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Techniques of blood group desensitization

Joseph Leo McDonald
University of Nebraska Medical Center

TECHNIQUES OF BLOOD GROUP DESENSITIZATION

Joseph L. McDonald

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INTRODUCTION

Landsteiner and Weiner originally discovered the Rh factor in human blood in 1940. They demonstrated that this factor was present in approximately 80 per cent of the population.[1]

Subsequent studies demonstrated the Rh factor as a cause for the vast majority of cases of erythroblastosis fetalis. Transplacental transfer of Rh positive (D) cells to the Rh negative (d) mother was intiminated as the cause for the production of her sensitization and antibody production with subsequent erythroblastosis in the infant.[1,2]

Numerous attempts of desensitization of the mother have been attempted without any method giving satisfactory clinical results.[3,4]

This study is an attempt to sensitize rabbits to human Rh positive (D) type O blood. After sensitization has occurred, desensitization by using small quantities of antigen was attempted. Subsequent large doses of the original antigen are used to suppress antibody production.

1 K. Landsteiner and A. S. Weiner, "Studies on Agglutinogen [Rh] in Human Blood Reacting with Anti-Rhesus Sera and with Human Antibodies", J., Exper., Med., 74:309, 1941.

2 P. Levine, L. Burnham, E. M. Katzen and P. Vogel, "The Role of Isoimmunization in Pathogenesis of Erythroblastosis Fetalis", Am. J. Obst. & Gynec. 42:925, 1941.

3 J. F. Shanaphy, "Rh Countersensitization", Am. J. Obst. & Gynec., 64:6 1261, Dec. 1952.

4 B. B. Carter, A. C. Williamson, Joseph Laughney and C. H. Ingram, "Evaluation of Rh Hapten", Am. J. Obst. & Gynec., 665, Sept. 1956.

Further support to this method of antibody suppression is obtained from studies on presence of antigen in serum correlated with presence of antibodies to antigen. It was conclusively demonstrated that as long as free antigen was present in the serum no antibodies were free in the serum.[5]

On a practical basis then, as long as the Rh negative (d) mother can be maintained with Rh positive (D) antigen in her serum, there are no Rh positive (D) antibodies present. If no Rh positive (D) antibodies are present in the serum, none are available to cross the placenta to damage the infant.

By the use of large doses of bovine albumin antigen in non immune rabbits, several interesting facts are brought to light. Among these is the fact that antigen clearage from the blood is very slow following such large dosage. There was an initial rapid phase of antigen removal from the blood stream followed by a subsequent plateau of antigen retention. Following this was the final clearance stage. This stage lasted as long as 108 days in some rabbits.

With desensitization of immune rabbits and subsequent massive antigen dosage, the curve of antigen removal is very similar, although, somewhat more rapid with antibody appearance within twenty days.

5 D. W. Talmage, J. J. Dixon, S. C. Buhantz and O. J. Dammin, "Antigen Elimination from Blood as Early Manifestation of Immune Response", J., Immunology 67:243 Oct. 1951.

This response to massive antigen dosage makes it unlikely that antibody is responsible for the final disappearance of antigen from the serum. If antibody were responsible for the removal of antigen from the serum in the desensitized animal, the antigen retention curves should show a sharp decline. The beginning of the sharp decline would correlate with the reappearance of antibody. Instead of the sharp decline of retained antigen which would result from antibody antigen combination, we see a very slow decline in retained antigen.

From these studies it appears first that antigen is removed from the immune and non-immune animals serum in much the same manner. Second, it appears that antibody is not responsible for this final removal of antigen. Third, no antibody is produced until all antigen is eliminated. This probably results from saturation of antibody formation sites by the antigen.[6]

By the use of large doses of Rh positive (D) antigen in the immune mother it may be possible to depress antibody formation.

The desirability of a relatively pure antigen for injection is immediately apparent. First, one does not wish to sensitize the individual to any antigens unnecessarily. Second, these minor factors may be a cause for reactions because desensitization against them may not be complete. The large amount of free hemoglobin produced from injection of large amounts of incompatible blood would be toxic to the kidneys. It was therefore deemed necessary

to produce an antige relatively free of hem lobin.
A. G. Johnson, D.W. Watson, W. J. Cromactie, Effect of massive Antigen Retention and Antibody Response", Proc. Soc. Exp. Biol. N. Y. 88 (3) P 421 Mar. 1955.

In line with this reasoning, it was decided to use antigen prepared from cell stromata in two groups of rabbits. Belkin and Weiner demonstrated that suspensions of cell stromata retained their antigenic properties.[7]

Antigen was prepared from type O Rh positive (D) cells. The packed cells were washed in isotonic saline until a clear supernatant was obtained. Distilled water was then added to lyse the cells. The cell walls were then washed in distilled water until the supernatant was light pink in color. Isotonic saline was then added to constitute a volume twice as large as the amount of cell wall stromata present. The strength of antigen was considered to be a 1:1 solution.

The volume of cell stromata derived from the lysed cells was equal approximately to seventy-five per cent of the volume of the packed cells.

Antigen for the third group of rabbits was prepared in a different manner. Lubinski and Portnuff demonstrated that heating human Rh positive (D) cells for ten to fifteen minutes at a temperature of 56°C. and Ph of 7.2 made the cells Rh negative.[8]

7 R. B. Belkin and A. S. Weiner, "Demonstration of the Properties A, B, M, N, and Rh in Red Cell Stromata", Proc. Soc. Exper. Bio. and Medicine 56:214, 1944.

8 H. H. Lubinski and J. C. Portnuff, "Influence of Heat and Formalin upon Rh Agglutinogen", J. Lab and Clin. Med. 32:1, 1947.

Murray and Clark then demonstrated that injection of this preparation into guinea pigs resulted in a production of good anti-D sera.[9]

From the results of this experiment, it appeared that this was a good method for the production of a relatively pure D antigen. Type O Rh positive (D) cells were washed until the supernatant saline was clear. An equal amount of saline adjusted to a Ph of 7.2 was then added. The cells were heated for seventeen minutes at 56°C. The cells were centrifuged and the supernatant was used as the 1:1 strength antigen. The heated cells were typed and found to lack the D antigen.

9 J. Murray and C. C. Clark, Production of Anti Rh Sera in Guinea Pigs from Human Erythrocyte Extracts", Nature, London 169:4, 24 May 1952, 886.

EXPERIMENTAL PLAN

Twelve white, two-thirds grown, male rabbits were selected for subjects. They were divided into three groups of four each.

It was decided to obtain one unit of human type O Rh positive (D) blood for each group of rabbits. Each group was then to be sensitized by the extract from one unit and desensitization was to be performed using extracts from the same unit. This procedure was used for groups II and III. It was impossible to follow this plan in group I where desensitization was carried out with antigen from a different unit of blood with the same genotype. Genotypes were determined on all three units of blood. Rabbit groups I and II are sensitized with cell stromata. Rabbit group III was sensitized with the heated preparation.

Sensitization was performed according to the method used by Scherer with some modification.[10] Three series of three daily intravenous injections four days apart were used. This was one more series than used by Scherer but was thought necessary because of the poor antigenicity of the Rh factors. One cc. of a 1:1000 dilution was used for sensitization in groups I and II. One cc. of a 1:100 was used in group III.

Twenty-eight days after starting injection, blood was drawn for determination of O positive (D) titers. It was found as suspected that some rabbits responded with higher titers than others. Only one rabbit failed to become sensitized. This rabbit was placed in group I.

10 W. F. Scherer, "Attempts to Inhibit Rh Antibody Production in Rabbits", Am. J. Med. Sc. 214:33, Jan. 1948.

The two rabbits with the highest titers were then selected for desensitization. These rabbits were tested with intradermal injections to determine if they demonstrated any skin sensitivity to the respective antigens. It was found that they were not sensitive so subcutaneous injections of antigen were given in an attempt to desensitize the rabbits.

It was impossible to produce anything but a slow rise in the rabbits titers using the subcutaneous method of injection. It was then decided to change to the intravenous method. Using the intravenous method, the antigen was found to be very toxic. In non-toxic doses, there was only a rise in the antibody titer. Higher doses resulted in death of the rabbits in groups I and II. In group III, rather large doses were non-toxic but resulted only in rising titers.

All titers were performed in both saline and albumin suspensions. A serial dilution of the rabbit serum was made in 0.85 per cent saline. To this was added a 2 per cent suspension of O positive (D) cells in saline or 22 per cent albumin. The cell suspensions were then incubated at 37°C. for one hour and were read macroscopically.

EXPERIMENTAL RESULTS

Initial albumin and saline titers were run on all the rabbits. It was found that rabbits number five, seven and eleven had titers to human type O Rh positive (D) blood. None of these rabbits developed sufficiently high titers to be used in the experiment. Sanford and Hooker discuss the presence of weak agglutinins for erythrocytes in rabbits.[11] They found them to have no relation to subsequent immunization since they neither affected the degree of immunization nor the incidence of immunity.

The rabbits were divided into three groups of four each. Into each group was placed one of the rabbits which had had an initial titer. All the rabbits were given three series of three daily intravenous injections of one cc. of antigen four days apart. Titers were then determined on the twenty-eighth day of the experiment.

Rabbits number one, two, four and five were placed in group I. They were injected as above with cell stromata of a 1:1000 dilution. The blood type from which this extract was prepared was type O with an Rh genotype of cDe/cDe.

The twenty-eight day titers were as follows:

RABBIT	SALINE	ALBUMIN
1	1:2	1:2
2	1:2	1:4
4	1:4	1:4
5	Negative	Negative

11 B. Sanford, L. Hooker, M. Anderson, "Weak agglutinins for Human Erythrocytes in Rabbits", J. of Immunology VI:419, 1921.

Rabbits number one and four were chosen for desensitization since they had developed the highest titers. Rabbit number one was given five cc. of a 1:1 dilution of antigen subcutaneously with no evidence of any sensitivity or reaction. This was given over a two day period at which time repeat titers were done. Saline was then 1:2 albumin 1:2. The subcutaneous dosage was then increased to eight cc. per day for two days. Titers taken at this time were saline 1:2 albumin 1:4.

It was obvious at this time that effective desensitization was not occurring by using this dosage subcutaneously. It was therefore decided to change to an intravenous method. Ten cc. of the antigen was then administered over a two to three-hour period. The rabbit exhibited no untoward effects at that time. However, the rabbit was found dead ten hours later.

At autopsy of the rabbit, the right ventricle was filled with clotted blood and appeared slightly dilated. The right auricle was also filled with blood and somewhat distended. The lungs, liver and spleen appeared slightly congested. On microscopic examination, the liver showed slight increase in inflammatory cells in portal areas to gether with slight increase in fibrous tissue and minimal proliferation of bile ducts. Some focal areas of liver demonstrated early necrosis in which cord cells are seen as shadows and tissue is infiltrated with numerous polymorphonuclear leukocytes. The lungs showed marked congestion and edema on microscopic examination. The spleen demonstrated focal zones of necrosis with acute and chronic inflammation. The heart was found to be normal except for a suggestion of mild edema.

Rabbit number four was given seventeen and one-half cc. subcutaneously in divided doses over a period of five days. At that time, titers were run and found to be saline 1:8 and albumin 1:8. Titers against O negative (d) were also run and found to be identical to the titers for O positive (D) blood. The animal was then given five cc. of antigen of 1:1 dilution over a six-hour period with no ill effects. Titers were run twenty-four hours later and found to be saline 1:4 and albumin 1:16. Since no lowering of the antibody titer was observed with this large dose, it was decided to discontinue desensitization procedures at this time.

Rabbits number three, six, seven and eight were placed in group II. They were injected on an identical schedule to that used for group I. Cell stromata was used in a 1:1000 dilution. The blood used to prepare the extract was type O with an Rh genotype of CDe/cDe. This group of rabbits developed considerably higher twenty-eight day titers than group I. This may have been because the group I antigen was slightly more dilute than 1:1000. Titers produced were as follows:

RABBIT	SALINE	ALBUMIN
3	1:16	1:8
6	1:32	1:8
7	1:4	1:32
8	1:32 and up	1:32 and up

It was then decided to use rabbits number six and eight for desensitization since they had the highest titers. Rabbit number six was given five cc. of a 1:1 dilution of cell stromata subcutaneously over a two day period. Titers at this time were saline 1:16 and

albumin 1:16. The dosage was then increased to eight cc. subcutaneously for two days. Titers at this time were saline 1:64 and albumin 1:64. No local evidence of sensitivity to subcutaneous injection of antigen was observed.

We then resorted to intravenous injection of antigen. Ten cc. of a 1:1 dilution of cell stromata was given over a two to three hour period. The rabbit developed considerable ataxia at the end of the first five cc. of antigen. He recovered from this, however, and appeared fairly normal after the final injection. Twelve hours later the animal was found dead. At autopsy, a marked dilatation of the right ventricle and auricle was found. Moderate congestion of the spleen and liver was present grossly. The lungs appeared quite pale. On microscopic examination, there was marked congestion of liver and spleen with moderate congestion of the lungs. The heart appeared essentially normal except for some autolytic changes.

Rabbit number eight was injected subcutaneously with seventeen cc. of a 1:1 dilution of cell stromata over a five day period. There was no evidence of local sensitivity to the antigen. Titers drawn at the end of this period were saline 1:8 and albumin 1:64. Against O negative (d) blood, titers were saline 1:4 and albumin 1:8. A change to intravenous method of injection of the antigen was then decided upon. The rabbit was given five cc. of a 1:1 dilution over a three hour period. He demonstrated a minimal ataxia after the injection from which he recovered shortly. The rabbit died approximately twelve hours later. At autopsy gross examination

showed slight congestion of the lungs, liver and spleen. The heart had a dilated right auricle and ventricle. On microscopic examination, the lungs showed marked congestion with early edema and moderate atelectasis. The liver demonstrated marked congestion with some increase in fibrous tissue in the portal areas. Marked congestion of the spleen was noted with the heart essentially normal.

Rabbits number nine, ten, eleven and twelve were placed in group III. They were injected on an identical schedule with groups I and II. The heated preparation of antigen was used in a 1:100 dilution. The blood used to prepare the antigen was type O with Rh genotype of CDe/cDe.

On the twenty-eighth day, the titers were drawn and results were as follows:

RABBIT	SALINE	ALBUMIN
9	1:32	1:8
10	1:4	1:4
11	1:4	1:8
12	1:2	1:2

The rabbits numbered nine and eleven were selected for desensitization.

Rabbit number nine was given, subcutaneously, seventeen cc. of the heated antigen preparation in divided doses over a five-day period. At the end of this period, the titers were salinel:64 and albumin 1:128. Titers against O negative (d) antigen showed saline 1:8 and albumin 1:8.

Rabbit number eleven was given five cc. of the 1:1 dilution of the heated antigen preparation in two days. Titers at this time were saline 1:16 and albumin 1:16. Seventeen cc. of 1:1 dilution were then given subcutaneously in two days in an effort to reduce

the antibody titer. At this time, titers were saline 1:32 and albumin 1:8. No evidence of any local sensitivity to the antigen was demonstrated.

Since subcutaneous injection was not producing desensitization, it was decided to use intravenous injections. Ten cc. of a 1:1 dilution of antigen were given intravenously for three days. Titers one hour after the last injection were saline 1:128 and albumin 1:64. Against O negative (d), titers were saline 1:8 and albumin 1:32.

It then appeared that it was impossible to reach a level of antigen injection which would desensitize the animal so injections were discontinued. The animals had never demonstrated sensitivity to injection of the heated preparation of the antigen.

CONCLUSIONS FROM EXPERIMENT

Desensitization or lowering of the antibody titers was not successful in this experiment. The subcutaneous injection produced only an increasing titer even when large doses of antigen were given. This indicated that some antigen was stimulating antibody production. However, the antigen reaching the antibody in the serum was not enough to reduce the antibody titer. Apparently, the antigen was inactivated before it combined with the antibody.

Intravenous injection was also unsuccessful. This was especially true of group III where very large doses only resulted in a rapidly increasing titer. There was no evidence of anaphylactic reactions in group III. This would indicate that a shocking dose was never achieved. It would appear that a considerable amount of the antigen is destroyed in the heated preparation. In order to achieve shocking doses, it may be necessary to concentrate the antigen. This may be accomplished by centrifugation as outlined by Lubinski and Portnuff.[12]

In groups I and II, it appears that shocking doses of antigen were achieved. The marked congestion of the viscera mainly in the lungs, liver and spleen as well as the marked dilatation of the right ventricle are compatible with an anaphylactic process. The fact that death was a delayed process makes it unlikely that the cause was the immediate products of the combination of antigen and antibody

[12] H.M. Lubinski and J. C. Portnuff, Influence of Heat and Formalin upon Rh agglutinogen. J. Lab and Clin. Med. 32:1,1947.

Waatkes, Weissbach, Bozicenich and Undefried[13] demonstrated that seratonin and histamine were released when anaphylaxis was produced in the rabbit. The maximum level of these substances was reached in one to two minutes with a rapid fall to normal. These substances may be responsible for the early ataxia observed in these rabbits. However, it seems unlikely that they are responsible for the death of the animal since death occurred ten to twelveshours after injection.

In view of the marked dilatation of the right ventricle in the rabbits at autopsy, it appears that the mechanism of death is probably due to spasm of the pulmonary artery or arterioles. The fact that the spasm did not occur until hours after the injection obscures the pathophysiology to this examiner.

It must be considered that these animals may have died of sepsis since it was subsequently discovered that the antigen preparations contained bacteria. Streptococcus fecalis was isolated from antigen number I. Aerobacter aerogenes was isolated from antigen number II. It does appear, however, that some reaction to the subcutaneous injection of these bacteria would have occurred if they were present at the time of injection. No reaction of this nature was observed. The immediate ataxia also gives support to an anaphylactic basis for the rabbits' death since sepsis could not have occurred in that short of a period.

13 T. P. Waatkes, H. J. Weissbach, J. Bozicenich and S. Undefried, "Seratonin and Histamine Release During Anaphylaxis in the Rabbit", J. of Clin. Investigation, 36(7) July 1947, 1115.

It would appear that antigens I and II were strong enough to produce anaphylactic shock. Therefor, small intravenous doses over an extended period might produce desensitization. However, the very delayed type of anaphylaxis demonstrated made it very difficult to know when a sublethal level was reached. As demonstrated in rabbits number one and eight, minimal reaction or no reaction at all at the time of injection may be followed by a fatal outcome. Sublethal dosage as demonstrated in rabbit number four resulted in no significant changein antibody titer.

One of the interesting facts observed in the experiment was the very minimal amount of antigen needed to produce sensitization. In groups I and II only one rabbit failed to become immunized using approximately 0.009 cc. of antigenic material. In group I, the antigen was dryed at one stage in the preparation which resulted in less antigen being present. Sensitization, however, was still produced. In group III 0.09 cc. of antigen was used. From these observations it appears that only a very few cells crossing the placental barrier may be all that is necessary to produce maternal sensitization. This may be as little as 36,000 cells in divided doses since antigen from this number sensitized eleven out of twelve rabbits.

The heated preparation used to sensitize group III was used in the hopes that it might be a pure Rh antigen. This was tested for by contrasting the titers obtained against type O negative (d) in groups I and II with those obtained in III. Group I demonstrated

equal titers against O negative (d) and O positive (D) blood. This indicates that considerable antibody was produced against the O portion of the cell. In group II the O negative (d) titers were less than O positive (D) with an average of two tubes difference. In group III there was an average of three tubes between the O negative (d) and O positive (D) titers. Adding the difference in O positive (D) and O negative (d) titers between rabbit groups I, II and III gives an average difference of one tube in those rabbits sensitized with cell stromata (groups I and II) and a three tube difference in those sensitized with the heated preparation (group III). This suggests that the heated antigen preparation is somewhat less O antigenic than is the cell stromata antigen. It may well be that the heated preparation is a pure Rh antigen. The slight titer against O antigen may be due to the few O cells which were undoubtedly in the preparation.

SUMMARY

Numerous attempts at desensitization of mothers to prevent erythroblastosis have been performed without success. The present work is an experiment in which twelve white, two-thirds grown, male rabbits were sensitized to type O Rh positive (D) human blood.

Sensitization was performed using repeated doses of small quantities of two types of antigen. Two groups of four rabbits each were sensitized with cell stromata. A third group was sensitized using a preparation obtained by heating the cells at 56°C. for seventeen minutes at a Ph of 7.2.

After sensitization was produced, attempts were made to lower the antibody titer by using large quantities of antigen. Subcutaneous injection of antigen was first attempted but found to produce only rising titers. Intravenous injection of relatively large doses of antigen was then used. In groups I and II, sensitized with cell stromata, it was found that sublethal dosage of antigen did not lower the antibody titer. Lethal intravenous dosage was followed by immediate ataxia and death within twelve hours, probably on the basis of pulmonary artery spasm.

In group III, in which the heated preparation of antigen was used, subcutaneous dosage again produced only a rising titer. Intravenous injection of antigen even when used in very large dosage did not reach a shocking level in these rabbits.

It would appear that once sensitization has occurred it is very difficult to inject sufficient antigen to result in a decrease in the titer. High doses of antigen also appear quite toxic.

By running parallel titers against O positive (D) and O negative (d) blood an attempt was made to distinguish between antibodies against O antigen and the Rh or (D) antigen. From these studies it would appear that the heated preparation is relatively a much better big D than O antigen. It may well be a pure (D) antigen with some type O cell contamination.

Also of interest was the very minute amount of antigen necessary to produce sensitization when given in divided doses. It appears that antigen from as little as 36,000 cells sensitized the rabbits used in this experiment.

CONCLUSIONS

1. Sensitization of rabbits to human type O Rh positive (D) blood extracts is easily accomplished.
2. Only very minimal quantities of blood extract are needed to produce sensitization when given in divided doses.
3. Desensitization using larger doses of antigen is very difficult to accomplish. The subcutaneous method is entirely ineffective in producing desensitization. It would appear that the antigen is inactivated at the injection site.
4. The intravenous method for desensitization is difficult to control and very dangerous. Lethal doses of antigen may produce only mild ataxia or no evidence of toxicity immediately, and yet end fatally within twelve hours.
5. Cell stromata produces antibodies of about equal strength against the O and Rh antigens. The heated preparation of antigen results in antibodies primarily against the Rh antigen. It would appear that the heated preparation is a relatively pure Rh antigen.

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