F.R.C.P., F.R.C.PATH.
D. G. COOPER,‡ F.I.M.L.T. ; VIOLET I. RAWLINSON,§ M.SC.

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Summary: To determine the best method for the production of high-titre anti-D serum primary immunization was carried out in two groups of Rh-negative male volunteers with washed group O R_2R_2 cells. The first group of six men were given 5 ml. of packed cells, and the second group of five men were given 0·5 ml. of packed cells, in each instance by intravenous injection. Only one individual in each group failed to develop anti-D following the primary inoculation, and it has been concluded that 0·5 ml. of packed R_2R_2 cells is probably a satisfactory dose for this purpose.

There was a delay of several weeks before anti-D could be shown to have developed. The initial antibodies which appeared in the serum comprised 7S γG immunoglobulins, with, in about half the cases, a minor 19S γM component.

Introduction

It is now well established that the administration of anti-D immunoglobulin to mothers at delivery will effectively prevent their immunization to the Rh(D) antigen (Clarke, 1968). Use of anti-D immunoglobulin in this way will in the future lead inevitably to a steady reduction in the number of women whose sera contain anti-D of sufficient strength either to produce the immunoglobulin or for use as typing serum. Thus the most important source of anti-D for both these purposes at the present time will be gradually eliminated.

There will exist for a considerable period of time a certain number of women whose sera contain a weakly reacting anti-D, the titre of which can be increased by the administration of Rh(D)-positive cells. It was, however, thought to be important to start a programme of investigations to determine the most successful method for the immunization of Rh-negative male volunteers with Rh(D)-positive cells, in order to produce anti-D of high titre. By this means large quantities of anti-D immunoglobulin and typing serum could be obtained by carrying out plasmapheresis on relatively few immunized donors.

The initial results of the immunization of 11 Rh-negative male volunteers with Rh(D)-positive cells are described below.

Materials and Methods

Selection of the Immunizing Donor

It was decided to use group O R_2R_2 red cells because, in general, the D antigen of these cells is more strongly reactive than that of cells with other Rh genotypes (Renton and Hancock, 1956). A 59-year-old male donor was selected. He had donated blood regularly since 1948 on 27 occasions. The

*Consultant in Charge, Transfusion Centre, Lancaster.
†Director, Regional Transfusion Service, Manchester.
‡Chief Technician, Transfusion Centre, Lancaster.
§Research Assistant, Regional Transfusion Centre, Manchester.

recipients of his last nine donations developed no untoward symptoms after transfusion of his blood, a fact elicited either by direct conversation with the patients several months or even years after the transfusion or from the follow-up records of the hospital.

Results of the blood-grouping tests done on the donor's cells were as follows: O, R_2R_2, MNss, P_1 negative, Lu(a−b+), kk, Le(a−b+), Fy(a−b+), Jk(a−b+), Wr(a−), V^w negative, Sd(a+), Bg(a−), Vel positive, I positive.

The Berger–Kahn test on his serum was negative and no reaction was found with the hepatitis associated (Australia-SH) antigen.

Selection of Donors for Immunization

Groups of four to seven group A, B, or AB Rh-negative (rr) men were invited to the Transfusion Centre, Lancaster, and the aims of the scheme explained to them. They were appraised of the possible risks to which they would expose themselves if they volunteered, and any questions were answered in the fullest detail. They were encouraged to discuss the matter with their relatives and their family doctor before making a decision. Those who agreed to take part in the scheme were asked to sign a form of consent.

All the volunteers were between the ages of 30 and 50 and had enjoyed good health and had never received a blood transfusion. Haemoglobin concentration, packed cell volume, and white cell and platelet counts were within normal limits. The presence of atypical blood group antibodies in their sera was excluded by tests against a panel of cells containing antigens from all the major blood group systems, and against cells from the immunizing donor. Tests were done with the indirect antiglobulin method, agglutination tests with cells suspended in human serum-albumin mixture and saline at 37° C. and at 16° C. with saline-suspended and papainized cells.

Immunization Procedure

From the immunizing donor 100 ml. of blood was collected into 35 ml. of A.C.D. solution (2% dextrose + 2% dihydrogen citrate) in a sterile manner. The red cells were washed three times with 200 ml. of pyrogen-free physiological saline, aseptic precautions being used. After the final wash the cells were suspended in approximately their own volume of physiological saline.

A small quantity of this final suspension was removed from the bottle and its packed cell volume was determined. By this means the actual dose of cells given to the volunteer could be accurately measured. The antigenic dose is expressed as the actual volume of packed red cells inoculated—for example, if the final suspension of cells was 50%, then to achieve a cell dose of 5 ml. an injection of 10 ml. of this cell suspension was given.

Intravenous injections of the cells were given within six hours of collection of the blood. Cultures of the cell suspension at room temperature and at 37° C. were done routinely after the injections, and these remained sterile on each occasion.

Investigation of Sera after Immunization

Serological Investigations.—After immunization, samples of blood were collected at about 14-day intervals. The general serological methods used in the investigations were those described by Stratton and Renton (1958). Use of a capillary technique, however, incorporating Löw's papain based on that described for Kell typing by Lewis *et al.* (1958) was found to be advantageous for the early detection of anti-D. The layering technique for the antiglobulin test (Stratton, 1967), especially after concentration of the serum by ultracentrifugation, was also used in the investigations of weakly reacting Rh antibodies.

Determination of Nature of Anti-D Immunoglobulin.—This was done by various methods which have been described previously—namely, by the use of specific antiglobulin reagents (Stratton *et al.*, 1962), filtration through G 200 Sephadex (Stratton *et al.*, 1968) (obtained from Pharmacia Ltd.), separation on diethylaminoethanol cellulose (Tomasi and Kunkel, 1964; Stratton *et al.*, 1965) (obtained from Whatman's Ltd.), and by sucrose density gradient ultracentrifugation (Stratton *et al.*, 1968). Since the early Rh antibodies formed were of low titre the methods had to be modified from a quantitative point of view to allow recovery of the antibody in the effluents of the chromatographic and ultracentrifugal separations.

Experimental Observations

The results obtained from the immunization of two groups of volunteers are shown in the Table. The first group (Volunteers 1 to 6) were given 5 ml. of group O R_2R_2 cells and the second group (Volunteers 7 to 11) were given 0·5 ml. of the same cells. In both groups there was a delay of several weeks before anti-D was found in their sera, but there was no material difference in the length of this period between the two groups. Only one man in each group failed to develop anti-D.

Two individuals in each group produced Rh antibodies with specificity other than anti-D. The other antibody components were anti-E in each instance, and one volunteer (No. 4) developed anti-G in addition. In two instances (Volunteers 4 and 10) the anti-E was demonstrated in the same sample as the initial anti-D, but in Volunteers 2 and 7 it was detected 14 days after the anti-D. The anti-G in Volunteer 4 was not apparent until 44 days after his anti-D was found—that is, 126 days after the immunization. Apart from one individual in the first group who developed a cold-reacting antibody with anti-H specificity, which disappeared from his serum in a few weeks, no antibodies of other specificities were found.

With freshly collected Rh-positive cells the anti-D was initially detected by the capillary method with Löw's papain or by means of papainized cells in the slide test. In most cases,

Results of Immunization of 11 Rh-negative Volunteers with Group O R_2R_2 Cells.

	Dose of Group O R_2R_2 Cells Injected										
	5 ml.						0·5 ml.				
Rh-negative volunteers:	1	2	3	4	5	6	7	8	9	10	11
First appearance of antibody (days)	37	63	77	82	119	Nil (210)	63	79	83	130	Nil (150)
Specificity of antibodies	Anti-D	Anti-D, anti-E	Anti-D	Anti-D, anti-E, anti-G	Anti-D		Anti-D, anti-E	Anti-D	Anti-D	Anti-D, anti-E	
Nature of anti-D immunoglobulin	7S γG + 19S γM	7S γG	7S γG + 19S γM	7S γG + 19S γM	7S γG		7S γG + 19S γM	7S γG + 19S γM	7S γG + 19S γM	Not fully tested (see text)	
Maximum titration score of anti-D (papainized cells—slide test)	21	16	6	26	9		16	4	5	<1*	

* These antibodies were detected by the capillary method only.

however, it was possible to obtain a positive antiglobulin reaction, though in some instances this test was positive only after previous concentration of the serum. The strength of the anti-D increased to its maximum in 14 to 21 days after it was first detected. With one exception (Volunteer 10), at this time the anti-D could easily be demonstrated by the indirect antiglobulin test and with cells suspended in human serum-albumin mixture. The anti-D in the serum of Volunteer 10 was demonstrable only by the capillary method. Only two men in the first group and one in the second developed saline-agglutinating anti-D. It can be seen from the Table that the maximum titration scores of the anti-D, done by the slide method and using papainized cells, tended to be higher in the first group than in the second.

With the exception of Volunteer 10, the nature of the anti-D immunoglobulin was shown to be 7S γG by the methods detailed above. In three instances from each group it was accompanied by a minor 19S component detected in effluents rich in γM globulin, from filtration through G 200 Sephadex, chromatography on diethylaminoethanol cellulose, and sucrose gradient ultracentrifugation. Hence, though it was not possible to obtain agglutination with specific anti-γM sera, it was assumed that these components were 19S γM immunoglobulins. Similarly, the anti-E in Volunteer 2 was apparently a 19S γM immunoglobulin, and a mixture of 7S γG and 19S γM immunoglobulins in Volunteers 4 and 7. The anti-D in Volunteer 10 was detected only by the capillary method, and though it was successfully eluted from the 7S protein peak after filtration through G 200 Sephadex its nature could not be confirmed by other tests. The anti-E component of this man's antibody was found in both the 7S and 19S protein peaks by this method. In only one serum (Volunteer 3) did the immunoglobulin nature of the anti-D change during the period of observation, and in this case the 19S γM component disappeared 28 days after the initial appearance of the antibody.

Discussion

Nine out of 11 Rh-negative volunteers developed anti-D following primary sensitization with R_2R_2 cells. Previous studies (Wiener, 1949; Freda *et al.*, 1963; Zipursky, *et al.*, 1963, 1965) indicate a certain correlation between the probability of immunization and the concentration of circulating antigen in the primary sensitizing dose of cells. There are, however, many factors involved, including the immunogenic effectiveness of the D antigen of the immunizing cells, and the sensitivity of the tests used to detect the presence of anti-D. It is difficult, therefore, to compare the results of our study with previous ones.

Each of these groups has received a second inoculation of 0·5 ml. of R_2R_2 cells, and Volunteer 6, who apparently did not respond to the primary sensitization, developed anti-D 14 days after the booster dose. Further investigations are in progress to try to detect anti-D in samples taken before the second inoculation, since it is considered that technical limitation is the principal factor for our failing to find this antibody. Anti-D was not detectable in the serum of Volunteer 11 (21 days after the booster dose).

It appears, from these results, that a high proportion of Rh-negative men are capable of responding to a stimulus of Rh-positive cells. The antibody titres developed by the second group were undoubtedly lower than that developed by the first, but we have concluded that 0·5 ml. of packed R_2R_2 cells is probably sufficient as a primary inoculation. This does not mean that all the men will necessarily produce anti-D of high titre and avidity.

Attention should be drawn to the interval between inoculation and the appearance of anti-D. In this study the shortest was 37 days, and in two instances there was a delay of 119 and 130 days. This finding has important practical applications, since one should not conclude that an Rh-negative individual has failed to respond to the primary stimulation until some 130 days have passed. Moreover, the formation of anti-D by an individual in considerably less than five weeks from the time of inoculation strongly suggests that the response is a secondary one and not the result of primary sensitization. This is well demonstrated by the response of Volunteer 6 to a second inoculation.

It is clear that there must be a reason for the long delay in the antibody response following this type of immunization. It may be associated with the long survival of the Rh-positive cells in an Rh-negative recipient whose blood does not contain antibodies against the immunizing cells. Benacerraf (1968), in a recent review, drew attention to the physical state in immunogenicity. If phagocytosis is an initial requirement of an immune response then it is evident that the inoculation of an Rh-negative individual with Rh-positive cells involves a slow protracted phagocytosis of the foreign cells as they become effete.

This is contrary to experience with animals inoculated with erythrocytes from another species, where the antibody response occurs within a few days, presumably owing to the rapid sequestration of the immunizing cells. Moreover, the initial antibody produced by such an animal is a 19S γM immunoglobulin. It is clear from the investigations detailed above that the initial antibodies detected in the male volunteers were 7S γG immunoglobulins together with, in about half the cases, a minor 19S γM component; a finding also noted by Pollack *et al.* (1969). This frequent appearance of 7S γG antibodies may be a result of the protracted removal of the Rh-negative cells from the circulation, since this could lead to a telescoping of the primary and secondary responses. Also, it is interesting to speculate that this phenomenon may contribute to tolerance rather than the production of antibodies.

If the above hypothesis has any substance then the practical implication is that efforts to increase phagocytosis of the Rh-positive cells should decrease the time in which the Rh-negative individual will respond and it may also affect the immunoglobulin nature of the initial antibody. Investigations to test this theory are in progress.

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