Experimental Hemolytic Anemia in Rabbits. Protective Role of Sensitization to the Species-specific Protein of the Heteroimmune Hemolytic Serum

BY ISRAEL DAVIDSOHN, DAVID HERMONI AND ELEANOR G. HANAWALT

HEMOLYTIC ANEMIA was one of the first diseases of blood produced artificially and studied in animals. Much valuable information has been gathered in this way. However, some moot points remain which require elucidation. In this paper experiments will be described which add to elucidation of the nature of a hitherto not fully understood phenomenon in the natural history of experimental hemolytic anemia.

The standard procedure for the production of experimental heteroimmune hemolytic anemia is to select animals of two different species; one for the actual hemolytic experiment, the other as the source of hemolytic serum.

Various systems have been employed by various authors since 1898, when Belfanti and Carbone introduced the use of heterologous immune serum for the study of experimental hemolytic anemia in animals. Dameshek and Schwartz stimulated renewed interest in this field by reporting a systematic study of the experimental disease, including minute technical details, and by pointing out the similarity with human disease. Their studies have thrown much light on the disease in animals and in man.

In the present study, heteroimmune hemolytic serum was produced in guinea pigs injected with erythrocytes of rabbits. The hemolytic serum produced in this manner was then injected into rabbits.

Clinic of Experimental Hemolytic Anemia

Using properly standardized immune serums we observed, in accord with previous reports, three distinct varieties of the disease, depending on the dose used:

(1) Hemolytic shock.
This was a fulminant shock-like condition terminating fatally in a matter of hours. The size of the dose needed for the production of this form of the disease varied for different lots of serum, but was the largest for each lot.

(2) Acute fatal hemolytic anemia, resulting in death in from two to five days, followed injection of a smaller dose (fig. 1).

Rabbit No. 142 was injected intravenously with 0.5 ml. per Kg. of hemolytic serum No. 1. Red cell count and hemoglobin dropped precipitously to 2,000,000 and 3.7 Gm., respectively, within three days. The spherocytes rose to almost 100 per cent, the normo-
blasts to 2062 per cu.mm. There was no significant reticulocyte response. The direct antoglobulin test was positive when first tested on the second day within 24 hours after injection. For the antoglobulin test, an immune serum produced in rabbits injected with normal guinea pig serum was used. Details regarding this test will be presented later. The animal died on the third day. The autopsy showed an acutely congested spleen. The urinary bladder was widely distended with bloody urine. On microscopic examination of the urine only very occasional red cells were seen. The color of the urine was caused by hemolysis.

The course of events in rabbit No. 142 was representative of acute hemolytic anemia, as seen in all our animals given an adequate dose.

In later experiments the antoglobulin test was positive as early as four hours after injection of hemolytic serum, which was the earliest this test was tried.

(3) Subacute hemolytic anemia with recovery.

![Graph](image-url)  
**Fig. 1.—Acute hemolytic anemia**
When smaller doses were injected singly or repeatedly the course was different (fig. 2).

In rabbit No. 159 there was a moderate drop in the red cell count after the first intravenous injection of 0.25 ml. per Kg. of a hemolytic serum which produced a rapidly fatal hemolytic anemia in control animals with 0.5 ml. per Kg. Following the second injection of the same dose on the sixth day, the hemolytic effect was intensified and the anemia reached the low level of 2.6 million red cells and 6 Gm. of hemoglobin on the ninth day. Spherocytes appeared within hours after the first injection of serum and continued to rise steadily until the peak of 86 per cent was reached on the seventh day. The antiglobulin test remained positive until the 14th day. At the time when spherocytes began to decline and reticulocytes to rise, the red cell count and hemoglobin began rising and gradually...
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returned to the preinjection level. The third injection of hemolytic serum on the 10th day failed to retard the upward trend and the fourth injection on the 22nd day showed no effect.

Additional details of hematologic and immunohematologic changes and the protecting effect of repeated injections of sublethal doses in another animal are recorded in Table 1.

The progression of the anemia until the ninth day and the steady improvement from then on in spite of another injection of hemolytic serum were typical of several other animals treated similarly.

The data presented in this table illustrate that the drop of the red cell count and hemoglobin, the spherocytosis and the positive antiglobulin test ran parallel. The subsequent return to normal of all these values was almost simultaneous. This behavior was the rule in all rabbits so treated. Normoblasts were also a sensitive indicator but they did not always appear as regularly and as persistently as in this rabbit. The increase of reticulocytes coincided with improvement of the anemia. Changes in the number of leukocytes similar to those reported by other authors were noted, but are not included in this report.

A single injection of a sublethal dose had a similar effect as had repeated doses. When given to rabbit No. 144, it was followed by an anemia of lesser intensity, with fewer spherocytes, with a shorter period of positive antiglobulin test and a more rapid recovery (fig. 3). However, the resistance to subsequent injections of the serum was just as clear-cut. When a fatal dose of serum was administered on the 20th day, the drop of red cells and hemoglobin and the rise of spherocytes

TABLE 1.—Protecting Effect of Repeated Injections of Sublethal Doses of Hemolytic Antirabbit Red Cell Serum Produced in Guinea Pigs

Rabbit #160—Weight 2.95 Kg.

<table>
<thead>
<tr>
<th>Immune hemolytic serum no.</th>
<th>ml/Kg i.v.†</th>
<th>Days after injection</th>
<th>RBC</th>
<th>Hb</th>
<th>Normoblasts per cu. mm.</th>
<th>Reticulocytes %</th>
<th>Spherocytes %</th>
<th>Direct antiglobulin test</th>
<th>Results</th>
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<tbody>
<tr>
<td></td>
<td>0.25</td>
<td>0.32</td>
<td></td>
<td>5.75</td>
<td>11.0</td>
<td>0</td>
<td>0.3</td>
<td>0.0</td>
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<tr>
<td></td>
<td>1.25</td>
<td>1</td>
<td>4.89</td>
<td>11.0</td>
<td>102</td>
<td>1.0</td>
<td>0.0</td>
<td>–</td>
<td>Incipient anemia</td>
</tr>
<tr>
<td></td>
<td>2.25</td>
<td>2</td>
<td>4.87</td>
<td>10.0</td>
<td>134</td>
<td>0.2</td>
<td>32.8</td>
<td>+</td>
<td>Peak of anemia</td>
</tr>
<tr>
<td></td>
<td>3.25</td>
<td>3</td>
<td>3.95</td>
<td>9.4</td>
<td>2828</td>
<td>0.0</td>
<td>41.0</td>
<td>+</td>
<td>Peak of anemia</td>
</tr>
<tr>
<td></td>
<td>4.25</td>
<td>4</td>
<td>3.14</td>
<td>7.1</td>
<td>4095</td>
<td>0.0</td>
<td>64.0</td>
<td>+</td>
<td>Peak of anemia</td>
</tr>
<tr>
<td></td>
<td>5.25</td>
<td>5</td>
<td>2.56</td>
<td>6.3</td>
<td>3927</td>
<td>0.2</td>
<td>62.8</td>
<td>+</td>
<td>Peak of anemia</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>6</td>
<td>2.60</td>
<td>6.3</td>
<td>1255</td>
<td>0.6</td>
<td>39.6</td>
<td>+</td>
<td>Peak of anemia</td>
</tr>
<tr>
<td></td>
<td>7.25</td>
<td>7</td>
<td>2.60</td>
<td>6.3</td>
<td>1255</td>
<td>0.6</td>
<td>39.6</td>
<td>+</td>
<td>Peak of anemia</td>
</tr>
<tr>
<td></td>
<td>8.25</td>
<td>8</td>
<td>3.19</td>
<td>6.6</td>
<td>2492</td>
<td>3.0</td>
<td>27.0</td>
<td>+</td>
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</tr>
<tr>
<td></td>
<td>9.25</td>
<td>9</td>
<td>3.80</td>
<td>9.4</td>
<td>945</td>
<td>12.2</td>
<td>10.6</td>
<td>+</td>
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<tr>
<td></td>
<td>10.25</td>
<td>10</td>
<td>4.00</td>
<td>9.4</td>
<td>490</td>
<td>19.0</td>
<td>18.2</td>
<td>+</td>
<td>Peak of anemia</td>
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<tr>
<td></td>
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<td>11</td>
<td>4.48</td>
<td>10.0</td>
<td>0</td>
<td>0.0</td>
<td>1.8</td>
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<td>Recovery</td>
</tr>
<tr>
<td></td>
<td>12.25</td>
<td>12</td>
<td>5.10</td>
<td>11.0</td>
<td>0</td>
<td>8.0</td>
<td>0.0</td>
<td>–</td>
<td>Recovery</td>
</tr>
<tr>
<td></td>
<td>13.25</td>
<td>13</td>
<td>5.20</td>
<td>11.0</td>
<td>0</td>
<td>5.0</td>
<td>0.0</td>
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<td>Recovery</td>
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<tr>
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<td>14.25</td>
<td>14</td>
<td>4.90</td>
<td>11.0</td>
<td>0</td>
<td>1.0</td>
<td>0.0</td>
<td>–</td>
<td>Recovery</td>
</tr>
<tr>
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<td>15.25</td>
<td>15</td>
<td>5.34</td>
<td>11.0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>–</td>
<td>Protection 4+</td>
</tr>
</tbody>
</table>

RBC = Red blood cells (million per cu. mm.). Hb = Hemoglobin (Gm. per 100 ml.). – = Negative. + = Positive.

* The dose of 0.5 ml. per Kg. injected intramuscularly was fatal in control rabbits in two days.

† Milliliters per Kg. of rabbit weight injected intravenously.
EXPERIMENTAL HEMOLYTIC ANEMIA IN RABBITS

Fig. 3.—Subacute anemia after single injection of sublethal dose; recovery.

were slightly less marked than after the first one-half dose and the antiglobulin test was positive only during a 48 hour period.

The modifying influence of the first injection on the effect of the second was distinct.

The phenomenon of resistance illustrated in the preceding two experiments has been known for many years. Muir and McNee wrote about it as early as 1911. They stated: "When a period of from five to six days is allowed to elapse after an injection, a degree of immunity seems to be established." Samaille et al. discussed the phenomenon of acquired resistance in a recent paper. They injected young dogs with normal rabbit serum and observed protection against subsequent injections of hemolytic rabbit serum. Arbouys and Eyquem found
that after resistance to hemolytic action had developed it could be overcome and anemia could be produced repeatedly when hemolytic immune serums produced in animals of different species were used for the subsequent injections. The last two reports of the French investigators showed clearly that sensitization to the species specific protein of the hemolytic serum was responsible for the resistance to the hemolytic action of later injections of the same serum.

The purpose of this presentation is to report observations which demonstrate how the resistance develops and how it can be removed without the use of a hemolytic serum of a different animal species. Our experiments are designed to explain the mechanism of the resistance that develops after injection of heterologous hemolytic immune serum.

**Experimental Methods**

I. Production of antirabbit erythrocyte guinea pig serum.

1. Preparation of rabbit red cells.
   Blood was obtained by cardiac puncture using 3.8 per cent sodium citrate as anticoagulant. The red cells were washed three times in about 10 times their volume of physiologic saline solution and a 20 per cent suspension in physiologic saline was prepared.

2. Immunization of guinea pigs.
   Five consecutive daily intraperitoneal injections of 2.0 ml. of the cell suspension were given, followed by a sixth injection seven days later. One week later the hemolysin and agglutinin titers were determined. If the titer of complete hemolysis was at least 1:128, the animal was bled. The titer of agglutinins in such serum was, as a rule, from 1:512 to 1:1024. If the titer was lower, a second series of injections was given.

3. Standardization of hemolytic serum.
   a. Titration of hemolysin
      To 0.2 ml. of progressive twofold dilutions of guinea pig immune serum, inactivated at 56 C. for 30 minutes, 0.4 ml. of a 1:10 dilution of lyophilized guinea pig complement and 0.2 ml. of a 2.0 per cent suspension of rabbit red cells in normal saline were added. The test tubes were shaken and incubated at 37 C. for 30 minutes. The tubes were then centrifuged and the titer recorded in terms of complete and 2+ hemolysis.
   b. Titration of agglutinins.
      The test was set up exactly as described for hemolysin titration, except that complement was omitted. The rack was incubated for two hours at 37 C. and the titers recorded as the highest dilution showing 1+ agglutination visible with the naked eye.
   c. Guinea pig serums with satisfactory titers were pooled and hemolysin and agglutinin titers of the pooled serum were determined. The titer for 2+ hemolysis was usually from one to two tubes higher than for complete hemolysis.
   d. Pooled hemolytic serums, inactivated at 56 C. for 30 minutes, with titers of 1:128 to 1:256 for complete hemolysis were used in the experiments.

II. Anti-guinea pig globulin immune serum was produced in the rabbit according to the method of Wootton.

III. The following examinations were done prior to immunization to establish the normal values, and then repeatedly in the course of the experiments:

1. Hemoglobin as oxyhemoglobin. The blood was diluted in 0.01 N ammonium hydroxide in the Coleman Spectrophotometer.
2. Red and white blood cell counts.
3. Differential counts and red cell morphology.
4. Reticulocyte counts.
5. Direct antiglobulin test (Coombs test).
6. Quantitative direct antiglobulin test. Progressive twofold dilutions of the anti-
globulin serum were mixed with equal volumes of 2 per cent suspensions of red cells. The highest dilution of the serum giving a microscopically visible agglutination was recorded as the titer.

7. Tests for precipitins:
   a. Sensitization of rabbits with normal guinea pig serum.

   Normal guinea pig serum was injected into normal rabbits by intravenous or intramuscular routes. The titers of precipitins were determined in sera of rabbits prior to administration of the hemolytic guinea pig serum or of normal guinea pig serum and subsequently at intervals. Rabbit serum was inactivated at 56 C. Normal guinea pig serum was used as antigen. For each serum a row of 12 small serologic test tubes was set up; 0.2 ml. of undiluted test (rabbit) serum was overlain with 0.2 ml. of progressive twofold dilutions of normal guinea pig serum; the mixture was incubated for from one to two hours at 37 C. and the results recorded as the highest dilution of normal guinea pig serum showing a ring of precipitation at the surface of contact.

   b. Desensitization of sensitized rabbits:

   Four series of injections were given consecutively to each animal, as follows:
   1) Four intracutaneous injections of 0.5 ml. of normal guinea pig serum at 15 minute intervals, a total of 2 ml.
   2) Four intramuscular injections of 0.5 ml. of normal guinea pig serum at 15 minute intervals, a total of 2 ml.
   3) Five intraperitoneal injections of 1.0 ml. at 15 minute intervals, a total of 5 ml.
   4) Eight intravenous injections, at 15 minute intervals, of 0.1, 0.1, 0.3, 0.5 of a 1:10 dilution of normal guinea pig serum, and of 0.1, 0.3, 0.5 and 1.0 ml. of undiluted normal guinea pig serum, a total of 2 ml.

   The total amount of serum given was 11 ml. A precipitin test was run on blood withdrawn immediately after desensitization. No changes in the blood picture and no other changes or abnormalities were noted during or following the injection of 11 ml. of the desensitizing serum.

   As a rule, the same lot of standardized hemolytic immune serum was used for all animals of each experiment. The hemolytic activity of the serum was determined by injecting several rabbits. The animals used in each experiment were of approximately equal weight.

Normal Blood Findings in Rabbits

In the study of 40 normal, healthy rabbits weighing approximately the same as our experimental animals, the following values recorded as maximum ranges were found:

   Red blood cells: 5.12 to 7.36 million per cu. mm.
   Hemoglobin: 10.7 to 15.1 Gm. per 100 ml.
   Hematocrit: 35 to 50 per cent
   Reticulocytes: 0.0 to 3.0 per cent

Results

Experiment 1

The purpose of this experiment was to see if rabbits sensitized with normal guinea pig serum became resistant to subsequent injections of hemolytic immune serum produced in guinea pigs.

One-half ml. per Kg. of body weight of normal guinea pig serum was given intravenously to rabbit No. 200. Similar and considerably larger amounts of normal guinea pig serum had no effect on the blood of control rabbits. Ten days later the precipitin titer against normal guinea pig serum was 1:1024. Fifty-two days after the injection, precipitins could not be detected. Thirteen days after a second injection of 0.3 ml. per Kg. of normal guinea pig serum
serum, the precipitins rose to a titer of 1:2048. The following day, 1.0 ml. per Kg. of hemolytic serum No. 7 was injected intramuscularly. This dose of the serum produced a rapidly fatal hemolytic anemia in normal control (fig. 4).

Only an insignificant lowering of the red cell count to 4,130,000 from the pretreatment level of 5,000,000 was noted on the 17th day. Such mild degrees of anemia have been observed in untreated animals subjected to repeated removal of blood for tests. Only occasional spherocytes were seen. The reticuloocyte response was not significant. The antiglobulin test was negative throughout.
TABLE 2.—Sensitization by Normal Guinea Pig Serum with Resulting Protection Against Hemolytic Serum

Rabbit #197—Weight 3.33 Kg.

<table>
<thead>
<tr>
<th>Sensitization</th>
<th>Immune hemolytic serum</th>
<th>RBC</th>
<th>Nor-</th>
<th>Reticulo-</th>
<th>Sphero-</th>
<th>Direct anti-</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGPS ml/Kg.</td>
<td>Day after injection</td>
<td>Precipitin</td>
<td>Day after injection</td>
<td>Hb</td>
<td>no-</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0.2 i.v.</td>
<td>0</td>
<td>7.09 14.3</td>
<td>0</td>
<td>0.4</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.4 i.m.</td>
<td>20 1:512</td>
<td>6.43 13.1</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>21 1:2048</td>
<td>22 3* 0.35</td>
<td>6.06 12.9</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
<td>Sensitization 3+</td>
</tr>
<tr>
<td>23</td>
<td>1 4.99 12.0 87</td>
<td>4.0 12.6</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2 4.36 10.7</td>
<td>1.4 31.0</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>7 5.75 11.4</td>
<td>2.8 Oec.</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
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<td>37</td>
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<td>19 5.74 12.7</td>
<td>0</td>
<td>2.4</td>
<td>0</td>
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</tr>
<tr>
<td>46 1:512</td>
<td>46 1:2048</td>
<td>4 5.23 11.0 535</td>
<td>4.8</td>
<td>6.6</td>
<td>0</td>
<td>-</td>
<td>Protection 4+</td>
</tr>
<tr>
<td>50</td>
<td>5 5.94 11.4 176</td>
<td>4.6 12.8</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>5 5.03 10.7</td>
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<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
<td></td>
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<td>57</td>
<td>11 5.75 12.0</td>
<td>0.6 Oec.</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

NGPS = Normal guinea pig serum. RBC = Red blood cells (million per cu. mm.). Hb = Hemoglobin (Gm. per 100 ml.). i.v. = Intravenous. i.m. = Intramuscular.
* Immune hemolytic serum #3: The dose of 0.4 ml. per Kg. injected intravenously was fatal in control rabbits in one day.
† Immune hemolytic serum #4: The dose of 0.6 ml. per Kg. injected intramuscularly was fatal in control rabbits in five days.

Detailed changes in the blood picture in another similarly treated rabbit are shown in table 2.

Two injections of normal guinea pig serum were followed in rabbit No. 197 by sensitization to guinea pig protein as manifested by development of precipitins. No hemolytic effects were observed following injection of normal guinea pig serum. Intramuscular injection of immune hemolytic serum was followed by minimal changes limited to a slight and transient drop in the red cell count and hemoglobin and moderate spherocytosis. A third injection of normal guinea pig serum combined with the effect of the first injection of hemolytic serum increased the titer of precipitins and the degree of protection, as evidenced by a minimal reaction to injection of a fatal dose of another hemolytic serum.

Interpretation: These and other similar experiments showed uniformly that rabbits injected with normal guinea pig serum became resistant to injections of antirabbit red cell immune hemolytic serum produced in guinea pigs.

Experiment 2

The purpose of this experiment was to see if resistance following sensitization with normal guinea pig serum could be removed by desensitization with normal guinea pig serum.

Rabbit No. 219 (fig. 4) was treated identically as rabbit No. 200. It was sensitized with normal guinea pig serum and developed a high titer of precipitins. It was then desensitized with 11 ml. of normal guinea pig serum given in frac-
tional doses. The precipitin test became negative. As stated previously, the same
and larger amounts of normal guinea pig serum had no effect on the blood of
control animals. One ml. per Kg. of hemolytic serum No. 7 was injected. A
rapidly fatal hemolytic anemia resulted.

Additional observations and details of hematologic and immunohematologic
findings are recorded in table 3.

In rabbit No. 195, a high titer of precipitins and a high degree of sensitiza-

| Table 3.—Sensitization by Normal Guinea Pig Serum with Resulting Protection. Desensitization Removes the Protection. |
|-------------------------------------------------|-------------------|----------------|-----------------|-----------------|----------------------|
| Sensitization | Immune hemolytic serum | RBC | Hb | Normal | Reticulocytes | Spherocytes | Direct anti-globulin test | Results |
| NGPS ml/Kg. | Day after injection | Precipitin | ml/ Kg. l.m. | Day after injection | | % | | |
| 0.3 i.v. | 0 | 7.79 | 15.5 | 0 | 0.5 | 0.0 | | |
| 14 | 1:1024 | | | | | | | |
| 0.4 i.m. | 20 | 6.57 | 13.1 | 0 | 0.8 | 0.0 | | Sensitization 3+ |
| 22 | 0 | 5.26 | 11.0 | 0 | 2.0 | 0.0 | | |
| 29 | 0 | 4.29 | 8.6 | 0 | 3.8 | 15.2 | | |
| 28 | 6 | 4.75 | 9.8 | 0 | 7.0 | 10.6 | + Protection 3+ |
| 25 | 4 | 5.10 | 9.7 | 0 | 6.0 | 10.0 | | |
| 29 | 7 | 5.21 | 10.0 | 136 | 3.0 | 8.2 | | |
| 0.58 i.m. | 0 | 5.89 | 11.0 | 157 | 0.2 | Occ. | | Sensitization 4+ |
| 65 | 1.0 | 6.13 | 12.7 | 0 | 2.4 | Occ. | | |
| 66 | 2 | 6.33 | 13.1 | 0 | 4.6 | 0.0 | | |
| 67 | 5 | 5.25 | 9.4 | 125 | 0.6 | Occ. | | |
| 66 | 11 | 5.55 | 11.0 | 0 | 0.4 | 0.0 | | Protection 4+ |
| 76 | | | | | | | | |
| Rabbit #195—Weight 3.8 Kg. | | | | | | | | |
| 0.2 i.v. | 0 | 6.54 | 12.7 | 328 | 0.2 | 0.0 | | Sensitization 4+ |
| 14 | 1:1024 | | | | | | | |
| 20 | 1:2048 | 6.67 | 13.8 | 89 | 0.3 | 0.0 | | |
| 21 | 3 | 0 | 6.44 | 13.1 | 0 | 2.4 | 0.0 | | |
| 22 | 1 | 5.94 | 12.7 | 0 | 1.8 | 0.0 | | |
| 23 | 2 | 6.20 | 12.7 | 0 | 6.0 | 8.6 | | |
| 28 | 7 | 6.05 | 12.4 | 0 | 0.8 | Occ. | | Protection 4+ |
| 63 | 1:2048 | | | | | | | |
| 65 | 1.0 | 6.92 | 13.8 | 94 | 0.4 | 0.0 | | Sensitization 4+ |
| 66 | 1 | 7.40 | 14.7 | 7567 | 1.2 | 32.2 | + Protection 0 |
| (Died within 24 hours of injection; cause: acute hemolytic shock) |

NGPS = Normal guinea pig serum. RBC = Red blood cells (millions per cu. mm.). Hb = Hemoglobin (Gm. per 100 ml.). i.v. = Intravenous. i.m. = Intramuscular.

* Immune hemolytic serum $3$: The dose of 0.4 ml. per Kg. injected intravenously was fatal in control rabbits in one day.

† Immune hemolytic serum $6$: The dose of 1.0 ml. per Kg. injected intramuscularly was fatal in control rabbits in four days.

‡ Desensitization with 11 ml. of normal guinea pig serum removed detectable precipitins.
tion resulted from repeated injections of normal guinea pig serum and was followed by complete protection when a fatal dose of hemolytic serum was administered.

On the other hand, a similar state of protection produced in rabbit No. 196 was overcome by desensitization which removed the precipitins and simultaneously made the animal susceptible to the hemolytic action of the immune serum. The hemolytic serums used in both rabbits were identical.

Interpretation: Observations made on the last three rabbits showed striking parallelism between the degree of sensitization and protection against the hemolytic serum, and, on the other hand, the parallelism of the degree of desensitization and resulting lack of protection.

This and other similar experiments demonstrated that following desensitization the animals became again susceptible to the hemolytic action of the immune serum. Complete desensitization removed the protection and made the animal behave like an animal not treated previously.

Experiment 3

The purpose of this experiment was to investigate further if there was a quantitative relation between the degree of sensitization to guinea pig serum and the resistance to the hemolytic action of immune antirabbit red cell guinea pig serum.

Rabbit No. 220 (fig. 4) was treated identically as the preceding four rabbits, but it produced a lower titer of precipitins (1:256). Following injection of a fatal dose of immune serum there was a drop in the red cell count to 1,700,000 in seven days, with a corresponding drop in hemoglobin, a spherocytosis reaching a peak of 84 per cent on the fourth day, and a positive antiglobulin test lasting from the third to the eighth day.

Interpretation: A comparison of results in rabbit No. 220 with those in rabbit No. 200, and other similar observations, demonstrated a direct relationship between the degree of sensitization and the protection against the hemolytic effect of the immune serum.

Variations in the effect of sensitization and desensitization have been observed, as illustrated by the following experiment.

Experiment 4

The purpose of this experiment was to study variations in the effect of sensitization on the inhibition of hemolytic activity of immune guinea pig serum.

One ml. per Kg. of hemolytic serum No. 10 produced in control rabbit No. 249 a severe and rapidly fatal hemolytic anemia (fig. 5). The animal died on the fourth day. The antiglobulin test became strongly positive in a matter of hours after the injection and remained positive as long as the animal lived. On the day before death the red cell count was 1,060,000, the hemoglobin 3.2 Gm., there were 21060 normoblasts per cu. mm., the reticulocyte count was 2.8 per cent, and the spherocytes 97 per cent.

Rabbit No. 246 was treated first with normal guinea pig serum, with a good precipitin response on the 20th day. On the 26th day the precipitin titer was 1:1024. Injection of 1.5 ml. per Kg. of hemolytic serum No. 10, a dose 50 per
Fig. 5.—Control for hemolytic serum No. 10 (rabbit No. 249). Inhibition of hemolytic action of immune guinea pig serum following sensitization with normal guinea pig serum (rabbit No. 246). Twice the fatal dose of immune hemolytic serum overcame the protection by sensitization (rabbit No. 248).

cent larger than the one used in control rabbit 249, resulted only in a slight drop of red cells from 5,060,000 to 4,100,000 on the fifth day, a spherocytosis with a maximum of 78 per cent on the ninth day, a reticulocytosis with a maximum of 4.6 per cent on the sixth day, and a maximum of 875 normoblasts per cu.mm. in the circulating blood on the ninth day. On the same day the red cell count and hemoglobin were normal and remained so throughout the period of observation. The antiglobulin test was negative throughout.
Only a slight anemia developed, to which the bleedings for blood testing may have contributed.

Interpretation: The protection was clear, especially in view of the fact that the dose was 50 per cent larger than the one capable of producing the fatal result in control animals. This may explain the presence of pronounced spherocytosis.

It is interesting that whereas the protection by sensitization was apparently sufficient to prevent a significant degree of anemia and the appearance of positive antiglobulin test, it did not interfere with development of spherocytes. The antiglobulin test was the more sensitive indicator of the protective influence of sensitization.

This and other experiments showed that the factor in hemolytic serum which is responsible for spherocytosis is less readily inhibited by sensitization than is the factor involved in the antiglobulin test.

Experiment 5

The purpose of this experiment was to study the effect of an excessively large dose of hemolytic serum on a sensitized and protected animal.

Rabbit No. 248 was first sensitized with normal guinea pig serum, produced a precipitin titer of 1:2048, and was then injected with 2 ml. per Kg., twice the fatal dose, of the hemolytic serum (fig. 5). There was a moderate anemia on the fifth day (red blood cells 3,200,000, hemoglobin 6.8 Gm.), a slight reticulocyte response (maximum 6.4 per cent), and a pronounced spherocytosis of 78 per cent on the seventh day. The antiglobulin test was positive but only for 24 hours. The animal recovered rapidly. Sensitization protected the animal against twice the fatal dose. The spherocyte response was considerably less inhibited than the antiglobulin reaction, in accord with observations discussed previously.

Interpretation: The inhibition achieved by sensitization was relative. It could be overcome by an adequate amount of a potent hemolytic serum.

Experiment 6

The purpose of this experiment was to see if sensitization with immune hemolytic guinea pig serum had a similar inhibitory effect as sensitization with normal guinea pig serum.

Two different hemolytic immune serums were used in this experiment. During the first half of the experiment, hemolytic serum No. 7 was used and rabbit No. 228 was the control (fig. 6). The animal died on the fourth day. The anemia progressed rapidly and reached a low level of 760,000 red cells and 2.4 Gm. of hemoglobin before death. The peak of spherocytosis was 90.4 per cent, the normoblasts were 9923 per cu.mm., and the reticulocytes 7.6 per cent, all on the last day.

Rabbit No. 250, the control for hemolytic serum No. 9, developed a severe hemolytic anemia with 1,300,000 red blood cells, 3.2 Gm. hemoglobin, and normoblasts 8,664 per cu.mm. on the fifth day, spherocytosis of 94 per cent on the third day, and a positive antiglobulin test lasting from the first to the sixth day (fig. 6). The animal recovered.

Rabbit No. 229 was injected with 0.5 ml. per Kg. of hemolytic immune
Fig. 6.—Control for hemolytic serum No. 9. Immune guinea pig serum has a similar protecting effect as normal guinea pig serum (rabbit No. 229).

serum No. 7, divided in three daily doses as follows: 0.2 ml., 0.1 ml., 0.2 ml. per Kg. (fig. 6). An anemia of 2,360,000 red blood cells and a hemoglobin of 5.6 Gm. were present on the fifth day, spherocytosis (59.4 per cent) on the fourth day, a positive direct antiglobulin test from the second to the fifth day, an early outpouring of normoblasts (6475 per cu.mm.) on the fourth day, and a reticulocytosis (12.8 per cent) on the fifth day. On the fifth day the precipitin titer was 1:128. Beginning with that day, the blood findings began to return to normal and the animal was apparently fully recovered on the 10th day. On the 21st day after the original injection the precipitin titer was 1:2048. At that time
the blood picture was normal. Two ml. per Kg. of hemolytic serum No. 9 were
given. The red cell count dropped from 6,270,000 to 4,290,000 and the hemo-
globin from 12 to 9 Gm. on the fourth day after the injection. There was spher-
ocytosis up to 64 per cent. The antiglobulin test was negative. The blood findings
returned to normal on the 10th day.

Interpretation: Sublethal doses of hemolytic immune guinea pig serum can
produce the same degree of sensitization as normal guinea pig serum and con-
sequentially, the same degree of protection against subsequent injections of hemo-
lytic serum.

Discussion

Rabbits were injected with single or multiple sublethal doses of antirabbit
red cell immune serum produced in guinea pigs. After they recovered from the
hemolytic anemia they became resistant to subsequent injections of the hemo-
lytic serum.

The same resistance could be produced by injections of normal guinea pig
serum. There was a direct relationship between the degree of sensitization as
manifested by the titer of precipitins and the protection against the hemolytic
action of the immune serum. Rabbits with high titters of precipitins showed less
pronounced hemolytic effects than did rabbits which had low titters of precipitin.

The resistance produced by treatment with immune or normal guinea pig
serum could be removed by desensitizing injections of normal guinea pig serum.

Desensitization with normal guinea pig serum removed the precipitins and
the hemolysis-inhibiting effect at the same time and made the animals suscep-
tible once more to the hemolytic action of the immune antirabbit red cell guinea
pig serum.

Incomplete desensitization, as manifested by remaining precipitin titters, was
followed by partial retention of resistance to the hemolytic action.

There was a quantitative relationship between the degree of sensitization to
the heterologous specific protein and the protection against the hemolytic action
of the heteroimmune serum, on one hand, and the degree of desensitization and
removal of the protection, on the other.

Sufficiently large doses of immune hemolytic guinea pig serum were capable
of overcoming the resistance to the hemolytic effect which resulted from pre-
vious injections of sublethal doses of the hemolytic serum or of normal guinea
pig serum.

The intravenous route was the most effective in demonstrating the potency
of hemolytic immune serums. It required the smallest dose to produce the hemolo-
ytic effect in the shortest time.

Spherocytosis and the antiglobulin test proved the most sensitive indicators
of the earliest action of the hemolytic serum. The antiglobulin test was a more
sensitive indicator of the protective influence of sensitization by the heterologous
protein. It was negative or only transiently positive in the protected animals.
Spherocytosis persisted long after the antiglobulin test became negative. This
suggests that the factor or factors responsible for the two phenomena are in-
fluenced differently or to a different degree by sensitization and the resulting
resistance to the hemolytic action.
Antiglobulin tests were done four hours after injection of hemolytic serum and later at intervals. Observation on antiglobulin tests done within minutes after injection of the hemolytic serum will be the subject of a separate report.

The possible role of complement in the phenomena described in this report was considered. The data collected thus far have not been sufficient to permit evaluation.

**Conclusion**

Inhibition of the hemolytic and anemia producing action of immune anti-rabbit red cell guinea pig serum in rabbits was shown to be the result of sensitization to guinea pig serum protein.

**Summario in Interlingua**

Esseva monstrate que le inhibition del action hemolytic e anemisante, que es effectuate in conilios per sero de porcos de India immun a erythrocytos de conilio, es le resultado de sensibilisation a proteina de sero de porco de India.

**References**

Experimental Hemolytic Anemia in Rabbits. Protective Role of Sensitization to the Species-specific Protein of the Heteroimmune Hemolytic Serum

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