Hemolytic Disease in Newborn Dogs

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Hemolytic Disease has been produced with regularity in pups born to bitches immunized by transfusion of dog erythrocytes of type A and mated with sires having red cells containing the canine A factor. Isoantibodies are not present in the serum of newly born dogs but are acquired from the mother's milk during the first day of life. Once hemolytic anemia develops in newborn dogs it is in many respects similar to that encountered in human babies.

The four antigenic factors of dog erythrocytes thus far studied most extensively have been arbitrarily designated canine A, B, C and D and the corresponding antibodies anti-A, -B, -C and -D in the order of their demonstration in this laboratory. The A factor has been referred to in previous reports as "Do." The in vitro behavior of dog isoantibodies is described in detail elsewhere and observations on hemolytic transfusion reactions due to canine anti-A have been reported separately. It is sufficient to state here that anti-B, -C and -D are "saline" agglutinins and cause no direct hemolysis in vivo or in vitro. Anti-A, on the other hand, acts as an hemolysin as well as an agglutinin and it fixes complement. Under proper conditions anti-A produces strongly positive indirect antiglobulin (Coombs) reactions; in fact, attachment of anti-A in some dog antisera to the A factor of some dog red cells is demonstrable only by means of the antiglobulin test. Cells reacting in this way are referred to as A' cells. It is considered possible that there exists a series of A' alleles comparable to the human D' alleles postulated by Stratton and by Race, Sanger and Lawler.

The object of this paper is to describe observations on 9 litters of pups that were studied in an effort to determine: (1) the manner in which isoantibodies are acquired by the pup from the immunized bitch, and (2) the serologic, hematologic and histologic alterations produced in newborn dogs affected with hemolytic disease. Preliminary reports on these observations have been published previously.

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This study was aided by contracts between the University of Rochester and the Office of Naval Research, Department of the Navy (NR 131-174) and the Public Health Service (RG 2014), and was supported in part by the Hochstetter Fund.
Previous Observations on Hemolytic Disease of the Newborn in Animals

Keeler and Castle in 1934 attempted to produce agglutination and destruction of the red cells of newborn rabbits by transfusing the mothers with rabbit cells containing agglutinogens designated H1 and H2. The mothers having red cells that lacked both agglutinogens developed agglutinins (called h1 and h2) and were then mated with H1 or H2 bucks. In the offspring from such matings, h1 and h2 agglutinins were found only in the serum of embryos or newborn rabbits having red cells that lacked the corresponding agglutinogen. Absence of the agglutinins in the rabbits having red cells that contained the corresponding agglutinogens was attributed to “neutralization in the circulation of the fetus.” There was no apparent agglutination or destruction of the fetal cells. Heard, Hinde and Mynors have recently reported similar observations and have demonstrated that the red cells of the young rabbits become sensitized for the Coombs antiglobulin reaction (employing anti-rabbit serum goat serum). There were only slight changes in the hematopoietic system of the offspring and no clinically apparent manifestations of hemolytic disease despite consistent sensitization of the red cells by isoantibody acquired from the mother.

Caroli and Bessis have studied naturally occurring hemolytic disease in newborn mules born to mares that have been mated with a breed of donkey found in the province of Poitou in France. These investigators have shown that the mares develop hetero-antibodies that are capable of lysing in vitro the red cells of the mule and the donkey, and Coombs has demonstrated that the red cells of the affected mule foals are agglutinated by antiglobulin serum. Caroli and Bessis have described in detail the serologic, hematologic and histologic abnormalities in affected mules and have compared these findings with those observed in human infants. Favorable results of exchange transfusion have also been described.

Naturally occurring hemolytic disease has been observed by Bruner and associates in newborn foals following transplacental isoimmunization of the mares by red cell antigens of the foals. These investigators have demonstrated that the antibodies reach the fetus from the mare through the milk and that the disease can be prevented by having the foals suckle only non-immunized foster mothers. Bruner et al. have more recently demonstrated that foals are capable of absorbing antibodies from the gut only during the first day of life. Coombs and associates have distinguished two types of isoantibodies in the serum of mares that have given birth to affected foals and have demonstrated the value of the antiglobulin test in detecting hemolytic disease in horses.

Bruner et al. produced hemolytic anemia in baby pigs that suckled a sow immunized with erythrocytes from the boar to which it was bred. Baby pigs appeared to be incapable of absorbing isoantibody after the first day of life. Bessis and Freixa have shown that in newborn rats the ingestion of rabbit anti-rat serum causes hemolytic disease up to about the twentieth day of life but not afterwards. Although well defined blood groups in rats have been described by Burhoe we are not aware of systematic attempts to produce hemolytic disease in rats by isoimmunization.
Mynors has attempted without success to produce hemolytic disease in baby guinea pigs by mating does to bucks from which the does have received transfusions. No isoantibodies have thus far been produced in this species. Eyquem has produced isoantibodies in cats by inoculation of pooled red blood cells from other cats. Hemolytic anemia was then produced in young cats by inoculation of incompatible isoantibody, but kittens born to isoimmunized mothers showed no hematologic or clinical evidence of hemolytic disease.

Eyquem has identified three types of immune isoagglutinins in transfused dogs and by subcutaneous or intraperitoneal inoculation of one of these antibodies he has produced hemolytic anemia and icterus in pups less than eight days of age. He observed reticulocytosis, spherocytosis, and erythroblastosis in the blood of affected pups and erythropagocytosis in the sinusoids of the liver and spleen. These findings were similar to those encountered in pups inoculated with anti-dog-erythrocyte rabbit serum, but nuclear-icterus was much more marked in the pups receiving hetero-immune serum. Eyquem has also reported that bitches immunized by transfusion and mated with donor sires of incompatible blood group gave birth to pups with erythroblastic anemia and slight icterus but clinical evidence of nerve cell damage was lacking.

To the best of our knowledge Abelson is the only investigator who has had opportunities to study hemolytic disease in newborn dogs (dachshunds and more recently in other breeds) the mothers of which had apparently developed isoantibodies as a result of natural immunization by pregnancy. The specificity and characteristics of the antibodies responsible for hemolytic disease in these litters have not been reported.

**METHODS**

Methods for titration of isoantibody and complement in serum, for the Coombs antoglobulin tests and for immunization of adult dogs have been described separately. Blood samples from both pups and adult dogs were drawn from external jugular veins with dry syringes and were used without delay. Blood for determinations of hemacrit (Wintrobe method), plasma bilirubin concentration and osmotic fragility of the erythrocytes was placed in 1.0 or 2.0 ml. amounts in mixtures of dried ammonium and potassium oxalates.

Samples of milk for determination of isoantibody titer were expressed manually from the dams' nipples into clean glass tubes measuring 100 x 13 mm. They were promptly centrifuged for ten minutes at 2500 r.p.m. after which the supernatant fat was removed with a cotton swab. Serial dilutions of the milk were then made with 0.85 per cent sodium chloride as a diluent and the diluted milk was routinely mixed in equal volumes (0.1 or 0.2 ml.) with 3 per cent suspensions of dog erythrocytes in fresh unheated autologous serum. In many instances duplicate titrations of isohemagglutinin content of milk were also made by using dog cells suspended in saline. The lower titers obtained by the latter method are not recorded in the figures of this paper.

Osmotic fragility of the erythrocytes of the pups in litter A was determined by the method of Shen, Ham and Fleming. Osmotic fragility of the red cells from other litters was measured by placing 0.02 ml. samples of oxalated blood into tubes containing 1.0 ml. amounts of hypotonic salt solution buffered to pH 7.0 with a buffer mixture containing 72 parts of M/12 Na₂HPO₄ and 28 parts of M/15 NaH₂PO₄. The cells were suspended in the solutions of buffered salt solution and allowed to stand fifteen minutes at room temperature. Hemolysis due to hypotonicity of the medium was then arrested by adding 9.0 ml. of 1.0 per cent buffered NaCl solution (75 parts of 1.2 per cent NaCl and 25 parts of the buffer...
mixture) to each tube. The tubes were then centrifuged for eight minutes at 1500 r.p.m. and the supernatant fluid poured off directly into colorimeter tubes. A drop of concentrated NH₄OH was added to each tube and the amount of hemoglobin in the tubes was measured with the Kromatrol photometer using a 540 filter.

In determining the proportions of the red cells that were nucleated 10,000 red cells were counted on cover slip smears of venous blood stained with Wright's stain.

**Experimental Results**

Certain essential facts concerning the nine litters reported in this paper are summarized in table 1. Six litters were born to dams immunized against the canine A factor and two litters to dams immunized against the canine C factor. The four pups in litter II were born to a non-immunized dam but were allowed to suckle the mother of litter G, as will be explained subsequently.

**Anti-A Titers in Dams' Sera and Milk and in Pups' Sera**

The anti-A titers in the dams' sera and breast milk and in the pups' sera are shown in figures 1, 2, 3 and 8 for litters A, C, D and E respectively. Titers demonstrated in the other dams and litters followed similar trends. In every case the isoagglutinin titer in the dam's serum, produced by transfusions as indicated, fell during pregnancy and did not rise after delivery unless a small immunizing transfusion was given. Transplacental isoimmunization of the dam by the A factor of fetal red cells was therefore not evident during these pregnancies.

Anti-A antibodies in the breast milk gradually disappeared during the first three weeks after delivery. Whenever samples of colostrum were obtained (litters B, D, E, F, G and I) on the day of delivery they were found to contain isoagglutinin in titers higher than those found in the serum drawn at the same time. All pups with red cells lacking the A factor had anti-A agglutinins in their sera for varying periods of time provided they suckled breast milk containing anti-A during their first day of life. The in vitro behavior of anti-A in the sera of the pups was similar to that described for anti-A in the sera of adult dogs; that is, these antibodies acted as both agglutinins and hemolysins, produced positive antiglobulin reactions and their agglutinative capacity was augmented by the presence of a heat-labile component of normal dog serum. In litters D and G, pups with red cells containing the A factor also had anti-A in their sera, presumably because they had sucked colostrum containing unusually high titers of anti-A.

During the course of this study it became apparent that anti-A was not acquired by the pups in utero. "A-negative" pups examined prior to suckling had no anti-A in their sera and "A-positive" pups' cells gave negative antiglobulin reactions when tested before the pups had suckled. Alling and Terry* had previously determined the electrophoretic pattern of pooled plasma from newborn pups and found little if any gamma globulin. Observations to be cited make it apparent, moreover, that the dog, like certain other mammals is capable of acquiring antibodies from breast milk during only a very short period of time after birth.

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* Personal communication.
Antiglobulin Reactions with Pups’ Red Cells

Positive antiglobulin reactions were obtained with the A or A' cells of all such pups that suckled breast milk containing anti-A during their first day. The length of time during which the antiglobulin test remained positive with A or A' cells of any surviving pups in each litter is given in table 1. Since quantitation of the antiglobulin reactions is difficult, no attempt will be made in this paper to present data pertaining to the strength of these reactions. It is sufficient to state that dilutions of anti-dog serum as high as 1:2560 were capable of agglutinating sensitized pups' cells. Agglutination of the red cells of the affected A-positive pups surviving after the third day gradually became weaker but the actual dilution of antiglobulin rabbit serum causing definite agglutination frequently remained high for several weeks. It then appeared that when high dilutions of rabbit serum failed to clump the cells, low dilutions also failed to do so. A' cells reacted fully as strongly as A cells and there was little correlation between the severity of the hemolytic disease and the degree of agglutination of the A cells produced by various dilutions of rabbit serum.

Hematologic and Serologic Observations on Litters A, C and D

The pups of litter A, referred to in a preliminary report, were first bled at various intervals after birth as explained in the legend for figure 4. Anemia was marked only in pup 1. The differences in osmotic fragility of the 4 affected “A-positive” pups and the 4 unaffected “A-negative” pups are apparent. For unknown reasons, spherocytes could not be demonstrated with certainty in smears and wet preparations of blood from the affected pups of this litter, while in subsequent litters spherocytosis was clearly demonstrable in the most severely affected pups especially on the second day of life when osmotic fragility was usually maximal. Nucleated red cells (figure 5) and reticulocytes were in general more numerous in blood smears of the affected pups of litter A and subsequent litters than in the A-negative pups, but in some instances the differences were not striking.

The only A-positive pup in litter C was normal except for a weakly positive antiglobulin reaction when first examined about one hour after birth. As shown in figure 6, however, this pup’s hematocrit fell sharply and the osmotic fragility of the red cells increased greatly during the first twenty-four hours of life. The Coombs test also became more strongly positive and this pup died at thirty-six hours. Blood from a normal litter mate was examined as shown in figure 6 for comparison with the affected pup.

Figure 7 reveals similar changes during the first sixteen hours of life in the pups of litter D. All whelps of this litter were first bled before they had had an opportunity to suckle. At the first bleeding all the cells gave negative antiglobulin reactions and all of the sera failed to agglutinate the sire’s red cells. It therefore appeared that all of the anti-A isoantibody found in the pups at sixteen hours had been acquired from the dam’s milk which had a titer of 1:4096. Pups 1, 2, and 3 were severely affected and died within forty-eight hours. The sera of these 3 type A pups at sixteen hours agglutinated the sire’s cells in a titer of 1:256
Table 1.—Summary of Information Pertaining to Nine Litters

<table>
<thead>
<tr>
<th>Litter</th>
<th>Sire’s number and blood type</th>
<th>Bitch’s number and blood type</th>
<th>Antibody titer in bitch’s serum on day of delivery</th>
<th>Number of pups having given blood type</th>
<th>Age of pups in hours at first suckling of bitch</th>
<th>Number of days antibody present in pups’ serum*</th>
<th>Number of days antibody present on pups’ cells*</th>
<th>Observations on pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>198 AC 1:4</td>
<td>47-184 C</td>
<td>Anti-A</td>
<td>4 AC</td>
<td>0</td>
<td>None</td>
<td>9</td>
<td>All affected in varying degrees. See figures 4 and 5. Pup 1 sacrificed on third day for histologic examination. Others recovered. All normal. One sacrificed on third day for histologic study.</td>
</tr>
<tr>
<td>B</td>
<td>1308 A’BD 1:8</td>
<td>1308 A’BD 1:8</td>
<td>Anti-C</td>
<td>2 AC</td>
<td>2 ABC</td>
<td>None</td>
<td>9</td>
<td>None affected.</td>
</tr>
<tr>
<td>C</td>
<td>198 AC 1:250</td>
<td>198 AC 1:250</td>
<td>Anti-A</td>
<td>1 AC</td>
<td>0</td>
<td>None</td>
<td>1</td>
<td>Died at 36 hours. See figure 6. None affected.</td>
</tr>
<tr>
<td>D</td>
<td>1308 BC 1:250</td>
<td>1208 BC 1:250</td>
<td>Anti-A</td>
<td>2 ABC</td>
<td>1 AC</td>
<td>1</td>
<td>1</td>
<td>All severely affected and died within 48 hours. See figure 7. No anemia or icterus; transient increase in osmotic fragility; both survived.</td>
</tr>
<tr>
<td>E</td>
<td>198 AC 1:64</td>
<td>43-381 C</td>
<td>Anti-A</td>
<td>6 AC</td>
<td>3; 16</td>
<td>None</td>
<td>22</td>
<td>Two pups that suckled own dam at 3 hours were severely affected and died; 2 pups that suckled foster dam at 3 hours were moderately ill but survived; 2 pups that started suckling own dam at 16 hours were unaffected. None affected. Four pups suckling at 3 hours acquired antibody in serum; 2 pups suckling at 16 hours acquired practically none.</td>
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<td></td>
<td>F</td>
<td>G</td>
<td>H</td>
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<td>198 AC</td>
<td>unknown</td>
<td>198 AC</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>1421†</td>
<td>48-202 CD</td>
<td>49F</td>
<td>48-152 C</td>
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<tr>
<td></td>
<td>Anti-C 1:8</td>
<td>Anti-A 1:512</td>
<td>None</td>
<td>Anti-A 1:32</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>6 AC 3 A/C</td>
<td>6 AC</td>
<td>2 AC</td>
<td>5 AC</td>
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<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>2-18</td>
<td>4</td>
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<td>None</td>
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</tr>
</tbody>
</table>

- All severely affected; 2 died without transfusion; 2 recovered without transfusion; 2 recovered rapidly after transfusion of A-negative blood. Unaffected.
- Two pups suckling mother of litter F during first day of life were severely affected but recovered.
- Two pups suckling mother of litter G during second day of life acquired no antibody and remained normal.
- One pup sacrificed at 4 hours, one at 24 hours, one at 72 hours. One affected pup recovered, one died at 48 hours.
- One pup never breathed. One pup sacrificed at 4 hours. One at 24 hours, 1 at 72 hours, all unaffected.

* The figures given for length of period in which isoantibody was demonstrable in pups' serum or on pups' erythrocytes are the maximum figures for the respective pups of each litter.
† The red cells of dog No. 1421 are not agglutinated by any of the available canine antisera.
(figure 3) and the pups' cells were strongly agglutinated by high dilutions of anti-dog serum rabbit serum. These results showed that the pups of this litter, like those of litter C, had absorbed unusually large amounts of antibody from the mother's milk.

**Fig. 1.**—Anti-A titers in sera of pups of litter A and in dam's serum and milk in relation to immunizing transfusions of type A blood. The anti-A titer was essentially the same in the sera of all of the 4 A-negative pups of this litter; hence only a single graph is shown. Milk was not obtained for anti-A titration until the ninth day after delivery.

**Fig. 2.**—Anti-A titers in sera of 4 A-negative pups of litter C and in dam's serum and milk in relation to immunizing transfusions of type A blood.

The red cells of the 2 A' pups in litter D also reacted strongly with antiglobulin serum and their sera agglutinated the sire's erythrocytes in a titer of 1:1024 (figure 3). The higher anti-A titers in the sera of the A' pups, as compared with the type A litter mates, was to be expected since A cells remove anti-A from serum more effectively than do A' cells. Sera from the A' pups continued for
Fig. 3.—Anti-A titers in sera of pups of litter D and in dam’s serum and milk in relation to transfusions. The solid dot at 256 in the top graph represents the anti-A titer in the serum of 3 severely affected A-positive pups that died on the following day. The 2 mildly affected pups in this litter were of type A'.

Fig. 4.—Hematocrit and osmotic fragility of the red cells in the pups of litter A at the time venous samples were first drawn. Pups 2 and 8 were bled at twelve hours, pups 1 and 6 at twenty-four hours, pups 3 and 5 at forty-eight hours and pups 4 and 7 at seventy-two hours. Initial examinations on all pups of this litter were made one hour after birth with blood samples obtained from the tip of the tail.
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Thirty-three days to be capable of agglutinating the red cells of the sire, and the red cells of the A' pups were agglutinable by antiglobulin serum for sixty-five days. The erythrocytes of the A' pups showed only a slight and transient increase in osmotic fragility and these whelps did not become anemic or icteric.

Observations on Litters E, G, H, and I with Regard to Absorption of Antibody from Breast Milk

The 12 pups of litter E were delivered at term by cesarean section. There were 6 A-positive and 6 A-negative whelps, all of which were normal from the hematologic standpoint at birth. Starting at three hours after birth, 2 A-positive and 2 A-negative pups were nursed by their own mother and 2 of each by the mother of litter D that had been delivered five days earlier. The remaining 2 pups of each blood type were fed cow's milk for sixteen hours and then allowed to suckle their own mother.

Figure 8 reveals that substantial anti-A titers were found in the sera of the 2 A-negative pups that nursed their own mother at three hours and somewhat lower titers in the 2 A-negative pups that suckled the immunized foster dam. No antibody was detectable at sixteen hours in the 2 A-negative pups that were fed cow's milk. In the blood of the 4 A-positive pups that suckled either dam at three hours all of the isoantibody presumably was attached to the red cells which

![Graph showing the percentage of red cells nucleated in smears of blood from pups of litter A at various ages.](image-url)
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gave strong antiglobulin reactions (or to other antigens in the body); the serum of these pups failed to agglutinate the sire’s erythrocytes. The osmotic fragility

![Graph](image.png)

**Fig. 6.**—Hematocrit and osmotic fragility of red cells of the only A-positive pup and an unaffected A-negative litter mate in litter C at birth (actually one hour after birth) and at twenty-four hours.

![Graph](image.png)

**Fig. 7.**—Hematocrit and osmotic fragility of red cells of pups of litter D at birth (before suckling) and at sixteen hours. The severely affected pups were of type A, the mildly affected pups of type A’.

of the red cells of these 4 A-positive pups was substantially increased at sixteen hours after birth. The 2 A-positive pups that suckled their own dam died within
forty-eight hours, while the 2 A-positive pups that suckled the foster dam were mildly affected and recovered. The cells from the 2 A-positive pups fed cow’s milk yielded negative antiglobulin reactions at sixteen hours.

The 4 pups of litter E that were fed cow’s milk during the first sixteen hours of life and were then allowed to suckle their own immunized mother acquired barely detectable amounts of anti-A. The cells of one of the A-positive pups in this group developed a very weak antiglobulin reaction while the cells of the other A-positive pup gave none. Neither pup developed anemia or increase in osmotic fragility of the red cells. The undiluted serum of 1 of the A-negative pups in this group weakly agglutinated the sire’s red cells; the serum of the other A-negative pup contained no demonstrable anti-A. The essentially negative findings in this group are more striking in view of the fact that the anti-A titer in the mother’s milk was 1:256 at the time of suckling.

Litter E

25 nursed

antibody 640 own dam

024 whelped

20 50 24 26 28 30 32 34 36 38

20 22 24 26 28 30 32 34 36 38

15 10 5 0 2 4 8 12 16 20 24 28 32 36 40

transfusions

weeks before birth

ml whole blood

days after birth

Fig. 8.—Anti-A titers in sera of 4 of the A-negative pups of litter E and in dam’s serum and milk. The 2 A-negative pups of this litter that first suckled their mother at sixteen hours acquired practically no anti-A.

Litters G and H were born during the same night and both had suckled their own mother by the time they were first found in the morning. The mother of litter G had been immunized by transfusion in the usual manner and mated with the sire of litters A, C, E and I. The mother of litter H had been acquired shortly before delivery and no transfusions had been given; the sire of this litter was not known. Two hours after the pups were found, 2 of the A-positive pups of litter H were allowed to suckle the mother of litter G, and twenty-four hours later 2 more pups from litter H were allowed to suckle the mother of litter G. The red cells of the two pups thus transferred on the first day of life developed strong antiglobulin reactions lasting ten days, and there was a marked increase in osmotic fragility of the red cells. The cells of the 2 pups transferred on the second day remained normal with regard to antiglobulin reaction and fragility. Figure 9 shows a lateral plot of the osmotic fragility of one member of each pair; results for the other members of each pair were nearly identical.

The pups of litter H were observed closely after being transferred to the
mother of litter G. These pups were well cared for by the foster dam and suckled as vigorously as the surviving members of litter G. Although the exact age at the time of the first transfer cannot be stated, it was less than eighteen hours and the pups could have been as young as two hours. The results obtained in this experiment are therefore in agreement with the previously described observations indicating that isoantibody can be absorbed by the pups only during the first day of life.

The 9 pups of litter I were delivered near term by cesarean section and were hematologically normal at birth. One A-positive and one A-negative pup were sacrificed by exsanguination about four hours after birth in order to permit histologic studies of the digestive tract and determinations of mechanical fragility of the red cells. Additional pairs of whelps were exsanguinated for similar purposes at twenty-four and seventy-two hours after birth. During the first three days of life no significant changes were grossly detectable in various regions of the digestive tract in the sacrificed animals. Microscopic examination, however, revealed evidence of increasing activity of the secretory cells of the stomach and upper small intestine during the first twenty-four hours of life.

Anti-A agglutinins were readily demonstrable in gastric contents of the 2 pups sacrificed at twenty-four hours. The contents were centrifuged at 3000 r.p.m. for ten minutes to remove curds and the supernatant fluid was neutralized by addition of 0.1 N. NaOH. The titer of the gastric fluid of the A-negative pup was 1:32 against the sire's A cells suspended in autologous serum, while that of
the A-positive pup was only 1:2. The lower titer in the A-positive pup might have been related to the fact that this pup was affected with hemolytic disease and had not been nursing vigorously. * The anti-A titer of the dam's breast milk was 1:128 at the time of these tests. Sufficient material could not be obtained from other portions of the digestive tract for determination of antibody content.

Other Observations on Litters Exposed to Canine Anti-A

The red cells of all of the 24 A-positive pups that during the first day of life suckled breast milk containing anti-A gave strongly positive reactions with high dilutions of antiglobulin serum. All showed increased osmotic fragility and in the most severely affected pups spherocytosis was demonstrable, being maximal at about twenty-four hours. Measurements of mechanical fragility were inconclusive. During the first two or three days of life the sensitized red cells of nearly all of the A-positive pups showed a tendency to agglutinate slightly in oxalated samples of blood and in freshly prepared saline suspensions. When the cells were centrifuged as part of the washing procedure they frequently agglutinated rather tightly in small clumps until after two or more resuspensions in saline.

The hemoglobin concentration in the plasma and serum of the affected pups was not measured quantitatively. Grossly detectable hemolysis was lacking, however, in most of the specimens even when drawn during periods of rapidly developing anemia. Hemolyzed specimens were equally distributed between the affected and unaffected pups and were thought to be attributable to difficulties in performing venipuncture. Although it seems likely that hemoglobinemia was slight or absent in the litters thus far examined, this aspect of the disease deserves further study.

The degree of anemia varied widely, even in affected pups of the same litter, but tended to be most severe in those pups suckling breast milk of high anti-A titer very soon after birth. The severely affected pups nursed poorly and became very pale within twenty-four to forty-eight hours. Hydrops was lacking in all of the pups thus far observed.

Jaundice was evident in the sclerae of only 2 of the pups, one of which had a maximum total serum bilirubin concentration of 3.4 mg per 100 ml. The lack of jaundice and hyperbilirubinemia in most of the affected pups presumably reflects the unusual capacity of the dog liver for excreting bilirubin.

Of the 24 exposed A-positive pups, 3 were sacrificed, 1 from litter A on the third day, 1 from litter I on the second day and 1 from litter I on the fourth day. Nine of the 24 pups died, all of them within seventy-two hours of birth after developing severe anemia. The 12 affected pups that survived beyond seventy-two hours made a complete recovery. Only 2 of the 12 pups that survived were transfused (on the second day) with fresh A-negative citrated whole blood from a normal, adult female donor dog. Eleven ml. of blood were withdrawn from the external jugular vein of one pup and 21 ml. of A-negative blood immediately transfused through the same needle. Nine ml. of blood were removed from the

* Soluble canine A factor has not yet been demonstrable in gastric juice of A-positive dogs.
other pup followed by transfusion of 13 ml. of A-negative blood. The pups were markedly improved after transfusion. A more complete exchange of blood was not attempted.

Autopsy of the 12 affected pups revealed varying degrees of splenomegaly and hepatomegaly but in some instances the liver and spleen were no larger than in unaffected litter mates. Extramedullary hematopoiesis was well marked in both normal and affected pups, but the erythroid cells of the marrow, liver and spleen were less mature in the affected pups. Erythrophagocytosis was a prominent finding in the sinusoids of the spleen, lymph nodes and liver of the severely affected pups. Gross examination of the brains of the affected whelps showed variable but slight yellow staining of the basal ganglia and grey matter of the medulla oblongata. Microscopic findings were inconclusive; however, in a few instances changes suggesting specific alteration in the nerve cells of the basal ganglia were seen. The placenta from each of the 21 whelps of litters E and I delivered by cesarean section was grossly normal.

**Observations on Litters B and F Suckling Milk Containing Anti-C**

Litters B and F born to dams immunized against the canine C factor showed no evidence of hemolytic disease despite the fact that from birth they suckled breast milk containing anti-C. All of the 12 pups in these 2 litters were C-positive. The anti-C titers of the maternal serum and milk were similar in the 2 dams; those obtained with the dam of litter F are shown in figure 10. There was no evidence of isoimmunization of either dam by fetal red cells during pregnancy.

Absence of anti-C in the serum of the pups of litters B and F might be attributable to either (1) lack of absorption of the antibody from the gut or (2) attachment of all absorbed antibody to the C-positive cells of the pups. Since C-positive cells exposed to anti-C in vitro are not agglutinated by anti-dog serum rabbit serum, the negative antiglobulin reactions obtained with the cells in these litters do not exclude some form of attachment of anti-C to the pups' cells. Lack of evidence of in vivo hemolysis in litters B and F might have been anticipated.
in view of the benign effects observed after transfusion of large volumes of anti-C into C-positive dogs.\textsuperscript{39}

Canine anti-B and anti-D behave in vitro like anti-C,\textsuperscript{1} and transfusion of large volumes of anti-B and anti-D into adult B-positive and D-positive recipients respectively has thus far produced no clear evidence of erythrocyte destruction.\textsuperscript{39} It is therefore unlikely that anti-B and anti-D will prove capable of causing hemolytic disease in newborn pups, but experiments to test this assumption have not yet been made.

**Discussion**

**Comparison with Hemolytic Disease in Newborn Human Babies**

The principal features of hemolytic disease of the newborn in dogs and human beings are summarized in table 2. Certain differences observed in these species are worthy of separate comment.

**Mode of Isoimmunization**

Transplacental immunization of pregnant bitches by canine A and C factors of fetal red cells has not yet been demonstrated in this laboratory. The effect of multiple pregnancies on this phenomenon has not been studied but it is significant that dams immunized by transfusions before pregnancy showed falling isoantibody titers during pregnancy. Abelson's report\textsuperscript{25} indicates that transplacental immunization may nevertheless occur in dogs, and it can therefore be hoped that dog breeders and veterinarians will be on the alert for this phenomenon, especially in bitches that have had multiple pregnancies. The importance of multiple pregnancies in producing transplacental isoimmunization of human mothers\textsuperscript{31} and mares\textsuperscript{46} is well established.

It is remarkable that the horse placenta, containing even more layers of cells than the dog placenta,\textsuperscript{32} does not form an impassable barrier to the passage of fetal antigens if such be conveyed to the mare's circulation in intact erythrocytes. This phenomenon has been considered by Levine\textsuperscript{33} who points out that in ruminants there are 4 layers of tissue cells (in addition to connective tissue) separating the fetal and maternal blood, (2 in dogs) while in rodents, apes and man there is a single layer of cells in addition to the fetal endothelium. The possible mechanisms of maternal isoimmunization during pregnancy have been ably discussed by Levine\textsuperscript{33} and Coombs,\textsuperscript{35} and their remarks serve to emphasize the need for further investigation of this phenomenon. The extent to which studies on dogs can contribute to an understanding of this problem remains to be determined.

**Mode of Transfer of Isoantibody from Mother to Offspring**

Since Ehrlich\textsuperscript{36} in 1892 investigated the passage in mice of anti-abrin and anti-ricin from mother to offspring by the placental and mammary routes, the transfer of antibodies from mother to young in various species has been the subject of considerable study. Mason, Dalling and Gordon\textsuperscript{46} showed that antibodies to tetanus and diphtheria toxins were not transmitted across the placenta in pigs and ruminants while substantial amounts of these antibodies were ac-
quired from colostrum during the first few hours or days of life. Little or no antibody of this type was obtained in utero by dog embryos but these authors demonstrated absorption of the antibodies from colostrum or from horse serum fed to newborn pups. These investigators and subsequent observers have correlated placental structure with the degree of transplacental passage of antibodies. The diagrams presented by Schneider and Szathmary are especially helpful in gaining an understanding of these relationships. In species such as the pig, cow and horse having

<table>
<thead>
<tr>
<th>Isoimmunization</th>
<th>Transfusion; ? transplacental</th>
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<tr>
<td>Transfer of isoantibody from mother to offspring</td>
<td>Breast milk during first day of life</td>
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<tr>
<td>Pathologic findings in affected offspring:</td>
<td></td>
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<tr>
<td>Fetal hydrops</td>
<td>Absent</td>
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<tr>
<td>Kernicterus</td>
<td>?</td>
</tr>
<tr>
<td>Erythroid hyperplasia of marrow and extra-medullary erythropoiesis</td>
<td>Present</td>
</tr>
<tr>
<td>Hematologic features of the disease in offspring:</td>
<td></td>
</tr>
<tr>
<td>Anemia</td>
<td>Variable</td>
</tr>
<tr>
<td>Hyperbilirubinemia</td>
<td>Mild or absent</td>
</tr>
<tr>
<td>Hemoglobinemia</td>
<td>Infrequent or absent</td>
</tr>
<tr>
<td>Erythroblastosis</td>
<td>Present</td>
</tr>
<tr>
<td>Spherocytosis</td>
<td>Frequently demonstrable</td>
</tr>
<tr>
<td>Osmotic fragility of red cells</td>
<td>Usually well marked increase</td>
</tr>
<tr>
<td>Antiglobulin reaction of red cells (Coombs test)</td>
<td>Regularly positive</td>
</tr>
</tbody>
</table>

a many layered placenta the young do not acquire antibodies prenatally and are therefore dependent on absorption of maternal antibodies from colostrum. In rodents, apes and human beings, on the other hand, antibodies readily pass across the placenta and absorption of antibodies from breast milk is generally considered to be of relatively little importance. Although the dog placenta is of intermediate complexity, Schneider and Szathmary found almost no antibody (antitoxin) in newborn dogs born to bitches immunized with diphtheria toxin, and like Mason, Dalling and Gordon they demonstrated that antibodies to diphtheria toxin were readily absorbed from colostrum by newborn pups.

It would seem paradoxical if intact red cells could pass from fetus to mother
across a many layered placenta, as in the horse and perhaps in the dog\textsuperscript{25} while antibody molecules could not be transmitted in the opposite direction. One possible explanation is that minute breaks in the integrity of the placenta might permit passage of the small numbers of fetal red cells required to immunize the mother,\textsuperscript{34} while maternal antibodies, passively acquired by the fetus through such channels, might not accumulate in the fetal circulation in quantities sufficient to be detected.

Absorption of antibodies from the digestive tract of a number of other mammals is known to be limited to a short period after birth. Bruner et al.\textsuperscript{16} have recently demonstrated that foals born to isoimmunized dams can be protected from development of hemolytic disease if nursing of the sensitized mare is postponed for thirty-six hours. These investigators have also shown that between the ages of 24 and 36 hours a newborn foal loses the ability to absorb antibodies from horse serum (anti-\textit{Salmonella abortivequina}) placed in its stomach in large quantities. The extent to which Rh antibodies are absorbed from breast milk by human babies has been the subject of considerable debate.\textsuperscript{39, 40} Cathie\textsuperscript{41} found that Rh antibodies were not readily destroyed by gastric juice but absorption of these antibodies from the gastro-intestinal tract of babies could not be demonstrated. Absorption of antibody during the first day of life, however, was apparently not investigated. It is pertinent to cite the recent studies of Vahlquist and Hogstedt\textsuperscript{42} who showed that only very small amounts of antibody to diphtheria toxin were absorbed from serum fed to newborns and older infants. Sugg\textsuperscript{43} had previously shown that in one instance the concentration of diphtheria antitoxin in human colostrum was one-third that of the woman's serum but fell very rapidly to 0.25 per cent of the concentration in the serum. The fall in titer of canine anti-A in the breast milk of bitches after whelping is less precipitous.

It seems likely that lack of antibody absorption from the digestive tract after the first day or two of life is attributable either to (1) a change in the absorptive capacity of the gut or to (2) destruction of antibodies by digestive juices. The only change detectable on gross and microscopic examination of the digestive tract of the newborn pup during the first twenty-four hours of life is an increase in the activity of the secretory cells. Antibody was readily demonstrable in the gastric contents of pups at 24 hours but unfortunately it was not feasible to obtain sufficient material from the small intestine for determination of antibody titer.

\textit{Pathologic Findings in Affected Offspring}

Development of hydrops (of the type associated with hemolytic disease) in the human fetus is dependent upon acquisition of isoantibodies by the fetus in utero. Since the pup and the foal acquire no antibody before birth it is not surprising that hydrops is lacking in these species even when the mother is strongly immunized.

The pathogenesis of kernicterus in human infants and in other species\textsuperscript{32, 44} is far from clear. Since this complication of hemolytic disease of the newborn is probably the most grave and the least understood, further observations on the central nervous system of experimentally affected animals are needed. No con-
elusions can be drawn concerning specific nuclear lesions in the A-positive pups thus far examined.

Erythroid hyperplasia of the marrow and extramedullary hematopoiesis are regular findings in hemolytic disease of pups, human babies and in other species. Massive enlargement of liver and spleen, such as may be encountered at times in affected human babies, was not observed in the litters described in this report.

Hematologic Features

The mechanisms by which red cells are destroyed in both canine and human hemolytic disease are not yet clear. Pickles considers it unlikely that Rh antibody has a direct hemolytic effect on infants’ erythrocytes, but she cites one case in which methemalbumin was present in the serum. Although Rh antibodies are not hemolytic in vitro when the usual technics are employed, Hill, Haberman and Jones have reported hemolysis in heparinized blood incubated forty-eight hours with Rh antibodies in the presence of complement.

Spherocytosis has not been described in hemolytic disease of human infants (due to Rh incompatibility) and definitive studies on osmotic and mechanical fragility of the red cells in this condition have not been reported. Of the blood smears and photomicrographs of smears from affected infants examined by the authors, only the photograph shown in figure 10 (p. 93) of Pickles’ monograph reveals many red cells having the appearance of spherocytes. Caution should be exercised in estimating degrees of spherocytosis from fixed smears, but it nevertheless seems reasonable to urge that the question of sphering and increased fragility of the red cells in this disease be thoroughly investigated by serial studies on affected infants during the first few days of life. It will be especially important to study these changes in the red corpuscles in relation to the specificity of the antibodies (anti-D, anti-A, etc.) causing the disease.

Although the osmotic fragility of the red cells is said to be slightly increased at times in infants with erythrocytes affected by Rh antibody, systematic determinations have not been described. Osmotic and mechanical fragility of the erythrocytes in cord blood from 5 mildly affected infants in this clinic were found to be normal, but repeated determinations on the infants’ blood were not made. It will be difficult to make the needed serial studies on blood of severely affected infants because of the current practice of carrying out exchange transfusion in such cases during the first day of life. Diamond has demonstrated destruction of a recipient’s Rh-positive red cells after transfusion of a small amount of potent anti-Rh serum, but we are aware of no reports describing in detail the effect of transfusing relatively large amounts of Rh antibody into Rh positive recipients. It would be of interest to compare such observations with those that have been made after the use of “dangerous” universal donors and after transfusion of A-positive dogs with plasma containing canine anti-A. Maximal sphering and increase in fragility of the recipients’ red cells at twenty-four hours after transfusion, rather than during the first few hours, has been a striking feature of the hematologic picture following transfusion of both human anti-A and canine anti-A.

Since the extent to which infants’ red cells may be agglutinated intravascularly
by Rh-antibody has been the subject of controversy, observations on the comparable phenomenon in newborn dogs are of particular interest. Postmortem tissue sections of the conventional type fixed in Zenker's solution and stained with hematoxylin and eosin did not reveal intravascular agglutination of the red cells in the pups thus far examined. As previously stated, however, the sensitized erythrocytes of most of the A-positive pups showed a tendency to agglutinate slightly in freshly drawn oxalated samples and when suspended in saline without being washed. In view of these observations and the fact that anti-A acts as both agglutinin and hemolysin in vitro, it seems likely that intravascular agglutination occurs in the pups and that it constitutes one stage in the process of destruction of some of the erythrocytes. The extent to which agglutinated cells may impair circulation in organs such as liver and brain is difficult to estimate, but this is a problem deserving further attention.

Hemolysis of dog red cells sensitized with anti-A in the presence of complement occurs rapidly in vitro and well marked hemoglobinemia is readily produced in A-positive adult dogs by transfusion of sufficient anti-A plasma. Lack of hemoglobinemia in affected pups may be attributable in part at least to the fact that anti-A is obtained from breast milk over a period of hours and is brought into contact with the red cells over a longer period of time than in the case of the transfused dogs. In any event, it should be emphasized that canine anti-A is a much more potent hemolysin in vitro than is human anti-Rh in conventional tests and it is therefore likely that these two antibodies exert their effects in vivo in somewhat different ways.

The Coombs antiglobulin test appears to be a sensitive and reliable indicator of coating of red cells with either canine anti-A or human anti-Rh in the two respective species. It is of interest that the degree of hemagglutination produced by various dilutions of antiglobulin rabbit serum does not appear to be correlated with the severity of the hemolytic process in either canine or human hemolytic disease of the newborn. In our experience the strength of the antiglobulin reaction is also unrelated to the rapidity of red cell destruction in patients with chronic "acquired" hemolytic anemia. These observations indicate that much remains to be learned about quantitation of antiglobulin reactions and about the factors other than erythrocyte-bound antibody that are concerned in certain hemolytic processes.

It is noteworthy that although the red cells of the A' pups gave strongly positive Coombs tests, these cells showed no definite spherocytosis and only minimal increase in osmotic fragility, and they were not rapidly destroyed. Comparable findings have been obtained after transfusion of anti-A plasma into A' adult dogs. In view of these findings further observations on hemolytic disease in D' infants and on hemolytic transfusion reactions involving D' cells will be awaited with interest.

**Summary**

1. Bitches with erythrocytes lacking the canine A factor were immunized with intravenous injections of A-positive dog cells and mated with A-positive sires.
All A-positive pups born to such dams developed hemolytic disease provided they suckled the immunized dam during the first day of life. There was no evidence of transplacental isoimmunization or of transfer of antibody across the placenta from mother to pup. Anti-A could not be demonstrated in the blood of pups at birth and was not acquired by pups that first suckled an immunized bitch after the first day of life. A-positive pups born to a non-immunized bitch developed hemolytic disease after suckling an immunized foster dam on the day of birth.

2. Of 24 affected A-positive pups, 3 were sacrificed, 9 died within three days of birth and 12 recovered, 2 with the aid of transfusions of A-negative blood. Autopsies revealed varying degrees of hepatomegaly, splenomegaly, erythroid hyperplasia of the bone marrow, extra-medullary erythropoiesis, and questionable evidence of specific injury of the nerve cells of the basal nuclei of the brain.

3. The degree of anemia in the A-positive pups varied widely, the minimum hematocrit for the group being 10 per cent at forty-eight hours after birth. Erythrobiosis, reticulocytosis and spherocytosis were noted in most of the severely affected pups and osmotic fragility of the red cells was substantially increased in all of the A-positive pups exposed to anti-A.

4. The concentration of bilirubin in the serum of most of the affected pups was only slightly increased. The relatively small increases in serum bilirubin, compared with those in hemolytic disease of human infants, are presumably attributable to the unusual capacity of the dog liver for excreting bilirubin.

5. The red cells of all affected A-positive pups gave antiglobulin (Coombs) reactions and in surviving pups the cells were agglutinable in antiglobulin rabbit serum for as long as twenty-two days in A pups and sixty-five days in A' pups. The degree of reactivity with antiglobulin serum was not correlated with the severity of the hemolytic process. Erythrocytes of the very mildly affected A' pups were strongly agglutinated by antiglobulin serum but showed no definite spherering and only slight increase in osmotic fragility.

6. Serum of the 20 A-negative litter mates examined in this study contained anti-A for periods as long as thirty-two days while the red cells of these pups remained normal. A few of the A-positive pups also had anti-A in the serum. The in vitro behavior of anti-A in the pups' sera was identical with that of anti-A in the maternal sera.

7. Twelve C-positive pups in 2 litters born to bitches immunized against the canine C factor showed no evidence of hemolytic disease.

8. Hemolytic disease of newborn dogs is compared with that of human infants and the need for further investigation of certain aspects of the disorder in both species is stressed.

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Hemolytic Disease in Newborn Dogs

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