AN EXPERIMENTAL STUDY OF HAEMOLYTIC DISEASE OF THE NEWBORN DUE TO ISOIMMUNIZATION OF PREGNANCY

I. AN ATTEMPT TO PRODUCE THE SYNDROME IN THE RABBIT

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(With 1 Figure in the Text)

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INTRODUCTION

In 1946 our attention was drawn to the work of Nachtsheim and Klein on a hydropic condition, accompanied by erythroblastosis, which they found in some newborn rabbits of a particular strain.

They recognized the possibility that the aetiology might be analogous to that of haemolytic disease in the human infant due to isoimmunization of pregnancy, and this condition, along with certain serological investigations made by Dahr, is fully described by Nachtsheim & Klein (1947) and by Dahr (1947).

Conditions in Germany in 1946 made it difficult for Nachtsheim to continue this work and to maintain this strain of rabbits, and as he was anxious to preserve it, certain of the rabbits were sent to us to be used in our investigations.

At that time a team of workers in the Department of Pathology, University of Cambridge, was engaged in studies on haemolytic disease of the newborn due to isoimmunization of pregnancy, and it was considered that an investigation of the condition in the rabbit reported by Nachtsheim should be continued, mainly from the serological aspect. The mechanism of inheritance of the condition described by Nachtsheim is obscure and we have been unfortunate in not yet finding young from Nachtsheim's stock in which we can recognize the condition he described. However, because of the obvious importance of producing evidence of isoimmunization of pregnancy in a laboratory animal, which might then be used to investigate some of the problems of isoimmunization in man, particularly isoimmunization of pregnancy, it was decided to attempt to produce this syndrome experimentally in the rabbit.

In this paper we describe experiments which show two main results:

(1) In certain litters, from does who had been successfully immunized by the whole blood of a buck to which they were mated, the young may show *in vivo* sensitization of their red cells by maternal antibody. However, despite having sensitized red cells the young rabbits appear unaffected clinically,

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and haematological and histological examination of the young at various ages supports this clinical observation. As yet it has not been proved whether the maternal antibodies belong to the H_1H_2 or to the K_3K_5 system of rabbit blood groups, nor whether they pass into the offspring by way of the placenta or the colostrum or both.

(2) Rabbits immunized with rabbit whole blood may show, in addition to antibodies which directly agglutinate red cells, antibodies of the 'nonagglutinating', 'sensitizing' or 'incomplete' variety.

EXPERIMENTAL STUDIES

I. *Experimental procedure and collection of material*

The rabbits used in this experiment were a group of twenty females, does of mixed ancestry, and eight bucks fairly closely related to each other but also of mixed strains.

The does were immunized with citrated whole blood from the bucks and later mated. Once a doe had been immunized with the blood of a buck she was mated only to him or reimmunized only with his blood throughout the whole experiment.

Three months later the does (with the exception of one, doe A, Table 1) were reimmunized and then all were remated. After another 5 months they were all remated without further reimmunization.

The does were bled before immunization and at intervals thereafter, and their sera examined for antibodies against the red cells of the buck. The details of immunization and the times of bleeding are given in Appendix I.

The offspring from these three sets of litters were examined for *in vivo* sensitization of their red cells by maternal antibody and for any clinical manifestation of illness resulting from this. A proportion of the young were further examined by the haematological and histological methods for changes or lesions resulting from *in vivo* sensitization of their red cells.

A number of control matings between untreated rabbits were made and experience of the serological, haematological and histological findings in normal baby rabbits was obtained at ages comparable to those at which young from the experimental group were examined. The sera of the does used for these control matings were tested for antibodies against the red cells of the buck to which they were mated.

II. *Methods*

All the methods we used were direct adaptations of standard procedures used for the examination of similar human material, and we therefore propose to give here brief descriptions of the tests we employed, together with any important modifications we found necessary, and to refer the reader to an appendix for a more detailed account.

(*a*) *Immunization of a group of does*

Blood from the chosen buck was collected with sterile precautions from the marginal ear vein into 3·4 % citrate solution and the mixture was injected immediately into the doe.

The details of immunization are given in Appendix I, but for the first course the injections were given intravenously and most animals had two inoculations of the equivalent of 7–8 ml. whole blood at intervals of 5 to 6 days. For the second immunization four injections, all by the intraperitoneal route, were given at twice weekly intervals; the amount of blood given was 0·5, 1·0, 1·5 ml., and finally 2·0 ml.

(*b*) *Methods used to test rabbit sera for antibodies against rabbit red cells*

Rabbit sera were tested for (i) direct agglutinins by simple direct agglutination of the appropriate red cells, and (ii) for 'non-agglutinating', 'sensitizing', or 'incomplete' antibodies by both an indirect sensitization test, using a goat anti-rabbit globulin serum, and by a modification of Diamond's albumin tube test (Diamond & Denton, 1945). These tests are all direct adaptations of the comparable methods fully described in the Medical Research Council's Memorandum (1948) on the *Rh* blood groups.

The most important modification which we made and maintained throughout this whole work was to use 3·33 % Analar magnesium sulphate ($MgSO_4$—$7H_2O$) as a diluent for cells and sera instead of 0·9 % sodium chloride solution. We found when we first began to work with rabbit red cells that we often obtained weak non-specific agglutination of red cells in 0·9 % saline solution, which could be extremely confusing. D. J. R. Lawrence, who did the initial serological work on Nachtsheim's rabbits when they first arrived in Cambridge, found that this non-specific agglutination could be overcome by using magnesium sulphate as a suspension medium.

Other procedures which we kept constant were to store all our sera without sterile precautions at − 20° C., to inactivate them by heating to 56° C. for 30 min. before use and to use a 1 % suspension of red cells in the tests.

Direct agglutination. The exact method for testing a serum and titrating its antibody strength is given in Appendix II. Briefly the serum was put up with an equal volume of a 1 % suspension of the appropriate red cells at 37° C. for 2 hr. and then read macroscopically and microscopically.

If the undiluted serum agglutinated the red cells it was titrated in a standard manner, using doubling dilutions of serum and equal volumes of a 1 % red cell suspension. These preparations were incubated

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at 37° C. for 2 hr. and then read macroscopically and microscopically.

Indirect sensitization test for 'non-agglutinating' antibodies using goat anti-rabbit globulin serum. If a serum failed to show direct agglutination of the appropriate red cells it was tested by allowing the serum to sensitize the cells for 2 hr. at 37° C., washing the sensitized cells three times and adding a suitably prepared goat anti-rabbit globulin serum. The cells were shaken up and allowed to settle at 37° C. before reading macroscopically and microscopically. This procedure was also adopted for the titration of a serum. The cells were sensitized with doubling dilutions of serum and then treated as above. This test is a direct application of that described by Coombs, Mourant & Race (1945). The details of the procedure are given in Appendix II and preparation of the antiglobulin serum is described in Appendix III.

Tests for 'non-agglutinating' antibodies using bovine albumin solution. Armour's bovine albumin (30 %) solution for *Rh* testing undiluted was used and diluted to give 20 % of albumin. The cells were allowed to be sensitized by the serum at 37 °C. The supernatant fluid after sedimentation of the cells was removed and replaced by the albumin solution. The cells were shaken up and then allowed to settle for 1 hr. at 37° C., centrifuged for 1 min. and the results read microscopically.

(*c*) *Method used to show* in vivo *sensitization of baby rabbit red cells by maternal antibody*

The direct sensitization test. Red cells taken from 1- or 2-day-old rabbits into a solution of 0·6 % sodium citrate in 3·33 % magnesium sulphate solution were washed three times and then the goat anti-rabbit globulin serum referred to previously, and in Appendix III, was added: the cells were later examined for agglutination. Again this test is a modification of the direct sensitization test of Coombs, Mourant & Race (1946), and the detail of our technique is given in Appendix IV.

(*d*) *Haematological examinations*

The baby rabbits were anaesthetized with ether: the thoracic cavity was opened and blood, taken from the heart by syringe before the circulation had stopped, was collected in a bottle containing a suitable amount of oxalate crystals. For haemoglobin estimation 0·1 ml. oxalated blood was diluted with 20 ml. alkaline solution (0·4 % ammonia solution was used) and examined with a photoelectric colorimeter. Imprints were taken from the liver and spleen, and marrow smears were made from the upper end of the femur. These imprints and smears and the blood smears were stained with Leishman and Giemsa: reticulocytes were stained with brilliant cresyl blue.

Differential counts were made on between 100 and 200 white blood cells from peripheral blood smears of each rabbit, and on 500 marrow cells from each smear. A differential count of the erythroid cells in the splenic imprints was made on 200 cells from each slide. The liver imprints were examined for evidence of haemopoiesis and particularly for early members of the erythroid series.

(*e*) *Histological techniques*

An autopsy was commenced as soon as the blood samples had been taken.

Pieces of liver, spleen, kidney, suprarenal and, in a few cases, brain were taken for histological examination from the freshly killed animal. They were fixed in 10 % formol-saline for 6–12 hr., followed by formol sublimate for 12–24 hr. The blocks were dehydrated in alcohol, cleared in chloroform and embedded in paraffin. Sections were stained in the routine manner with Ehrlich's haematoxylin and eosin after treatment with picric alcohol, Lugol's iodine and 5 % sodium thiosulphate.

Femurs were fixed in 10 % formol-saline for 6–12 hr., followed by formol-Zenker for 8–12 hr., and decalcified in Custer's fluid for 1–48 hr. according to age. Unfortunately, the preservation of cytological detail in the haemopoietic cells was unsatisfactory, despite several modifications in technique.

Sections of liver, spleen, and bone marrow were tested for the presence of iron by the Prussian blue reaction and counter-stained with 0·5 % safranin. Mallory's modification of Turnbull's method (Russell, 1939) was also used in a few cases and gave comparable results, except that the blue tended to be deeper and the granules larger and more irregular—probably an artefact (Gomori, 1936).

Barrett's bone-marrow stain (Barrett, 1944) was used on the liver, spleen, and bone marrow of a large proportion of the cases.

Counts of the haemopoietic cells of the liver were made in every case. The average was taken of counts from two strips, 0·3 × 0·01 cm., examined under the 1/12 objective.

RESULTS

As has been described above, we immunized twenty female rabbits, each with the whole blood of the male to which they were mated; eight males in all were used. We examined the does' sera over a period of 10 months for direct agglutinins and 'incomplete', 'sensitizing' or 'non-agglutinating' antibodies against the red cells of the appropriate buck.

We also examined the offspring for evidence of fixation of these maternal antibodies on the red cells carrying the appropriate paternal antigen. In addition, we examined the young for any lesion or effect resulting from this sensitization, which could be

demonstrated by the haematological and histological techniques given above.

Our results thus fall into two categories, the findings in the mothers' sera as a result of immunization and the effects of such an immunization on their offspring.

I. *Results after immunization of female rabbits with rabbit whole blood*

(*a*) *Effects on general health of the does*

Of our group of twenty rabbits one died whilst having the second injection of her first course of immunization. Otherwise the animals remained in good condition throughout the experiment and their breeding performances did not differ significantly from similar but untreated does of the department stock.

(ii) *Results of tests on the nine antisera formed.* We found two main types of antibody; one produced direct agglutination of red cells (six examples), and the other was demonstrated only when the antiglobulin indirect sensitization test was used (three examples). Of the six antisera which produced direct agglutination there were three which directly agglutinated cells only undiluted or in their lower dilutions, but which, at higher dilutions, could be shown by the antiglobulin indirect sensitization test, to be still sensitizing cells without agglutinating them.

We tested our antisera with Armour's bovine albumin solution for *Rh* testing but with the method we used we always had negative results. We have not yet explored this finding further.

(iii) *Variation in titre level of antibody in the nine antisera.* Table 1 shows a summary of the titre levels

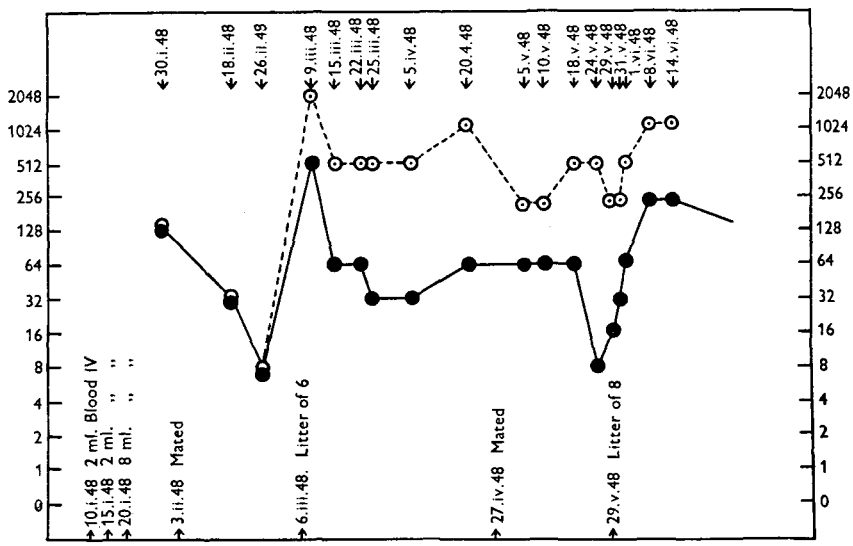

Fig. 1. Titre levels of the antibody in the serum of doe A over her first two pregnancies. Vertical columns express the titre of antibody found in the serum. Horizontal columns express the times at which, (i) sera were collected, (ii) the animal was inoculated and mated. —●—, titre of antibody shown by the direct agglutination reaction; - - -⊙- - -, titre of antibody shown by the indirect sensitization test.

(*b*) *Serological findings*

(i) *Number of animals forming demonstrable antibodies.* As we lost one animal so early in the experiment our working group may be considered to be nineteen animals. Of these nineteen, nine formed antibodies demonstrable by our tests. We tested the red cells of the remaining ten does and the bucks to which they were mated with all the antisera we had available and found that five does could have formed antibodies to the buck's cells, whilst five did not appear to be incompatible with the buck. We did not find any trace of a naturally occurring antibody against the buck's cells in the pre-inoculation serum of any of our does.

of antibody we found in our antisera over the course of the experiment, and Fig. 1 gives the results in detail in one animal, doe A, over two pregnancies.

It can be seen that there was considerable variation in the strength of the antisera and the length of time during which the antibody could be demonstrated. We found that during pregnancy there was a tendency for the antibody level to drop, only to rise usually 4–5 days post-partum, and that this was most marked when the offspring inherited paternal red cell antigens not possessed by the mother; for instance, see doe F, 3rd pregnancy, doe D, 2nd pregnancy, and doe A, 1st and 2nd pregnancies.

It will be noted also that there was much variation

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Table 1. *Summary of findings in sera of the nine does who formed antibodies over the course of the experiment related to times of inoculation, pregnancies and findings in offspring*

Doe	10 day after 1 course injectio 30. i. 4		1 week before xpected 1st rturition		1st litter		3–4 days post-partum		Prior to 2nd course of inocula-tions		10 days after 2nd course of inocula-tions, 29. iv. 48		1 week before expected 2nd parturition		2nd litter		3–4 days post-partum		Prior to 3rd mating, 5. viii. 48		1 week before expected 3rd parturition		1		4 days post-partum	
	(1)	(	1)	(2)	(3)	(4)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(3)	(4)	(1)	(2)	(1)	(2)	(1)	(2)	(3)		(1)	(2)
A	128	1	32	32	*6**	*6*	512	2048	(Rabbit not reinjected)				8	512	*8**	*2 at least*	64	512	64	256	0	0			32	128
B	—	-	2	2	*7*	.	4	4	1	16	1024	1024	32	512	*7**	*2 at least*	32	128	16	256	4	256			8	512
C	—	-	–	—	*No litter*		0	0	—	—	256	512	16	64	*4**	*4*	32	128	16	32	4	16			0	0
D	—	-	–	—	*5*	.	—	—	—	—	—	—	—	1	*8**	*4*	—	16	—	—	—	—			1	4
E	—	-	–	—	*6*	.	—	—	—	—	—	—	—	—	*No litter*		0	0	—	Trace	—	1			—	8
F	—	-	–	—	*3*	.	—	—	—	—	128	128	8	—	*1*	.	16	16	4	4	2	2			256	256
G	—	-	–	—	*No litter*		0	0	—	—	16	16	—	—	*No litter*		0	0	—	—	—	—			—	—
H	—	-	–	—	*6*	.	—	—	—	—	—	2	—	1	*No litter*		—	1	—	—	—	—			2	2
I	—	-	–	—	*10*	.	—	—	—	—	2	2	—	—	*6*	*1*	—	—	—	—	—	—			—	—

Horizontal col give data relating to a particular doe.
Vertical colum -=no antibody found but test made; 0=no test made; * susceptible offspring showed *in vivo* sensitization of the cells.
Figures in col 1)=titre of antibody reacting to direct agglutination test at date or period indicated.
Figures in col 2)=titre of antibody reacting to indirect sensitization test at date or period indicated.
Figures in col 3)=total number of offspring in 1st, 2nd, or 3rd litters (as indicated at head of column).
Figures in col 4)=total number of offspring in 1st, 2nd or 3rd litters (as indicated at head of column) whose red cell antigens were i atible with their mother's a .

in the time after immunization when the presence of an antibody was first demonstrated.

II. *Results of examination of the offspring*

The examinations of the offspring of the nineteen does from the three litters they each had during the experiment are considered together. The length of gestation, the average litter-size, the clinical appearance of the newborn rabbits and the neonatal mortality were similar to what we found amongst the young of the stock-breeding does of the department.

(*a*) *Results of direct antiglobulin sensitization test on the red cells of offspring*

We found that four does (does A, B, C and D in Table 1) had, in seven litters, offspring whose red cells were positive to the direct antiglobulin sensitization test. The litters were counted and were examined for abnormal young, stillbirths, or deaths as soon after birth as was practicable; the babies' red cells were tested within the first 48 hr. Doe A had three litters of six, eight and six offspring, all well and all with sensitized cells. Doe B had two litters each of seven offspring, all well and all save one having sensitized red cells, but the red cells of that one did not react with its mother's antiserum. Doe C had one litter of four, all well and all with sensitized red cells. Doe D had one litter of eight, all well; four had sensitized red cells, and also carried a paternal antigen incompatible with their mother's antiserum, the other four had cells negative to the direct sensitization test and also to their mother's antiserum. In addition doe E had one litter of four in which the offspring's cells were weakly sensitized.

In our experience once a doe had produced offspring with red cells sensitized by maternal antibody the susceptible red cells of the young of subsequent litters also showed sensitization.

All the other offspring tested for *in vivo* sensitization of their red cells, whether the cells reacted to maternal antibody or not, were negative; as also were the red cells of the young of all does who had no demonstrable antibody in their serum to the father's red cells.

(*b*) *Results of haematological and histological examination of offspring*

The offspring whose red cells gave a positive direct sensitization test were clinically indistinguishable from other rabbits of a similar age at all stages, and this was so whether the young were from the first or subsequent litters in which the young showed *in vivo* sensitization of their red cells. To substantiate this clinical impression by haematological and histological examinations, we killed rabbits aged 3 days and 24 days, taking two babies at a time from a litter whenever possible. We chose these dates with the findings of Sabin and her colleagues in mind (Sabin, Muller, Smithburn, Thomas & Hummel, 1936). These workers have shown in their studies of the blood and bone marrow of young rabbits that erythropoiesis is extremely active throughout the first 3 weeks of life and is at its height at the end of the second week. We accordingly took 3 days from birth as being a particularly vulnerable point and 24 days from birth as one by which the haemopoietic climax is normally over.

The rabbits can be classified into the following four groups:

(1) Babies whose red cells were positive to the direct sensitization test.

(2) Babies whose red cells were negative to the direct sensitization test, but whose mothers had formed antibodies. This group included some who were litter mates of 1.

(3) Babies whose mothers had not formed demonstrable antibodies.

(4) Babies from normal rabbits.

The numbers taken from each group and their ages are given in Table 2.

Table 2. *Number of baby rabbits* (3–24 *days*) *for each group examined both haematologically and histologically at* 3, 17 *and* 24 *days old*

Group	3 days	17 days	24 days
I	8+2*	(4)	5+(4)
II	6	—	4
III	6	(2)	2+(2)
IV	15	(7)	4+(3)

( )=peripheral blood examination only.
* Histological examination only.

Group I. Babies showing *in vivo* sensitization of their red cells by maternal antibody.

Group II. Babies whose mothers formed antibodies but whose cells were not sensitized.

Group III. Babies from mothers who were inoculated but who did not form demonstrable antibodies in their sera.

Group IV. Normal baby rabbits.

(i) *Haematological findings.* The results of the peripheral blood examinations are set out in Table 3, in which the average figure and the range of findings are given for each group at weekly intervals. The haemoglobin is reported as g. per 100 ml.; the red blood count as millions per cu.mm. and the reticulocyte count as a percentage of the red blood cells.

It will be seen that there is no significant difference in the figures from the different groups. Normoblasts (Gilmour, 1941) were always found in the 3-day-old blood smears in numbers ranging from a few hundred to nearly three thousand per cu.mm. The high figures were not confined to any one group: both high and low figures were found in

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Table 3. *Haematological findings, giving average figures and the range for each group of baby rabbits at* 3, 17 *and* 24 *days old*

	Haemoglobin		R.B.C.		W.B.C.		Reticulocytes	
	Average	Range	Average	Range	Average	Range	Average	Range
At 3 days:								
Group I	11·7	8·1–16·0	3·8	2·7–4·7	1695	738–4375	16·0	8·6–25·8
Group II	12·3	10·3–15·8	4·1	3·5–4·8	1222	578–2194	9·3	6·7–14·7
Group III	12·1	10·8–14·2	4·2	3·6–4·7	1637	1092–3010	10·9	10·0–11·7
Group IV	14·7	12·9–16·7	4·1	3·6–4·6	1843	750–3119	12·1	8·5–22·3
At 17 days:								
Group I	12·1	11·8–12·5	5·7	5·4–6·2	4642	3870–5300	12·2	8·2–16·2
Group II	14·2	12·6–15·0	5·6	4·8–6·2	4000	2900–4900	11·2	5·5–23·2
Group III	14·0	13·9–14·1	5·6	4·9–6·4	4700	4600–4800	—	—
Group IV	13·4	12·9–14·0	5·5	4·9–5·7	3700	2450–5802	9·1	5·2–14·1
At 24 days:								
Group I	12·4	10·2–14·6	5·9	4·9–7·1	4949	1700–8600	7·3	4·2–10·4
Group II	11·1	10·6–12·4	4·9	4·3–5·6	2653	1150–4750	6·3	4·5–9·1
Group III	13·1	11·9–14·4	5·8	5·2–7·0	2850	1300–5300	3·1	0·9–4·6
Group IV	11·6	10·8–12·2	4·9	4·1–5·5	3603	686–8300	7·9	4·7–12·1

Haemoglobin expressed as g./100 c.c. Red blood cells (R.B.C.) expressed as millions per cu.mm. White blood count (W.B.C.) expressed as absolute figure per cu.mm. Reticulocytes expressed as a percentage of the red blood cells.

Group I and several of the high counts amongst the normal rabbits of Group IV. Two 3-day-old Group I rabbits whose splenic imprints are separately reported below were altogether exceptional in having normoblastic counts of 5000 and 7000 respectively per cu.mm. Apart from these two cases there were none in which a high normoblast count could be related to erythropoietic activity elsewhere. The reticulocyte count was no higher in rabbits with thousands of normoblasts in their peripheral blood than in those with very few. In just under half the blood smears taken at 3 weeks normoblasts were still to be found, but not more than 260 and usually less than 100 per cu.mm. In the peripheral blood no erythroid cells more primitive than normoblasts were seen.

The differential white blood counts showed in each group the expected increase in the lymphocyte count from 40 to 80 % of the total in the interval between the 3-day and the 24-day counts, and the polymorphonuclear leucocyte count fell proportionately.

The differential marrow counts at 3 days old showed a great predominance of erythroid cells: 92·5 % of the marrow cells in each group belonged to this series. At 24 days old this figure had fallen to 71 % in Groups I and IV and to 80 % in Groups II and III. Again there appears to be no significant difference between the groups.

The liver imprints from the 3-day-old rabbits of all groups showed great erythropoietic activity with erythroblasts at all stages of maturity as well as early and late myeloid cells, but no differential counts were made. At 24 days no nucleated erythroid cells were found in either liver or splenic imprints.

The splenic imprints from the 3-day-old normal rabbits (Group IV) showed a fairly consistent picture: there were either no nucleated erythroid cells or numerous pyknotic normoblasts. Imprints from the spleens of the other groups showed a varying quantity of intermediate or late erythroblasts which formed up to 12 % of the differential erythroid counts, the remainder being normoblasts. Further, the imprints in which the higher erythroblast counts occurred were crowded with nucleated cells and suggested considerable erythropoietic activity in the spleen. This activity was not confined to Group I, though all its members showed it to some extent and the three most conspicuous examples came from this group. Even these three examples, however, do not show activity as pronounced as may be seen in the splenic imprints from 1-day-old rabbits, apart from the two following cases, both from the first litter of doe A. The splenic imprints from these two 3-day-old rabbits showed some peculiar features. In them a quantity of large phagocytic cells was seen containing up to eight erythrocytes embedded in the cytoplasm, as well as granules giving a positive Prussian blue reaction. Erythroid cells were very numerous in these imprints, and 30 % of them were more primitive than normoblasts. These two rabbits gave the lowest figures in the range of haemoglobin values and total red blood cell counts for Group I and indeed for any 3-day-old rabbits examined, and are the two whose splenic sections are separately discussed later.

(ii) *Histological findings.* In the majority of organs examined histologically (kidney, suprarenal, bone marrow and brain) no significant difference can be detected between those from the offspring of immunized does and those from the offspring of

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normal rabbits. Certain differences in the findings are, however, present in some of the livers and in two of the spleens.

The livers of ten of the eleven Group I babies (see Table 2) differ in their iron content from all other 3-day-old livers examined (thirty-eight) with one exception, and six of these ten livers differ also in their content of haemopoietic tissue.

Haemopoietic activity is greater in the livers of these six 3-day-old Group I rabbits than in any other liver of the same age, and reaches the level seen in 1-day-old rabbits, of which twenty were examined. Counts of haemopoietic cells were made to confirm this visual impression. Moreover, in these six animals erythropoiesis is markedly in excess of myelopoiesis, as it is also on the first day of life, whereas in all other livers examined at 3 days old there is either only a slight erythroid predominance or erythropoiesis and myelopoiesis are approximately equal.

Iron, as shown by the Prussian blue reaction, is present in the liver of rabbits in two forms, which vary in their relative proportions: (i) as a diffuse pale blue staining of the cytoplasm, and (ii) as fine to moderately coarse dark blue granules. Both forms may be present in the same cell, but granular iron is never found in the absence of diffuse iron. Experimental evidence shows that diffuse iron appears in the cell first, granules only forming when it has become saturated with diffuse iron. In all the 3-day-old livers examined, apart from the ten exceptions in Group I already mentioned, granular iron is conspicuous, often very abundant, extending nearly to the centre of the lobule. In the ten exceptional rabbits (including the six with unusual liver haemopoietic activity) granular iron is much less abundant, being present only in traces or small amounts round the portal tracts. The separation into two classes is, however, not absolutely distinct and occasional livers showing an intermediate picture are seen. The amount of diffuse iron is variable, probably partly owing to slight deviations in the staining procedures. It does not always run parallel with the granular iron content, but in general appears to cover the same range in all groups. Kupffer cells containing iron are not present in any of the animals examined.

Neither iron nor haemopoietic tissue is present in the livers of any 24-day-old animals.

Immature haemopoietic cells are not easy to recognize in sections of the spleen and it is difficult to assess their relative abundance in different specimens and the degree of maturity of individual cells. In the whole series of 3-day-old spleens there appears to be considerable variation in the numbers of immature haemopoietic cells, without however any constant correlation with the haemopoietic activity of the liver or uniformity within the experimental groups. Cells of the erythroid series predominate: erythroblasts appear to be scanty, though this may be due to difficulty in their recognition.

The presence of immature blood cells in the spleen is not merely due to their presence in the peripheral blood, but is an indication of active local haemopoiesis, since they are present not only in the sinusoids but also form small haemopoietic foci in the pulp. There is an approximate correlation between the frequency of these foci and the abundance of nucleated blood cells in the sections.

The spleens of four of the six Group I animals, already mentioned as showing unusual haemopoietic activity in the liver, are not distinguishable from others of the same age. In them the number of nucleated blood cells reaches as high a level as any by the series, but similar levels are seen in normal control animals and are not in them associated with undue haemopoietic activity in the liver. The spleens of the remaining two of these six animals, however, are distinguished from all the rest by their conspicuously greater content of haemopoietic cells, of which a higher proportion are erythroblasts, and by the presence of erythrophagocytosis and haemosiderosis. The last two features are much more conspicuous in one animal of the pair than in the other. It is to be noted that although these two animals fall into the group of six, showing an unusual degree of haemopoietic activity in the liver, in them it does not reach as high a level as in some of the other four.

As already stated, we have been unable to find any abnormality in the kidney, suprarenal, bone marrow or brain of animals from any of our four groups.

DISCUSSION

The work reported in this paper forms part of the preliminary experiments we have made in an attempt to approach the fundamental problems of isoimmunization of pregnancy. These problems are of course being actively explored by many groups of workers, who are tackling the problem as it affects man and also endeavouring to find the effects of isoimmunization in animals, both as a condition which occurs naturally and as a condition which can be induced experimentally.

The rabbit has not yet provided any results from which new and definite conclusions can be drawn, but we think there are some findings which are important and with further investigation would give profitable results. The most important is the evidence of *in vivo* sensitization of baby rabbit red cells by maternal antibody, and the fact that this occurs in rabbits which are clinically well and remain so is surprising. Very similar results have been reported by Keeler & Castle (1933, 1934*a*).

The antiglobulin direct sensitization test is a definite and repeatable reaction which remains

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positive in our experience sometimes for a few days but often as long as 2–3 weeks after birth. We have never found it positive, or weakly positive, if the doe had not got antibodies to the buck's red cell antigens in her serum, and we have tested a large number of young apart from any referred to in this paper. It is positive when the baby's red cells are of the same blood group as the father's, and negative when they are of the mother's group. It can thus be closely compared with the analogous test for direct sensitization of human infant red cells by maternal isoantibody (Coombs *et al.* 1946) and also with the direct sensitization test for the red cells of foals of mules and thoroughbred horses (Coombs, Crowhurst, Day, Heard, Hinde, Hoogstraten & Parry, 1948).

We have tested a few baby rabbit red cells which have been shown to be sensitized, using a modification of Diamond's albumin tube test, but our results are quite inconclusive. We cannot comment on any of our findings, using bovine albumin either directly on babies' cells or on adult rabbit red cells sensitized *in vitro* with rabbit antisera, without further work; for instance, a thorough exploration of the effect of magnesium sulphate which we used as a suspension medium and diluent.

We have examined the serum from a few babies with sensitized red cells, but though the mother's antibody appeared to be present, again we cannot comment on this yet, for we are still working out the specificity of the antibodies in our antisera: many of them are clearly mixtures of antibodies.* Keeler & Castle (1934*a*) found that maternal antibody to the H_1 and H_2 antigens could be present in newborn rabbits, but only in those whose red cells did not react to the antibody in question by direct agglutination.

From the results of the direct sensitization test we conclude that a positive direct antiglobulin sensitization test on baby rabbit red cells means that maternal antirabbit red cell antibody is attached to the cell.

We examined the blood and organs of newborn rabbits who showed *in vivo* sensitization of their red cells to determine whether there was evidence of abnormal blood destruction, increased haemopoietic activity, or any other lesion which might differentiate these animals from the young of normal does, or of the other does in our experimental group. The results have already been given, and it is seen that in the group of eleven newborn rabbits, showing *in vivo* sensitization of their red cells, certain features occur, not necessarily all present in any one animal, which might suggest a disturbance of the haemopoietic system. These features are: an unusual degree of haemopoietic activity in the livers of six of the eleven rabbits; a high proportion of immature haemopoietic cells in the splenic imprints of all eleven rabbits and erythrophagocytosis and haemosiderosis in the spleens of two. It is therefore necessary to discuss them in more detail in an attempt to arrive at a correct interpretation of their significance in relation to *in vivo* sensitization of red cells by maternal isoantibody. The question is whether these observed discrepancies, which are of a quantitative rather than a qualitative nature, can be regarded as physiological or are indicative of a pathological process.

The unusually high haemopoietic activity in the livers of six of the eleven Group I rabbits is not associated with any evidence of excessive blood destruction or any abnormality in the other blood-forming organs, apart from the exceptional findings in spleens of two animals which will be considered later. Furthermore, the amount of haemopoietic tissue as well as the type and maturity of its cells are comparable with those in many of a series of twenty 1-day-old livers examined, although among the latter there are also examples with even greater haemopoietic activity, showing that there is considerable individual variation on any given day during this period of declining liver haemopoietic activity. It is therefore not surprising to find an overlap in pictures separated only by 48–72 hr. These facts combine as strong presumptive evidence that the cases with higher levels of haemopoietic activity than the mean are due to a slight non-pathological variation in the rate at which haemopoiesis disappears from the liver.

The paucity of liver iron, associated with unusually high haemopoietic activity in six of the Group I animals, might have been dismissed as due solely to an increased demand for iron for haemoglobin formation, especially as an equally small quantity of iron is present in the majority of livers from 1-day-old animals. But the abundant iron in the livers of four of the latter, from two litters, associated with the extreme haemopoietic activity usual on this day, and conversely its extreme scarcity in the remaining four Group I rabbits and in one Group II rabbit (litter-mate of two of the former) show that this is not the only factor. The exceptions tend to occur in litter-mates, but an investigation of some of the possible controlling factors—size of litter, diet and number of experimental bleedings of the doe—failed to reveal any correlation. Neither do these deviations from the more usual level of liver iron appear to be associated with a generalized disorder of iron metabolism, since in none of the animals examined on the 1st and 3rd day of life, with the exception of the two showing erythrophago-

* We have not been able to find any antisera, whose specificity is known, to compare with our sera, though Dr P. B. Sawin of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and Dr M. Bessis of Paris have been very helpful in sending us samples of their own rabbit antisera to compare with ours.

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cytosis in the spleen, is there more than a very occasional minute trace of iron in the bone marrow or spleen. As these variations in liver iron occur irrespective of haemopoietic activity, both in normal newborn rabbits and in offspring of immunized mothers, it seems reasonable to conclude, from these results alone, that they are probably not related to the presence of sensitized red cells but are, more likely, the net result of the interplay between a number of undetermined biological factors.

The splenic imprints suggest that there is greater erythropoietic activity in some of the rabbits of Group I than in their contemporaries. This is neither confirmed nor denied by the evidence from the splenic sections, but the cells are difficult to recognize in sections and differential counts are more reliably made from the imprints. But this enhanced activity in the spleen does not coincide with that in the liver except in one case, and 1-day-old rabbits have spleens more active still. The imprints show a continuous gradation in haemopoietic activity, and although the extremes are widely separated, it is difficult to be sure of a pathological cause for this at a time of great physiological variation.

Less easy to explain is the finding of erythrophagocytosis in the spleen of two rabbits. Laude (1928), from his study of a series of animals, including the rabbit, concluded that erythrophagocytosis is an abnormal finding. It occurs in two rabbits only, 3-day-old litter-mates from the first litter of doe A in which the erythrocytes of the offspring showed *in vivo* sensitization by maternal antibody, and here it is almost certainly of pathological significance, since it is associated with haemosiderosis and a mild anaemia. We killed and examined two more 3-day-old babies from doe A's next litter, but these do not show any of the unusual features found in the first litter, though their red cells gave as definite a positive reaction to the direct sensitization test, and the level of antibody in doe A's serum was comparable. The above findings can be interpreted as either due to a haemolytic process severe enough to cause anaemia, or to an abnormal condition of the erythrocytes, intrinsic or acquired, rendering them more liable to destruction by phagocytosis. If the latter was the case it would be tempting to correlate this abnormal state of the erythrocytes with their sensitization by maternal antibodies. Such a finding would then be expected in all animals with sensitized cells, but in its absence we cannot prove such a relationship from two cases alone, especially when, as in them, the haemolytic process appears to be limited to the spleen, the other organs examined giving rise to no special comment.

From the above arguments, and in view of the very mobile condition of haemopoietic activity in the newborn resulting in a wide range of normal values, we feel it permissible to assume that the apparent discrepancies with regard to the haemopoietic activity of the liver and spleen might individually be accounted for as examples lying near one limit of the physiological range and as such relatively uncommon. But before they can be dismissed as of no consequence in the present experiment, it remains to explain their appearance almost exclusively in Group I animals. Here they appear to have a random distribution, they are not found uniformly throughout the group, neither are they constantly associated in any one animal as would be expected if they were part of a common pathological process. We therefore think we should be cautious in regarding their more frequent appearance in Group I as more than coincidental.

We hope that future work and more material may make clearer these indications of a possible pathological process; but the results of this experiment do not provide definite evidence of such a process occurring in baby rabbits showing *in vivo* sensitization of their red cells by an experimentally produced maternal isoantibody, comparable to that seen in the naturally occurring haemolytic disease of the newborn in human babies and foals due to isoimmunization of pregnancy. This suggests the question of how the rabbit's physiological make-up protects it from the clinical syndromes found in the human infant and the thoroughbred horse and mule foals. One of the important problems of isoimmunization of pregnancy in humans is the exact explanation of the different syndromes which can be presented by the infant. Though it is dangerous, especially at this early stage of investigation, to theorize and to apply findings from one animal species to another, further work on the reactions of the rabbit and the comparison between it and other animals should be valuable.

We cannot yet explain why all the susceptible offspring of the does who formed demonstrable antibodies in their serum did not show *in vivo* sensitization of their red cells. This does not seem to be only a matter of the height of the titre of antibody in the maternal serum, as is shown by a comparison (see Table 1) of the titres found in doe F, whose offspring could have had sensitized red cells but in fact did not, with the titres found in an animal such as doe A or doe D, whose offspring had sensitized cells. An important point we must establish, before further discussion on this question is possible, is to which of the systems of rabbit red cell antigens our antisera relate, for it has been shown by Fischer (1935) that the K_3 and K_5 antigens are found not only on red cells but also in extracts from organs and tissues, whilst the K_1K_2 antigens are found only on the red cells.

We did find, however, that all the does whose offspring showed *in vivo* sensitization of their red cells had antibodies which were demonstrated by the in-

direct sensitization test, though in three cases these were detected only in high dilutions of the sera and showed, as well, marked direct agglutination of red cells. In a previous experiment (Coombs *et al.* 1948) it was found that antibodies in the sera of thoroughbred mares, whose foals showed haemolytic anaemia with *in vivo* sensitization of their red cells, were demonstrated also by the appropriate indirect sensitization test, though again often only at the higher dilutions of the serum. These findings can only be noted now, and cannot be discussed without further investigation.

Since 'incomplete', 'blocking', 'non-agglutinating' and 'albumin' *Rh* antibodies were first demonstrated, the importance of the different forms in which the antibody occurred and their relationship to the severity of the disease in the baby have been discussed and investigated, and it is very possible that some facts on the significance of the form in which isoantibodies may be found could be learnt from the rabbit.

A general, but not constant, finding which has previously been described by Keeler & Castle (1934*b*) was a fall in the titre of a doe's antibody during pregnancy and a rise after parturition, reaching a peak about 3–5 days post-partum. The degree of post-partum rise was most marked when the red cells of the offspring were incompatible with their mother's antiserum and was found whether the offspring's red cells showed *in vivo* sensitization or not. Further work on this subject might give important results and might show, for instance, whether—in the rabbit—immunization of a mother by her foetus occurs at parturition.

SUMMARY

1. Nineteen rabbit does were inoculated each with the whole blood of a buck to which they were later mated.

2. They were observed over a period of 10 months which included three pregnancies: (*a*) for the appearance and behaviour of anti-red cell isoantibody in their sera; and (*b*) for the effect of such an antibody on the offspring, especially those inheriting a paternal red cell antigen incompatible with their mother's antiserum.

3. The does' antisera were examined for antibodies capable of directly agglutinating red cells and for 'incomplete' antibodies which might sensitize cells without causing agglutination. The latter were identified by an indirect antiglobulin sensitization test.

4. Nine does formed antibodies in their sera, six of these antisera directly agglutinated the appropriate red cells, but in three cases the antibodies were demonstrated only by the indirect antiglobulin sensitization test. Of the six antisera which directly agglutinated red cells three had antibodies which caused direct agglutination of red cells undiluted or in their lower dilutions, but in higher dilutions were shown to be capable of sensitizing cells without agglutinating them.

5. Four does, who had produced antibody in their sera, had between them seven litters in which the red cells of the young inheriting a paternal antigen incompatible with their mother's antiserum reacted to a direct antiglobulin sensitization test. This reaction is discussed and it is concluded that a positive direct antiglobulin sensitization test means that maternal isoantibody is attached to the infants' red cells. The offspring whose red cells reacted positively were, nevertheless, clinically normal.

6. A proportion of the young were killed at 3 days and 24 days old. A full peripheral blood examination was made and imprints taken from the liver and spleen. The bone marrow was also examined. Specimens were taken from liver, spleen, suprarenal, kidneys, femur, and, in some cases, brain for histological examination after an autopsy had been performed. Fifteen normal rabbits were similarly examined at 3 days old and four at 24 days old.

7. The range and average figures for the various haematological examinations made are given and the findings in the experimental animals compared with the normals.

8. A few differences were found between the experimental and the normal animals as a result of this experiment. The significance of these is discussed.

CONCLUSION

After inoculation of nineteen female rabbits with the blood of male rabbits some does produced iso-antibodies against their respective bucks' red cells. The does were then mated with these bucks and the young observed for manifestations of haemolytic disease of the newborn. In some cases *in vivo* sensitization of the red cells of the young rabbits by the maternal antibody resulted. Although it has not yet been proved whether the maternal antibody passed to the offspring by way of the placenta, or by the colostrum or by both routes, it is of interest that this *in vivo* sensitization took place without apparently affecting the baby rabbits clinically. Haematological and histological examinations suggested that *in vivo* sensitization may be accompanied by slight changes in the haemopoietic system, but we consider, from the results of this experiment, that the differences we found between the experimental and the normal animals were within the physiological range.

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* Reference not verified.

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APPENDIX I

Immunization of a group of female rabbits with rabbit whole blood

All the does were bled before inoculation for sera. Blood for immunization was collected sterilely from the marginal ear vein into a measured amount of a sterile solution of 3·4 % trisodium citrate, allowing two parts citrate solution to one of blood. This mixture was immediately injected.

For the first course of immunization seventeen animals were given two intravenous injections 5–6 days apart, of the equivalent of 7–8 ml. whole blood. The remaining three were given three intravenous injections at 5-day intervals of amounts which varied from 2 to 10 ml. whole blood. After the last injection the does were mated as soon as possible, but they were all bled 10 days after their last injection. They were all bled again a week before their expected parturition and again 3–4 days post-partum. One animal (doe A), who developed demonstrable antibodies in her serum 10 days after her inoculation, was bled weekly throughout pregnancy.

For the second course of inoculations, which was exactly 3 months after the first course, the procedure was kept constant and modelled on the treatment given to doe A. The animals were given four intraperitoneal injections of whole citrated blood at twice weekly intervals in doses of the equivalent of 0·5, 1·0, 1·5 and 2·0 ml. whole blood; the does were all bled for serum 10 days after their last inoculation and then mated as soon as possible. Any doe who formed an antibody in her serum was bled weekly throughout her pregnancy and for the first 3–5 days post-partum, and at varying intervals thereafter.

Four months after these injections the does were bled again and later remated without further immunization. Serum was collected a week before the expected parturition and 4 days post-partum.

APPENDIX II

Detail of the methods used to test a serum for antibody and for titrating the strength of the antibody

To test for the presence of an antibody in a serum the pre-inoculation and post-inoculation sera were put up in triplicate with the red cells against which the animals had been immunized. A drop of undiluted serum was delivered by means of a fine pasteur pipette into a rimless precipitin tube, 7 × 50 mm., and a similar sized drop of a 1 % suspension of red cells, washed three times in 3·33 % $MgSO_4.7H_2O$ solution, added. The tubes were incubated at 37° C. for 2½ hr. They were then read macroscopically and a drop examined from the first pair of tubes under the 2/3 objective of a microscope. If the tube containing post-inoculation serum was negative the cells in the second pair of tubes were rapidly washed three times in magnesium sulphate solution. After the third washing the supernatant fluid was removed completely and a drop of a suitable dilution of the anti-globulin serum added (see Appendix III). The cells in the third pair of tubes were tested (if the direct agglutination test was negative) by a modification of Diamond's albumin tube test, using Armour's bovine albumin solution for the *Rh* testing. The supernatant fluid was thoroughly removed from the

cells and a drop of the albumin solution put on. The usual concentration used was 20 % of albumin, but 30, 10 and 15 % were also tried. The cells were shaken up and allowed to settle for an hour at 37° C. and then spun for a minute and read microscopically. Other controls used were the cells put up alone in magnesium sulphate solution, and with the antiglobulin serum; a positive control on the antiglobulin serum once a serum had been found which could sensitize cells without agglutinating them; and a positive control on the albumin solution using an 'incomplete' *Rh* antiserum with the appropriate serum. Most sera were tested at room temperature as well as at 37° C.

The titration of the antibody strength of a serum was made by putting up doubling dilutions of serum beginning at a 1 in 1 or 1 in 2 dilution. The dilutions were made using a syringe and conventions of usage were made and strictly adhered to, to minimize errors due to carrying over one dilution to another. These dilutions were made in sufficient amounts to allow 0·1 ml. of each dilution to be put into each of three tubes. Then 0·1 ml. of a 1 % suspension of red cells was added. The titration was thus set up in triplicate. The tubes were incubated at 37° C. for 2½ hr. and then one set was read macroscopically and microscopically for direct agglutination; one set was washed and treated with antiglobulin serum and the third set was treated as previously described with bovine albumin solution. The last dilution giving a definite and strong agglutination of cells which we recognized as a 2+ reaction was taken as the titre of the serum. The controls set up were the same as described above. The sera were stored at − 20° C. until several from one animal could be titrated together.

APPENDIX III

Preparation of goat antirabbit globulin serum

A goat, after bleeding for serum, was immunized with pooled rabbit serum, until a precipitating antibody could be demonstrated in its serum.

The antiserum was inactivated at 56° C. for 30 min. and then absorbed with rabbit red cells until the serum no longer agglutinated the cells of any rabbit used in the experiment. The cells for absorption were washed six times in magnesium sulphate solution to remove all traces of serum globulins.

The serum was used in a dilution of 1 : 8 until a rabbit serum which would sensitize rabbit cells without agglutinating them was found, when cells sensitized with such a serum were used to titrate the strength of the antiglobulin serum. Then the dilution of antiglobulin serum used depended on the titre of the antibody. Dilutions were made immediately before use and the absorbed serum was stored undiluted and without sterile precautions at − 20° C.

APPENDIX IV

The direct antiglobulin sensitization test

Blood was obtained from baby rabbits by snipping the extreme tip of the ears, collecting the blood with a Pasteur pipette and transferring it to 0·6 % sodium citrate dissolved in isotonic magnesium sulphate solution. The cells were rapidly washed three times in 3·33 % magnesium sulphate solution. One drop of a 5 % cell suspension was mixed on a tile at room temperature with one drop of a suitable dilution of the antiglobulin serum, and the tile gently rocked. Agglutination, if the cells were sensitized, appeared within 2–5 min. The test was also put up in a tube when one drop of a 2 % cell suspension was mixed with a drop of the diluted antiglobulin serum and incubated at 37° C. until the cells settled. In all the cases which we have reported as having *in vivo* sensitization of their red cells the tile test was positive as well as the tube test, but a few cases showed a weak tile test but a strong tube test. A positive as well as a negative control for the antiglobulin serum was always set up. The positive control has already been described in Appendix II and the negative control was the cells of the father and the mother of the baby rabbit put up with the antiglobulin serum.