THE RECOGNITION OF FOUR RED CELL ANTIGEN-ANTIBODY SYSTEMS IN THE RABBIT

By D. H. HEARD

Department of Pathology, University of Cambridge

INTRODUCTION

In this paper an account is given of iso-antibodies which will allow four red cell antigen-antibody systems to be identified in the rabbit. Three of these appear to form an allelomorphic series, the fourth seems to be inherited independently. This work developed out of an investigation in which an attempt was made to produce haemolytic disease of the newborn in the rabbit experimentally (Heard, Hinde & Mynors, 1949). All the serological characteristics of the human disease could be reproduced, but the baby rabbits remained well. This experiment was first made as long ago as 1933 by Keeler & Castle, who proved that while maternal antibody could pass from the mother to the foetus, the baby rabbits suffered no ill effects and must have 'neutralized' the antibody. In 1948 we used an adaptation of the anti-globulin direct sensitization test (Coombs, Mourant & Race, 1946), and by this means could demonstrate that though the baby rabbit did not appear ill, maternal iso-antibody was affixed to its red cells.

Although the rabbit may not be very useful in the study of haemolytic disease of the newborn, the injection of red cell iso-antibody into newborn rabbits made them anaemic and jaundiced and the antibody could be demonstrated attached to their red cells. The signs and symptoms shown by the baby rabbits varied with the dose of antibody injected. The difference between rabbits which had large doses of incompatible serum given parenterally and were sick, and well rabbits in which the antibody reached the red cells by natural channels, may have been one of dosage; had the dose of antibody been larger these rabbits might have been sick too, as were those described by Kellner & Hedal (1953*b*). However, the factors determining the amount of antibody on the rabbit's red cell are probably rather complicated. In another paper (Heard, 1955) it is shown that the cells of some rabbits absorb more homologous antibody than others, and that this may be due to a variation in the number of antigen sites available. The amount of antibody which red cells will absorb from a serum will depend on their own absorptive power and on the amount of antibody in the serum.

In this paper four immune rabbit iso-antisera each containing a single antibody are described. No study has been made of naturally occurring iso-antibodies, which are found in the rabbit but are weak and relatively rare. An iso-agglutinin found in the serum of a female adult rabbit, which has never been inoculated with rabbit blood, may be not one of the weak, rather rare iso-agglutinins analagous to the naturally occurring human anti-*A* and anti-*B* agglutinins, but an immune antibody formed as a result of iso-immunization of pregnancy. In the author's experience this is uncommon. In one such case the red cells of a litter of apparently

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normal baby rabbits used as controls for the anti-globulin direct sensitization test, were weakly positive to the test and the mother's serum showed an antibody titre of 8–16. In over fifty other normal families the cells of all of the young were negative to this test, and no agglutinins or incomplete antibodies were found in the mother's serum. On the other hand, Kellner & Hedal (1953*a*) found natural iso-immunization of pregnancy in five out of ten rabbits. Natural iso-immunization of pregnancy produces weak agglutinins in the mother's serum soon after delivery and weak direct sensitization of the baby's corpuscles by maternal iso-antibody.

SURVEY OF PREVIOUS WORK ON THE BLOOD GROUPS OF THE RABBIT

Robertson & Rous discovered immune iso-agglutinins of the rabbit in 1922. They were studying the effects of daily transfusions on rabbits which led to massive agglutination of the animal's own blood even whilst it was being shed; they separated this phenomenon into the agglutination of donor cells still circulating in the recipient by immune iso-antibody, and agglutination of the recipient's cells by auto-agglutinins, which were effective at room temperature and below but inactive at 37° C. They made some interesting investigations into the auto-agglutinin but left the immune iso-antibody without further study.

In 1929 Levine & Landsteiner published a paper describing experiments made some time previously when they had transfused rabbits with rabbit blood. Some of the recipients developed immune iso-agglutinins active at 37° C., from which the authors deduced a number of individual differences in the blood of rabbits. A second paper in 1931 gave more detail concerning the earlier history of work on iso-agglutinins in the rabbit and described improvement of immunization by inoculating with haemolysed blood.

At the same time, Fischer & Klinkhart were searching for naturally occurring iso-agglutinins and injecting rabbits to produce immune iso-agglutinins. Their first experiments were unsuccessful, but later they succeeded in finding immune iso-agglutinins, and their work is summed up in a paper by Fischer (1935).

Altogether, Fischer found five blood group antigens, which he labelled K_1 (earlier A), K_2 (earlier B), K_3, K_4 and K_5. K_1 and K_2 were found first and allowed him to identify four blood groups: K_1 plus K_2, K_1 alone, K_2 alone, and a group carrying neither antigen. These occurred independently of K_3, K_4 and K_5.

K_1 and K_2 were found only on the red cells and not in the serum nor on other tissue cells; they were destroyed by heating to 70° C. for 15–20 min. They were able to fix complement, but this property too was destroyed by heat.

K_3 and K_5 were found on the tissue cells as well as on the red cells but not in the serum. They were insoluble in alcohol and resistant to boiling.

Keeler & Castle (1933) described how they received some serum and stock rabbits from Levine & Landsteiner. The serum they were able to match by injecting washed red cells which could be agglutinated by the serum into rabbits whose cells could not be so agglutinated; this serum they called anti-H_1 and its antigen H_1. They collaborated with Fischer & Klinkhart whose sera they used to demonstrate an

antigen which they called H_2. Finally, they showed that H_2 corresponded with Fischer's K_1 and H_1 with K_2 (Keeler & Castle, 1933, footnote, p. 410).

Castle & Keeler (1933), and also Keeler & Castle (1934*a*, *b*), using their antisera H_1 and H_2 demonstrated four blood groups in the rabbit: those carrying the antigens H_1 and H_2, H_1 only, H_2 only, or neither. They considered that H_1 and H_2 were allelomorphs and behaved as dominant characteristics obeying Mendel's laws.

Keeler & Castle (1933) also showed that the antigens are present on the red cells of 15-day-old rabbit embryos and that they are present, in adult animals, only on the erythrocytes and not on other tissue cells. In addition, they considered that the antigens were incapable of crossing the placenta, while the antibodies could and did.

Knopfmacher in 1942 described another antigen, H_6, which was inherited independently of H_1 and H_2. He also stressed, as did Kellner & Hedal (1953*b*), that individual rabbits' cells vary in their agglutinability, and that strongly reacting cells will absorb antibody out of a serum more efficiently than poorly reacting cells. He found by statistical methods that the homozygous state was associated with strongly agglutinating cells and the heterozygous with cells giving weak reactions. He observed also that the antigens H_1, H_2 and H_6 were destroyed by heating to 70° C. for 15–20 min.; also that chloroform, acetone, ethyl alcohol and the enzymes trypsin and steapsin destroyed the antigenic effect of H_1 cells.

Thus much work on rabbit blood groups was done before the 1939–45 war. When the subject was reopened after the war it was found that the sera and the rabbits whose blood groups were known had perished; a fresh beginning had to be made. In 1953 Kellner & Hedal published two important papers (1953*a*, *b*), in one of which (1953*a*) they described the isolation of two allelomorphic antigens which they called '*G*' and '*g*'.

Post-war workers can use adaptations of methods developed during and after the war to recognize 'incomplete' or non-agglutinating forms of iso-antibodies. Kellner & Hedal found their antibodies both in the agglutinating form and also in an 'incomplete' form which could be detected by using guinea-pig anti-rabbit-globulin serum, by treatment of the antigen with trypsin and by haemolytic tests using guinea-pig complement. Albumin and normal rabbit serum as diluents were disappointing and detected the incomplete forms of the antibody very badly. The antigens '*G*' and '*g*' were found only on the red cells and not on tissue cells or in serum.

EXPERIMENTAL STUDIES

Antisera which clearly contained antibodies of different specificities were first chosen for examination. After some preliminary experiments with methods elaborated in recent years for testing human red cell iso-antibodies, it was decided that, for the first analysis of an antiserum, information could be obtained most economically as follows. The antiserum was first tested against the cells of a panel of rabbits by both the direct agglutination test and the antiglobulin sensitization test. It was then titrated against all the red cells with which it had reacted. Lastly, samples of the antiserum were absorbed, each with the cells from one rabbit

chosen from the panel. The absorbed serum samples were then tested by both the direct agglutination test and the anti-globulin sensitization test against all the red cells on the panel which had been agglutinated by the original serum. The material and methods for this examination are described below, with the results and conclusions which can be drawn from them.

MATERIALS

(*a*) *Antisera.* Each rabbit yielding antiserum received blood from one rabbit only. Six to eight intraperitoneal injections of citrated whole blood in doses increasing from 0·5 to 2·0 ml. were given on alternate days and the animals were bled 10 days after the last injection. In some cases blood was collected during the first 10 days following parturition, because a rise in the titre of maternal antibody had been frequently observed at that time in experiments on the production of haemolytic disease of the newborn.

Samples of serum taken from a rabbit at different bleedings were pooled, provided that their titres were at least 5.

In this way five sera were obtained in quantity sufficient for the experiments to be described and were designated serum I, serum 1275, serum 5, serum 1561 and serum 1471.

Table 1 records the laboratory numbers of the eight donor and recipient rabbits producing these five antisera; Table 2 shows the five sera and the donor cells used to produce them.

Table 1. *Summary of origins of sera analysed*

Cells of rabbit no.	13	were injected into rabbit	1	and so produced serum	I
Cells of rabbit no.	17	were injected into rabbit	1275	and so produced serum	1275
Cells of rabbit no.	1471	were injected into rabbit	5	and so produced serum	5
Cells of rabbit no.	1511	were injected into rabbit	1471	and so produced serum	1471
Cells of rabbit no.	1511	were injected into rabbit	1561	and so produced serum	1561

Table 2. *Reactions of rabbit red cells to the sera investigated*

Cells from rabbit no.	Reactions of red cells to native sera				
	I	1275	5	1561	1471
1	−	W	+	−	−
1275	−	−	−	×	×
5	+	+	−	×	×
13	+	+	+	−	W
17	+	+	−	−	−
1471	−	−	+	−	−
1511	−	−	−	+	+
1561	+	+	+	−	−

× = not tested. Rabbit died before sera was ready for use.
W = reacted weakly with serum used neat.

Antisera were inactivated at 56° C. for 30 min. and stored at − 20° C.

(*b*) *The panel of rabbits.* The panel consisted of a collection of fifty-two rabbits which was maintained throughout the duration of the experiment; they were of

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mixed breeds and of necessity a number of them were close relatives. It was not, unfortunately, possible to collect a panel of fifty rabbits which were a random sample of a population.

EXPERIMENTAL METHODS

The experimental methods used were adaptations and modifications of the standard techniques used in human blood-grouping work. The most important departure from common practice is that the diluent used throughout this work, unless otherwise stated, was a 3·33 % solution of magnesium sulphate ($MgSO_4.7H_2O$). In physiological saline solution, but not in 3·33 % $MgSO_4$, rabbit red cells often show weak non-specific agglutination with controls which appear 'sticky' when examined microscopically (Heard *et al.* 1949).

The direct agglutination test (D.A.T.). One drop of serum of the chosen dilution was mixed with one drop of a 2 % suspension of thrice-washed red cells in a rimless precipitin tube, 5 × 50 mm. (hereafter referred to as an *Rh* tube) and incubated at 37° C. for 1 hr. The test was then read macroscopically and microscopically. Control reactions both negative and positive were also set up.

The indirect sensitization test (I.S.T.). This test is an adaptation of the anti-globulin reactions of Coombs, Mourant & Race (1945), and was used to test the sera for 'incomplete' non-agglutinating or sensitizing antibodies. One drop of serum of the chosen dilution was mixed with one drop of a 2 % suspension of red cells in an *Rh* tube and allowed to stand for 30 min., or sometimes 1 hr., at 37° C. The sensitized cells were then washed three times in magnesium sulphate solution and the supernatant fluid was removed; one drop of a suitable dilution of goat anti-rabbit-globulin serum which had been absorbed with rabbit cells was added. After incubation at 37° C. for an hour the test was read both macroscopically and microscopically.

Titration of a serum. To titrate a serum the direct agglutination test and the indirect sensitization test were both used. Serial dilutions of the serum were made in bulk and distributed into sets of rimmed precipitin tubes 7·5 × 50 mm. (hereafter called complement-fixation tubes) in amounts of 0·1 ml. for each dilution so that the serum could be titrated against the red cells from 8 to 12 different rabbits at one time. To allow for comparison between results obtained on different occasions a serum was always retitrated against the cells from one rabbit used on a previous occasion. The dilutions were made with a syringe. The small error due to carrying over one dilution into another was kept constant and was considered to be unimportant. 0·1 ml. of a 2 % suspension of the appropriate cells was added to each tube and the tubes were well shaken. Two drops of the suspension in each tube were transferred to *Rh* tubes, allowed to settle at 37° C., and read for direct agglutination macroscopically and microscopically.

The remaining suspensions in the complement-fixation tubes were allowed to stand at 37° C. for an hour, then washed three times and the sensitized cells resuspended in 0·1 ml. of diluent. One drop was then transferred to each of two *Rh* tubes, one containing anti-globulin serum and the other diluent. The *Rh* tubes were shaken and allowed to stand at 37° C. until the contents had settled before reading microscopically and macroscopically.

Absorption of a serum. Samples of antisera were absorbed, each with red cells from a different animal. An equal volume of packed washed red cells was added to the undiluted serum and the two were well mixed. The serum was allowed to remain in contact with the cells for 10–15 min. at 37° C. It was then centrifuged for 10–12 min. The supernatant serum was removed and added to a fresh volume of packed red cells. The process was repeated until the cells used for the absorptions were no longer agglutinated by the serum, using either the direct or indirect sensitization tests.

RESULTS

Each of the sera chosen for analysis was subjected to the procedures described above; as a result, four antigen-antibody systems could be recognized and sera were prepared, each of which contained only one antibody. For ease in discussion and until their relationships with other red cell antigen-antibody systems in the rabbit are established, the four antigens have been temporarily labelled *Z*, *Y*, *X* and *W*.

The isolation of the Z antibody. Serum I was chosen for analysis first; it was considered that this serum might contain only one antibody or a commonly occurring mixture of antibodies, because five others behaving in the same way had been found. It reacted with the cells of thirty-six of the fifty-two rabbits on the panel. Samples were absorbed with the cells of seven different rabbits chosen at random; the cells of each of these rabbits removed all the antibody from the serum, which therefore seemed to contain only one antibody. Unfortunately, the titre of serum I was low; therefore, serum 1275 was investigated to see if it could be used as a source of the same antibody. The direct agglutination titre of serum 1275 varied, against different cells, from 16 to 256 and the indirect sensitization titre from 256 to 1024, but the serum reacted strongly with the cells of the same thirty-six rabbits which were agglutinated by serum I and very weakly with those of two others. After absorption with either of these weakly reacting cells, the other thirty-six were still strongly agglutinated. Fourteen samples of the serum were absorbed, each with cells from a different rabbit, chosen from these thirty-six, and all the antibody was removed from each sample. Serum 1275 was therefore judged to contain one high-titred antibody and one very minor one which could easily be removed by absorption or diluted out. The antigen with which the high-titred antibody reacted was called *Z* and the antibody anti-*Z*.

The isolation of the Y antibody. Serum 5 was next analysed. This serum came from a *Z*-positive rabbit inoculated with *Z*-negative cells and was therefore unlikely to contain *Z* antibody. The titre varied, according to the cells used, between 8 and 128 (D.A.T.) and 64 and 256 (I.S.T.). The cells from twenty-three animals out of fifty-two were agglutinated by it, and samples of the serum were absorbed with fifteen different kinds of cell. When the absorptions were thorough enough the antibody in the serum could be absorbed out by each kind of cell. As with serum 1275, the cells of a few reacted very weakly with undiluted serum 5; this very low-titred antibody could be absorbed out without affecting the major antibody. Serum 5 was therefore judged to contain one main antibody, which was named anti-*Y*.

The isolation of the antibodies for X and W. These antibodies will be considered together as they were both found in two sera. The cells of rabbit 1511 were selected as antigen because they were negative for both *Z* and *Y*; they were injected into rabbits 1561 and 1471. The resulting sera both appeared to contain two antibodies, one a direct agglutinin and the other in the 'incomplete' form; in addition, serum 1471 appeared to contain a third antibody in low titre. Absorption tests confirmed this; in both sera, by suitable differential absorption, the two major antibodies could be separated and antisera prepared containing only one antibody, either anti-*X* or anti-*W*. The third, low titre, antibody in serum 1471 could easily be absorbed out without affecting either of the stronger ones: its titre was, however, too low for it to be isolated by itself.

OBSERVATIONS ON THE PROPERTIES AND BEHAVIOUR OF *Z*, *Y*, *X* AND *W* ANTIBODIES

Before describing the properties of *Z*, *Y*, *X* and *W* antibodies a difficulty must be mentioned, which has been noted by other workers and is fully discussed in another paper (Heard, 1955). Rabbits vary in the susceptibility of their red cells to agglutination by homologous iso-antisera, some being strongly agglutinated and others poorly. It will be noticed that the titre of the anti-*Y* serum varied, according to the cell used for the titration, from 8 (D.A.T.) and 128 (I.S.T.) to 64 (D.A.T.) and 256 (I.S.T.). This would suggest the presence of two antibodies, if it were not that poorly agglutinated cells, if enough of them are used, can absorb out completely the antibody which agglutinates 'strong' cells. There is a stage during the absorption of serum by poorly agglutinating cells when it will no longer agglutinate 'poor' cells but will agglutinate 'strong' cells. Incomplete absorption of this kind can—and did—lead to much confusion as to the number of antibodies a serum really contains. It is suggested (Heard, 1955) that this difference between cells may be due to a difference in the number of antigen sites available for the antibody under consideration.

Rabbit red cell iso-antibodies can act either as direct agglutinins or as 'incomplete' antibodies. The former are subjected to the limitations imposed by the phenomenon mentioned above; the latter are well demonstrated with anti-rabbit-globulin serum such as the one used in these experiments, which was prepared in a goat. The titre of a direct agglutinin (cf. sera 1275 and 5) is usually augmented by the use of anti-globulin serum, and the difference between strongly and poorly agglutinating cells is usually reduced to not more than one dilution.

Immune haemolysis. The red cell antigens from different species differ considerably in their ability to show, when sensitized by antibody, haemolysis in the presence of complement. Human cells only lyse with difficulty, and the method is virtually not used in human blood-grouping work. On the other hand, bovine cells lyse so easily that haemolytic tests provide the usual method of demonstrating their antigens. Rabbit cells are intermediate, but nearer human than ox. Tests have been made using guinea-pig and rabbit complements, with saline as a diluent instead of magnesium sulphate solution, and the results may be summarized thus.

The antigens *X* and *W* cannot be detected by haemolysis with guinea-pig or rabbit complement. The antigens *Z* and *Y* can be detected by guinea-pig complement but not by rabbit complement. Guinea-pig complement, which on a sheep cell-rabbit-anti-sheep serum system had a titre of 80, had to be used at a dilution of 1 in 5 or 1 in 10 to show any degree of haemolysis.

Allowing complement to act on washed sensitized cells was a method which was a little more sensitive than letting antigen and antibody react in the presence of complement. *Y* was less sensitive than *Z* to haemolysis; for instance, with guinea-pig complement used 1 in 10 the titre of the antibody was only 8 when the serum was titrated for agglutination. Against the same red cells the D.A.T. titre was 128 and the I.S.T. 256. If complement was used at a suitably low dilution the titre of anti-*Z* serum on haemolysis was the same as that found by the direct agglutination test and there was a difference between poorly and strongly agglutinating *Z* positive cells. This finding is interesting and the explanation is likely to be complicated. An attempt can be made to explain it on the hypothesis, put forward in the accompanying paper, that the degree of haemolysis may depend on the number of available antigen sites as perhaps does the degree of agglutination. Strongly agglutinating cells at a given high dilution of serum may have more sites satisfied by antibody, and ready for complement to join on to, than poorly agglutinating cells have, and thus will lyse at a higher dilution of serum than 'poor' cells will.

Direct agglutination after treatment of the antigen with trypsin. Morton & Pickles (1947) showed how by treatment of the human *Rh*-positive red cell with trypsin it could be changed in such a way as to allow it to agglutinate when sensitized by an 'incomplete' *Rh* antiserum. Other enzymes can have the same effect, and therefore some experiments adapted from the methods of Morton & Pickles were made to find out the effect of trypsin on rabbit cells. A 1% solution of trypsin (British Drug Houses preparation dissolved in M/15-phosphate buffer at a pH of 7·2) was used. Cells were treated with the trypsin solution for 30 min. at 37° C., washed once and then allowed to react with serial dilutions of the serum at 37° C. Cells were also sensitized with serial dilutions of serum, washed once and allowed to resettle at 37° C. in the presence of a 1% solution of trypsin. The following results were obtained. The tests in which cells were treated with trypsin before antiserum were more sensitive by a dilution or so than the tests made by adding trypsin to the already sensitized cells. The titres of the sera against trypsin-treated cells were as high as, and often a tube higher than, the titres found with anti-globulin serum. There was no difference between strongly and poorly agglutinating cells. All cells behaved as strongly agglutinating cells. Thus trypsin is a good reagent for the detection of the systems *Z*, *Y*, *X* and *W*, as Kellner & Hedal (1953*a*) found when they were working with *G* and *g*. Kellner & Hedal (1953*a*), however, drew attention to irregular strong non-specific agglutination of rabbit cells when treated with trypsin in a saline medium. A comparison of the effects of using saline and magnesium sulphate as diluents has been made, and strong non-specific agglutination was often found with saline but not with magnesium sulphate solution.

Direct agglutination in the presence of bovine albumin. Diamond & Denton (1945) described a method for the detection of 'incomplete' *Rh* antibodies by allowing

the cells and sera to react in the presence of a 20% solution of bovine albumin. Bovine albumin proved a disappointing reagent to use in the detection of rabbit iso-antibodies. It had little effect save to augment slightly the titre found by direct agglutination. The incomplete anti-*W* antibody in albumin had a titre of four; using anti-globulin serum or trypsin it was 64. Kellner & Hedal (1953*a*) also had negative results from experiments using bovine albumin on the rabbit red cell antigen-antibody systems they handled.

Direct agglutination in the presence of normal rabbit serum. Some titrations were made using normal rabbit serum as a diluent for both serum and cell suspensions. The results were on the whole the same as with bovine albumin.

The effect of temperature on the demonstration of the antibodies. The titres of all antisera were higher by a dilution or so when the tests were incubated at 37° C. than at room temperature, as is usual for the immune antisera of red cell antigens.

OBSERVATIONS ON THE INHERITANCE OF THE ANTIGENS *Z*, *Y*, *X* AND *W*

The available rabbits did not allow a planned investigation of the inheritance of the antigens *Z*, *Y*, *X*, and *W*, but some observations made during the course of the work described above support the following observations. The four antigens, as is of course well known for the blood groups of the rabbit, are inherited according to Mendel's laws. *Z* and *Y* appear to be inherited as dominant characteristics; together they allow four blood groups to be recognized: *Z* positive, *Y* positive, *Z* + *Y* positive animals and animals carrying neither antigen, which for the moment may be called 'group *O*'. This suggests that *Z* and *Y* may be two of the ingredients making up an allelomorphic series of three. It is therefore important to discover whether *Z* and *Y* are on the same chromosome or if they are alleles. Group *O* offspring were only produced when both parents were group *O*, or when one parent was group *O* and the other carried *Z* or *Y* in the heterozygous state. A *Z* + *Y* parent has never been known to have group *O* offspring under any conditions. These facts suggest that *Z* and *Y* are alleles. This much was known when anti-*W* was found; amongst fifty-two rabbits tested with anti-*W* none had cells which carried all three antigens and conversely no cells lacked all three. '*O*' in the genotypes so far worked out with anti-*Z* and anti-*Y* can be replaced by *W* without any exceptions. 'Group *O*' animals can be considered as homozygous group *W*; *Z* + *Y* animals are invariably *W*-negative, so also are homozygous *Z* and homozygous *Y* animals. Thus the evidence so far strongly suggests that *Z*, *Y* and *W* are an allelomorphic series; more animals will have to be tested before the suggestion becomes a certainty. On the evidence so far available *X* appears to be inherited as a dominant characteristic independently of *Z*, *Y* and *W*.

SUMMARY

Four red cell antigen-antibody systems in the rabbit are described.

Three of these systems, *Z*, *Y* and *W*, appear to be inherited as an allelomorphic series. The fourth is inherited independently.

Some of the serological properties which allow these sera to be recognized are described and attention is drawn to a property of rabbit red cells, manifested by a variation in their susceptibility to agglutination by homologous antisera, which can complicate the separation of a mixture of antibodies in one serum.

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