Anemia with Positive Direct Coombs’ Test
Induced by Trypan Blue

By David V. Brown, Eva M. Boehni and Lorna M. Norland

Positive Direct Coombs’ tests in human anemia are generally regarded as evidence of autoimmune phenomena. The experimental production of positive antiglobulin tests by drugs and chemical agents, however, has suggested that nonimmunologic mechanisms may also be responsible for protein coating of erythrocytes. During attempts to produce tumors of the reticuloendothelial system in rats by injection of trypan blue, an anemia, characterized by increased erythrocyte destruction and a positive Coombs’ test, was noted following large injections of the dye. The present report describes this experimentally induced anemia and includes a discussion of the mechanisms involved in its production.

Materials and Methods

Experimental animals—The studies were carried out on male and female Wistar strain rats (Carworth farms) weighing from 250-350 Gm. The animals were housed individually in air conditioned quarters, and maintained on a diet of Purina Laboratory Chow plus a special supplement. Greens were given twice weekly and fresh water was available ad lib.

Dye administration—Trypan blue (National Aniline Co. Lot #16944) in aqueous solution was injected subcutaneously at two week intervals. In a preliminary study on the effect of various dosages, 148 animals were divided into seven groups, each group receiving a different dose from 2.5 to 40 mg./100 Gm. of body weight. An additional 150 animals were utilized for special studies as indicated.

Hematologic procedures—Microhematocrits were done in duplicate on blood obtained by snipping the end of the tail. Removal of equivalent amounts of blood from control animals at the intervals indicated was shown to have no effect on the hematocrit. Reticulocyte values were determined by counting the number present in 1,000 erythrocytes on slides pre-stained with brilliant cresyl blue. Hemoglobin determinations were done by the oxyhemoglobin method in the Coleman Jr. Spectrophotometer. Platelet counts were done in duplicate using Rees-Ecker diluting fluid. Peripheral blood smears stained with 0.2 per cent methyl violet in 95 per cent alcohol were examined for Heinz bodies. Osmotic fragility determinations were carried out at room temperature according to the method of Waugh and Asherman.

Serum bilirubin concentrations were measured by a micro-modification of the method of Malloy and Evelyn. Plasma hemoglobin levels were done using a modification of the method of Bing and Baker. Fecal urobilinogens were measured according to the method of Watson. The determinations of red cell survival time using radioactive Cr were carried out as previously described.

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Plasma dye concentration—The amount of dye in the plasma was determined by direct colorimetry with correction for hemolysis. The trypan blue content was expressed as mg. per cent. A set of color standards based on these determinations was prepared in capillary tubes so that the plasma dye level of the experimental animals could be estimated directly from the microhematocrit.

Agglutination of red cells—Direct macroscopic and microscopic examination of a drop of whole blood for agglutination was performed according to the method of Wasastjerna. Serum samples (0.5 cc.) from anemic animals were incubated with normal cells (0.1 cc. of a 2 per cent suspension) for 30 minutes and 2 hours and then examined microscopically for agglutination.11

Coombs' test—The antiglobulin serum was prepared by injecting pooled normal rat serum into rabbits at weekly intervals. The first injection, consisting of 2 cc. of serum, was given in the marginal ear vein and the following three injections in Freund’s adjuvant were given in the inguinal and axillary regions. After the fourth injection, when the precipitin titer was 1:10,240, the rabbits were bled and the anti-rat rabbit sera absorbed with pooled normal rat erythrocytes. One cc. aliquots were stored at —20 C. A second antiglobulin serum was prepared by injecting rabbits with serum from trypan blue treated rats in an effort to provoke a wider range of antibodies.12 Antiglobulin tests carried out with these two sera gave identical degrees of agglutination with positive cells.

Erythrocytes to be tested for protein coating were collected in buffered isotonic saline (M/100 PO₄, pH 7.3). Cells were washed three times in an excess of buffered saline at room temperature and resuspended to give a 2 per cent suspension. One drop of the cell suspension was added to one drop of a 1:4 dilution of antiglobulin serum in saline in a 75 x 8 mm. test tube, and the mixture centrifuged at 1500 rpm for one minute. The tube was then shaken gently to resuspend the cells and inspected for agglutination grossly and microscopically. Positive tests were stable for more than 30 minutes.

The indirect Coombs' test was performed by incubating 0.1 cc of a 5 per cent suspension of washed normal rat erythrocytes in amounts of serum from anemic animals varying from 1 to 2 cc. After incubation at 37 C. for 30 minutes, the cells were washed and tested in the same manner as in the direct procedure.

Cells giving a positive antiglobulin reaction were also examined for agglutination after incubation in 22 per cent human albumin. Trypsinized red cells were prepared according to Mollison.13

Eluates from Coombs' positive erythrocytes were prepared by two methods: an adaptation of the Landsteiner-Miller procedure as outlined by Muirhead et al.,2 and the precipitation of antibody protein with ethanol according to Weiner.14

Splenectomy—The animals were splenectomized under general ether anesthesia after basal preparation with intraperitoneal Nembutal. In the animals subjected to sham splenectomy, the abdomen was closed after inspection and manipulation of the spleen.

Histologic procedures—Autopsies were performed on the sacrificed animals and representative materials were fixed in 10 per cent neutral formalin. Bone marrow preparations and splenic imprints were prepared with Wright's and Giemsa stains. Microsections were routinely stained with hematoxylin and eosin. Gomori's iron stains were used when indicated. Methyl-green-pyronine stains were done in selected cases on Carnoy fixed materials from the spleen and lymph nodes.

Results

Relationship of Anemia to Dose of Trypan Blue and Timing of Injection

Preliminary observations in a series of 148 animals indicated that development of anemia was dependent on the amount of dye injected. Injection of a single dose of 20, 30, or 40 mg. of trypan blue/100 Gm. of rat produced a

*Cutter Laboratories, Berkeley, Calif.
severe anemia; however, doses of 2.5, 5.0 or 10 mg./100 Gm. had no effect on the hematocrit level even when given for eight bi-weekly injections. The 20 mg./100 Gm. dose was found to be the most effective and was adopted for the purposes of this experiment. A decrease in hematocrit was first noted on the 4th day following the initial dye injection, with a maximum depression by the 8th to 10th day and spontaneous recovery by the end of the 2nd week (fig. 1). When a second injection was then given, the anemic response was more rapid but less severe and with continued bi-weekly injections the hematocrit stabilized at about the 40 per cent level with only minor fluctuations. Cessation of dye treatment after a series of 8 injections resulted in a return of the hematocrit to a normal level.

Following the first two dye injections, the reticulocyte counts, even when corrected for the degree of anemia, were found to be elevated to 9 times normal (fig. 1). Subsequent doses resulted in a sustained reticulocytosis ranging from 2 to 7 times normal.

![Graphs](image)

**Fig. 1.—** Mean hematocrits, reticulocyte values and plasma dye concentrations in 5 rats receiving 8 injections of 20 mg./100 Gm. trypan blue. The individual Coombs' tests are represented by dots. Normal values and standard deviations: Hematocrit 51.0 ± 2.6, reticulocytes 2.0 ± 2.05. Roman numerals indicate the serial dye injections given every 2 weeks.
A thrombocytopenia developed following a single dye injection. The lowest platelet counts with a mean of 150,000 were recorded on the 8th and 10th days with a return to normal by about the 20th day.

A transient leukocytosis followed by a leukopenia was observed. The lowest white counts with a mean of about 6,000 compared with a normal mean of 14,000 were found on the 8th day following dye administration.

Heinz bodies were searched for in methyl violet-stained preparations of peripheral blood following one injection of 20 mg. of dye. A few erythrocytes contained intracellular bodies, but the percentage of altered cells was low, and they were found only in the first few days when the direct Coombs' test was still negative.

**Relationship of Hematocrit Values to Plasma Dye Concentration**

The amount of trypan blue in the plasma reached maximum levels in the first hour after the initial injection, with an abrupt fall in concentration within 24 hours (fig. 1). The disappearance of the anemia and positive Coombs' test after one dye dose was accompanied by a pronounced decrease in serum dye concentration. With successive dye doses the residual level increased so that the plasma clearance was not as rapid following the last injection as after the first.

**Erythrocyte Survival Studies**

The half-life (T/2) of chromium tagged autogenous erythrocytes in two animals made anemic by a 20 mg. dye dose was found to average 2.8 ± .49 days (fig. 2.). In 14 untreated normal control animals, the mean half-life was 17.1 ± 2.33 days.

A series of transfusions, consisting in each case of 1 cc. of Cr²⁺ tagged whole blood, was undertaken to study the factors responsible for the observed shortening of red cell survival. These observations are summarized in figure 3. The mean cell life of erythrocytes in normal animals was determined by transfusing 14 rats with chromium tagged cells, 8 with their own cells and 6 with cells from other normal animals. In acutely anemic animals the mean cell life of autogenous erythrocytes was found to be 5.5 days as compared to a mean of 44 days in normal rats. The survival time of normal red blood cells as well as cells from long-term dye treated animals was also shortened in the anemic recipients with means of 7.8 and 12.9 days respectively. When red cells were transfused from acutely anemic to normal animals, the moderately shortened mean cell life of 32 days suggested that the cells may have been altered by the dye treatment. There was only a moderate shortening of erythrocyte life span in the long term dye treated animals.

**Determinations of Osmotic Fragility**

Erythrocytes from four animals were tested on the 4th, 9th, 17th, and 28th days following a single 20 mg./100 Gm. dye injection. Increased osmotic fragility was found only on the ninth day when the anemia was most severe and these values together with the normal range are shown in figure 4.
Fig. 2.—Red cell half-life (T/2) in 2 trypan blue treated anemic rats compared with 14 normal control animals.

Agglutination and Hemolysis of Red Blood Cells

The conjunctival blood vessels were examined using a dissecting microscope (90X) and reflected light at intervals of ½, 1, 3, and 6 hours, and daily for 8 days following the injection of one dose of 20 mg. of dye. Intravascular erythrocyte agglutination was never observed. Daily microscopic examination of a drop of blood showed aggregation of red cells for the first time on the 7th day, and this was associated with an increase in the sedimentation rate. Erythrocytes from dye-treated animals were not agglutinated after incubation with autologous or normal serum. However, serum from some dye treated animals was found to agglutinate normal rat red blood cells, but the results were variable and there was no evidence of hemolysis.
Fig. 3.—Individual and mean red cell survival times are shown for 3 recipient groups consisting of normal, acutely anemic (1 dose 20 mg./100 Gm. of dye) and long-term dye treated animals (7–8 biweekly dye doses). The dye treated recipients were all transfused 3 days after the last dye injection. The source of the transfused cells is indicated.

The serum bilirubin remained at normal levels and determinations of plasma hemoglobin at various intervals after the dye injection were negative. Determinations of fecal urobilinogen showed elevated values in some animals on the 8th, 9th and 10th days following dye treatment.

Effect of Splenectomy on Development of Anemia

Splenectomy was carried out on 6 animals. Blood counts, done 1 month after the surgical procedure, revealed no significant change in any of the formed elements of the peripheral blood. A single subcutaneous dose of 20 mg. of trypan blue/100 Gm. of body weight was then given to 3 splenectomized, 3 sham splenectomized, and 3 normal animals. Erythrocyte changes were evaluated on the 8th day following dye treatment and compared with similar determinations on 8 normal and 6 splenectomized non-dye treated animals (table 1). Removal of the spleen did not have an ameliorating effect since anemia of equal severity developed in all dye treated animals. A reduction in
mean cell volume was accompanied by microcytosis and anisocytosis. The splenectomized animals responded to the dye injection with a thrombocytopenia and leukopenia similar to that observed in intact animals.

**Direct Coombs' Tests**

The results of tests done at 2 to 6 day intervals in a representative group of 5 rats given a series of 8 injections are shown in figure 1. Weakly positive transient tests, first noted on the 3rd or 4th day after dye injection, could be made strongly positive by incubation of the erythrocytes in an excess of either autologous or normal rat serum for 30 minutes at 37 °C. Control determinations using normal cells were negative. The first clearly positive Coombs' tests were obtained on the 5th day, and the strength of the reaction was greatest between the 9th and 11th days. The tests became negative in 15 to 21 days, but the second dye injection induced a more prompt response. When the dye administration was stopped after the 8th injection, the Coombs' test remained positive for as long as 40 days. Coombs' positive erythrocytes were not agglutinated in 22 per cent albumen or by absorbed normal rabbit serum. It was not possible to elute the responsible agent from positive erythrocytes.
Table 1.—Hematologic Findings in Splenectomized and Normal Animals Before and Eight Days After Trypan Blue Treatment

<table>
<thead>
<tr>
<th>Animals</th>
<th>Hct. (%)</th>
<th>Hgb. (Gm.%)</th>
<th>R.B.C.</th>
<th>M.C.V. (μ3)</th>
<th>M.C.H. (γγ)</th>
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<tr>
<td>8 Normal</td>
<td>50 ± 1.1*</td>
<td>15.5 ± 0.78</td>
<td>8,142,500 ± 735,000</td>
<td>60.9</td>
<td>19.1</td>
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<tr>
<td>6 Splenectomized</td>
<td>51 ± 1.7</td>
<td>15.7 ± 0.63</td>
<td>7,971,666 ± 1,216,800</td>
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<tr>
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<td>19</td>
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* ± Refers to standard deviation.

and to transfer it to normal cells. The smaller dye doses (2.5, 5.0, 10.0 mg./100 Gm.) did not produce either anemia or positive Coombs' tests even after a prolonged series of injections. The 15 mg./100 Gm. dose was effective but the results were not as consistent as with the 20 mg./100 Gm. dose. Dye treated splenectomized animals developed positive Coombs' tests and showed the same pattern of response as intact animals.

Serial Dilution and Neutralization of Coombs' Serum

With serial dilutions of the Coombs' serum, the highest titer (1:128) was found on the 10th day following a single dye injection (table 2). Neutralization was carried out by adding one drop of a 2 per cent suspension of washed Coombs' positive erythrocytes to a mixture of equal amounts of the standard 1:4 Coombs' serum in saline and serial dilutions of pooled normal rat serum. The lower dilutions of normal serum were found to completely inhibit the reaction.

Indirect Coombs' Test

Positive indirect Coombs' tests were obtained on the 9th, 10th, and 11th days at the time the direct tests were positive (table 2). Tests done before this time were either negative or weakly positive even when trypsinized erythrocytes were employed. The indirect Coombs' test was not abolished by inactivation of the serum at 56 C. for 30 minutes. Control determinations using absorbed normal rabbit serum were negative.
Table 2.—Serologic Results

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Neutralization of Coombs’ serum

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<td>1+</td>
<td>2+</td>
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In Vitro Studies of the Effect of Dye on Red Cells, Plasma and Whole Blood

A series of twofold dilutions of trypan blue in buffered saline (pH 7.0) were prepared over a concentration range of 2.5 to 320 mg. per cent. Three drops of a 50 per cent suspension of pooled washed normal rat erythrocytes were added to 1 cc. of each dye saline mixture and incubated for one hour at 37 C. After light centrifugation, conglomerate amorphous masses of dye and cells were seen in the 320 mg. and 160 mg. concentrations and slight agglutination in the 80 mg. per cent solution. There was no evidence of hemolysis, and cells incubated in these mixtures were Coombs’ negative. However, when these dye treated cells were washed and then incubated for 30 minutes with an equal amount of pooled normal rat plasma, a positive Coombs’ test was obtained. Erythrocytes which had been incubated in the 40 and 80 mg. per cent dye solution gave a stable 3+ Coombs’ reaction, those incubated in the 10 and 20 mg. per cent a 1+, and those in the 2.5 mg. per cent solution were negative. Attempts to elute the agent responsible for the positive Coombs’ test and transfer it to normal cells were unsuccessful. Dye treated cells incubated with saline instead of plasma yielded negative Coombs’ tests, as did omission of trypan blue from the system.

Dye was added to 1 cc. aliquots of pooled normal rat plasma (final concentration 5-320 mg. per cent), and after incubation for one hour at 37 C., 0.25 cc. of a 50 per cent suspension of normal erythrocytes were added, and the mixtures incubated for an additional 60 minutes. Amorphous masses were again noted in the higher concentrations but there was no agglutination. Cells incubated in each of these dye plasma mixtures gave negative Coombs’ tests.
Incubation of whole blood with trypan blue in the same concentrations for periods of 1, 3, 24 and 48 hours did not cause erythrocyte agglutination, nor did these cells give a positive Coombs’ test. Determination of the plasma hemoglobin content of the serum following incubation showed no significant degree of hemolysis.

Methyl violet preparations of erythrocytes from each of these in vitro mixtures showed variable numbers of intracellular basophilic bodies resembling Heinz bodies. Evaluation, however, was difficult because of the presence of crenated cells.

**Morphologic Changes in Intact and Splenectomized Animals**

Morphologic studies were carried out on animals given a single 20 mg./100 Gm. dose of trypan blue (table 3).

**Bone marrow**—Hypercellularity due mainly to an increase in myeloid cells was noted in sections and imprints of the vertebrae and sternum from the 4th day. Increased erythropoiesis did not appear until about the 8th day. Dye storage was evident by the 4th day and increased during the rest of the observation period. Hemosiderin deposits were not increased in either intact or splenectomized animals.

**Spleen**—Splenomegaly was a regular finding in the dye treated animals with a sevenfold increase in mean weight by the 16th day (table 3). Histologically there was reticular hyperplasia, increased hematopoiesis and erythropagocytosis (fig. 5). In the hematoxylin and eosin preparations, plasma cells were thought to be increased but methyl-green-pyronin stains were not convincing. There was an increase in the amount of stainable iron in the enlarged spleens of animals sacrificed after the dye injection.

**Lymph nodes**—Generalized lymphadenopathy was a constant finding in the dye-treated animals with a sevenfold increase in mean weight by the 16th day (table 3). Initially the follicular pattern was exaggerated, but after the 3rd day there was a loss of normal architectural pattern with cellular hyperplasia and mitotic figures (fig. 6). Plasma cells appeared by the 7th day and there was a strong pyroninophilic reaction with the methyl-green-pyronin stain. Erythropagocytosis and agglutination of red blood cells to dye phagocytes was evident as early as the 2nd day. Only a small amount of hemosiderin was found in the lymph nodes of intact animals. Splenectomy prior to the dye treatment resulted in an increased deposition of hemosiderin.

**Liver**—Hepatomegaly was not found in either intact or splenectomized animals (table 3). There was degeneration of parenchymal cells in the early specimens with centrilobular necrosis by the second day. Signs of regeneration then appeared and mitotic figures were most frequent in the liver cells of the animals sacrificed between the 9th and 16th days. Mononuclear cells accumulated in the periportal areas during the same period, and the dye was taken up by the Kupffer cells and by the liver cells. Hemosiderin was inconspicuous in the Kupffer cells of both intact and splenectomized dye treated animals.

**Kidney**—Of the non-reticuloendothelial organs, the kidney showed the greatest histologic effect of trypan blue treatment. As early as 6 hours after the dye
Table 3.—Summary of Morphologic Changes in Relation to Time After Dye Injection

<table>
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<tr>
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<th>Time after dye injection</th>
<th>30–60 min.</th>
<th>3–6 hr.</th>
<th>1–2 days</th>
<th>3–4 days</th>
<th>6–7 days</th>
<th>8–10 days</th>
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<td><strong>Bone marrow:</strong></td>
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<td>Overall cellularity</td>
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<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
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<td>Myeloid hyperplasia</td>
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<td>+</td>
<td>+</td>
<td>++</td>
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<td>-</td>
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<td><strong>Spleen:</strong></td>
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<td></td>
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<tr>
<td>Mean weight in Gm./100 Gm. rat</td>
<td>.21 ± .036*</td>
<td>.26 ± .059</td>
<td>.35 ± .055</td>
<td>.43 ± .098</td>
<td>.66 ± .273</td>
<td>1.11 ± .621</td>
<td>1.45 ± .602</td>
<td></td>
</tr>
<tr>
<td>Reticular hyperplasia</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Erythrophagocytosis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemosiderin deposits</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Lymph nodes:</strong></td>
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<td></td>
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<tr>
<td>Reticular hyperplasia</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
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<tr>
<td>Erythrophagocytosis</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Hemosiderin deposits</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Liver:</strong></td>
<td></td>
<td>4.48 ± 1.089</td>
<td>4.85 ± 1.217</td>
<td>4.60 ± .877</td>
<td></td>
<td></td>
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<tr>
<td>Intact animals</td>
<td></td>
<td>4.55 ± .032</td>
<td>5.0 ± .622</td>
<td>5.13 ± .615</td>
<td></td>
<td></td>
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<tr>
<td>Periportal cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>Dye staining</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemosiderin deposits</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Kidney:</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Tubular cell necrosis</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
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</tr>
<tr>
<td>Dye staining</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
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<tr>
<td>Hemosiderin deposits</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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*Standard deviation.
injection there was degeneration of the epithelium of the proximal convoluted tubules and these changes were progressive with frank cellular necrosis evident from the second through the 16th day (table 3). There was concomitant regenerative activity with frequent mitoses. Dye appeared in the epithelial cell cytoplasm 6 hours after injection with the maximum concentration on the 4th–7th days and some dye was still present at the 16th day. There were no
glomerular changes, vascular damage, or deposits of stainable iron. Urinalyses disclosed albumen, coarsely granular casts and occasional red blood cells as early as the 2nd day but these findings were most evident from the 3rd to the 7th day. Throughout this period the urine contained large amounts of amorphous blue material. The blood urea nitrogen level was not found to be significantly elevated following a single dye injection.
DISCUSSION

Several mechanisms were considered in an attempt to explain the action of trypan blue in producing anemia. The appearance of positive Coombs’ tests associated with anemia led to consideration of an immunologic mechanism. Two possibilities were envisaged; the dye may have altered the antigenic character of the red blood cells provoking the formation of auto-antibodies, or the dye, through its effect on cells of the reticuloendothelial system, may have stimulated the production of abnormal serum proteins which reacted immunologically with red blood cells. The 4 to 5 day delay in the appearance of the Coombs’ test after the first dye injection was consistent with the time required for antibody production in the rat. The accelerated response following the second dye injection was also compatible with an immunologic phenomenon. There were a number of observations, however, that made an immune mechanism unlikely. The uniform manner in which every animal reacted to the dye stimulus was not a typical immunologic response pattern. The active agent could not be eluted and transferred to normal cells. Also, it has been shown that trypan blue may inhibit antibody production, and this action has been observed with the dosage employed in this experiment. Finally, the in vitro production of a positive Coombs’ test with trypan blue made the immunologic explanation unnecessary.

It is recognized that positive Coombs’ tests may be produced by nonimmunologic pathways. Diverse chemical agents and drugs such as lead, tannic acid, metallic cations, bis-diazotized benzidine and phenylhydrazine have been found capable of inducing protein coating of erythrocytes. These substances are thought to act either by damaging the red blood cell membrane with exposure of protein groups, by causing adsorption of plasma proteins onto the altered cell surface, or by serving as prosthetic groups to bind nonimmune proteins to the cells. In our view, one of the latter two mechanisms best explains the action of trypan blue, since a positive Coombs’ test was obtained in vitro only when dye treated cells were subsequently exposed to serum protein. The failure to obtain positive tests when the dye was exposed to protein before the addition of erythrocytes was attributed to the preferential saturation of the protein binding sites by the dye. The sensitization of erythrocytes by tannic acid is similarly inhibited by protein.

Trypan blue, and its isomer Evans blue, bind firmly with plasma proteins. Although the dye in low concentrations is completely bound to albumin, with higher plasma dye levels the dye-binding capacity of the albumin may be exceeded, leading to binding with other protein fractions and possibly with red blood cells. In his work with Evans blue, La Veen concluded that the dye reacted with elements of the plasma proteins forming dissociable compounds which behaved according to the law of mass action. If it may be assumed that trypan blue behaves in a similar fashion, some free dye would always be present as a result of this dissociation constant. The anemia and positive Coombs’ tests produced by the larger doses of dye may be explicable on this basis and the failure of the smaller dye doses could be attributed to the low plasma-dye levels attained.
Several explanations could be offered to account for the 4 to 5 day interval between the initial dye injection and the appearance of positive Coombs' tests. The in vitro observations would seem to exclude the possibility that an in vivo alteration of the dye is necessary for its activation. The high serum dye levels recorded in the first few hours after injection make it unlikely that the delay was due to a paucity of dye altered erythrocytes. Since weakly positive tests, obtained on the 3rd day could be potentiated by incubation of the red cells in autologous or normal serum, the delayed appearance of strongly positive tests was regarded as more likely due to a deficiency of available protein in the plasma. The gradual recovery from anemia in the animals given repeated dye injections, together with their nearly normal red cell survival times, suggested the development of a resistant erythrocyte population. However, in cross transfusion studies, cells from these animals were rapidly destroyed in animals made acutely anemic by a single dye injection. These findings suggested that the decreasing effectiveness of the dye treatment was due at least in part to a reduced capacity of the long-term dye treated animal to destroy red blood cells. It is probable that the interaction of a number of factors may have been responsible including alterations in the dye-serum protein relationships, overloading or blockade of the reticuloendothelial system, rate of absorption of dye from the subcutaneous injection sites, as well as the compensating effect of the sustained reticulocytosis. The return of the hematocrit levels to normal following cessation of the trypan blue injections established the dye dependent nature of the anemia.

One of the most interesting observations was the finding of positive indirect Coombs' tests and there has been considerable speculation about the nature of the responsible agent. It is conceivable that it could be an antibody formed against trypan blue altered erythrocytes or proteins attached to these cells, and cross reacting with normal erythrocytes. A more likely explanation is that it consists of a dye-protein complex acting in a nonimmunologic fashion. Since this phenomenon was demonstrable in vivo only when the direct Coombs' test was most positive, the concentration of the agent in the serum was most likely dependent on the saturation of the animals' red cells.

In considering the leukopenia and thrombocytopenia that developed concomitantly with the anemia it was thought that the macromolecular character of the circulating dye-protein complex may itself have been injurious to these cells. Injection of a variety of macromolecular substances such as methylcellulose have been found to produce leukopenia and thrombocytopenia as well as anemia. Jandl has attributed the leukopenia accompanying acute immune hemolysis to the action on leukocytes of antigen-antibody complexes released from the hemolyzing red cell. It is tempting to speculate that the dye protein complexes in this nonimmunologic anemia act in a similar fashion.

Commercial trypan blue, such as that employed in this study, is composed of a major blue fraction and at least three red components as well as a number of impurities. A purified blue fraction prepared by butanol extraction of a different dye lot also produced anemia when administered at the same dosage level. The red components were ineffective. It is possible, although unlikely
because of the small amounts present, that remnants of the tetrazo tolidine and H acid used in the manufacture of the dye or other by-products may be the active agent.

The pathogenesis of the anemia induced by trypan blue differs from that produced by methyl cellulose or zymosan in several important ways. Protein coating of red cells has not been demonstrated with either of these substances and the erythrocyte destruction has been primarily attributed to hyperactivity of the reticuloendothelial system. In the anemia caused by methyl cellulose in rats the enlarged spleen is an essential feature, while in the zymosan induced anemia of mice splenectomy is followed by a compensatory reticuloendothelial hyperplasia with hepatomegaly. Although splenomegaly was a prominent finding in trypan blue induced anemia, this organ was not an essential site of red blood cell destruction since splenectomized animals became equally anemic and there was no compensatory hepatomegaly.

The observations made in this study suggest that trypan blue alters the erythrocytes in such a way as to render them susceptible to sequestration and destruction in the spleen, lymph nodes and liver. It thus appears that red blood cells coated with protein by this nonimmunologic means may be destroyed in the reticuloendothelial system in a manner similar to cells coated with incomplete antibody such as anti-D.

SUMMARY

An anemia with a positive direct and indirect Coombs' test was induced in Wistar strain rats by injection of the dye trypan blue. The anemia was characterized by a shortened red cell survival time, increased osmotic fragility and reticulocytosis. There was an associated leukopenia and thrombocytopenia. The positive Coombs' tests were regarded as a nonimmunologic phenomena and attributed to the protein binding properties of the dye. Splenomegaly and lymphadenopathy were constant findings in the anemic animals. Splenectomy did not alter the course of the anemia. The pathway of erythrocyte destruction was thought to involve the sequestration and destruction of protein coated cells in the spleen, lymph nodes and liver.

SUMMARIO IN INTERLINGUA

Un anemia con positive tests directe e indirecte de Coombs esseva inducite in rattos del racia Wistar per injectiones de blau trypan. Le anemia esseva characterisate per un reduce longevitate erythrocytic, un augmento del fragilitate osmotic del erythrocytos, e le disveloppamento de reticulocytosis. Esseva notate le phenomenos associate de leucopenia e thrombocytopenia. Le positive tests de Coombs esseva reguardate como phenomenos non-immunologic e attribuite al proprietates proteino-ligante del colorante. Splenomegalia e lymphadenopathia esseva uniformemente presente in le animales anemic. Splenectomy non alterava le curso del anemia. Esseva opinate que le processo per que le erythrocytos esseva destruite implica le sequestration (e disintegration) de cellulas revestite de proteina in splen, nodos lymphatic, e hepate.
TRYPAN BLUE-INDUCED ANEMIA WITH COOMBS’ TEST

ACKNOWLEDGMENTS

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cells altered by immune mechanisms.
and red-cell destruction. Brit. M. J.

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Anemia with Positive Direct Coombs’ Test Induced by Trypan Blue

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