

STUDIES IN EXPERIMENTAL ANEMIA: *II. The Effect on Rabbits of the Injection of Stool Extracts of Patients with Pernicious Anemia and Normal Individuals*

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J Clin Invest. 1927;4(4):529-538. <https://doi.org/10.1172/JCI100138>.

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STUDIES IN EXPERIMENTAL ANEMIA

II. THE EFFECT ON RABBITS OF THE INJECTION OF STOOL EXTRACTS OF PATIENTS WITH PERNICIOUS ANEMIA AND NORMAL INDIVIDUALS

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(Received for publication May 16, 1927)

The hypothesis that a toxic hemolytic substance could be recovered from the stools of pernicious anemia patients was investigated. Saline stool extracts of patients suffering from pernicious anemia and those of normal individuals were administered to rabbits by intravenous and subcutaneous injection over long periods of time. Stool extracts prepared by the special technique of Seyderhelm (1) were also employed. In addition, total mixed cultures grown aerobically and anaerobically from the fresh stool were fed to rabbits by mouth.

METHODS

Saline extracts. Stools were collected for 3 to 5 days, allowed to stand in water over night, filtered through gauze and cotton, and passed through a Berkefeld N candle. The concentration of the final solution represented one part of dried stool to 30 parts of water. It was adjusted to a pH 7.2 and a salt content of 0.85 per cent. The final product was a clear sterile solution. Tests of its hemolytic activity in vitro were variable. In a few instances no hemolytic power was demonstrated by incubating 1 cc. of extract with 1 cc. of a 5 per cent suspension of red blood cells for one hour. In others incomplete hemolysis took place with 1 cc. and with 0.5 cc. of extract, occasionally with 0.25 cc. of extract.

Seyderhelm's technique. The freshest possible feces were well mixed with about 10 times the quantity of distilled water, the suspension brought to a weak acid reaction by the addition of a little sulphuric acid and, after the addition of a 1 to 2 cm. layer of toluol, shaken for several hours. It was then filtered through a folded filter and the filtrate concentrated by evaporation to about one-half its volume in a water-bath at 50 to 60 degrees. Alkalinization as a result of the evaporation of the volatile acids was avoided by the addition of dilute sulphuric acid.

The concentrated fluid was diluted with an equal quantity of 96 per cent alcohol, shaken for several hours and filtered through a folded filter in such a way that the filtrate flowed down the walls of the vessel into an equal amount of 96 per cent alcohol. Either immediately or after a few hours a flaky precipitate was formed. In order to accelerate the formation of the latter, half the volume of ether was added. After standing for 24 hours, the precipitate was washed with 80 per cent alcohol. The filtrate contained the greater part of the substances having a hemolytic action in vitro, fatty acids, soaps, etc. The precipitate supposedly contained the anemia-producing toxic substance, which did not have a hemolytic action in vitro. The precipitate was now shaken up in 200 cc. of physiological salt solution, filtered through cotton or gauze. The final result was a neutral-reacting solution which was preserved in the ice-box with toluol.

The extract made by Seyderhelm's technique was not hemolytic in vitro. The saline extracts which frequently showed traces of hemolysis in vitro were incubated with Welch bacillus antitoxin and normal rabbit and normal human serum. The degree of hemolysis produced by the untreated extracts was usually diminished and at times disappeared under these circumstances, but the Welch bacillus antitoxin exerted no more effect than the normal sera.

The blood counts and the pathological sections were made as noted in the previous paper.

RESULTS

Twenty experiments in all were performed, 8 with saline extracts of pernicious anemia stools; 8 with similar extracts of normal stools; 2 with extracts of pernicious anemia stools according to Seyderhelm's technique and 2 with mass aerobic and anaerobic stool cultures.

Results of the administration of Seyderhelm's extracts. Two rabbit experiments were performed. Four extracts were prepared as closely as possible to the directions of Seyderhelm, although quantitative data were not always given, such as the amount of dried weight of stool in the final solution. Since intravenous injection of large amounts such as 30 cc. caused death of the animal, a smaller dose was given. One experiment will be reported as the other showed the same results.

Experiment 1. A rabbit was injected intravenously three times a week with 10 cc. of extract for a period of six weeks. The blood count was taken every week for this period and for three months thereafter. No sign of anemia appeared. At the end of the period of administration the red blood cells changed from 5.97 million to 7.10, the hemoglobin from 72 to 90 per cent, the color index from 0.60 to 0.63. The blood smear was normal throughout.

In two such experiments we were unable to induce an anemic condition in rabbits who received an intravenous injection of 10 cc. of Seyderhelm's extract three times a week for six weeks.

Results of the administration of saline extracts from pernicious anemia stools

Eight rabbit experiments were performed with saline extracts of stools from pernicious anemia patients. In 2 rabbits anemia was produced by the intravenous injection of extract. In 3 rabbits anemia

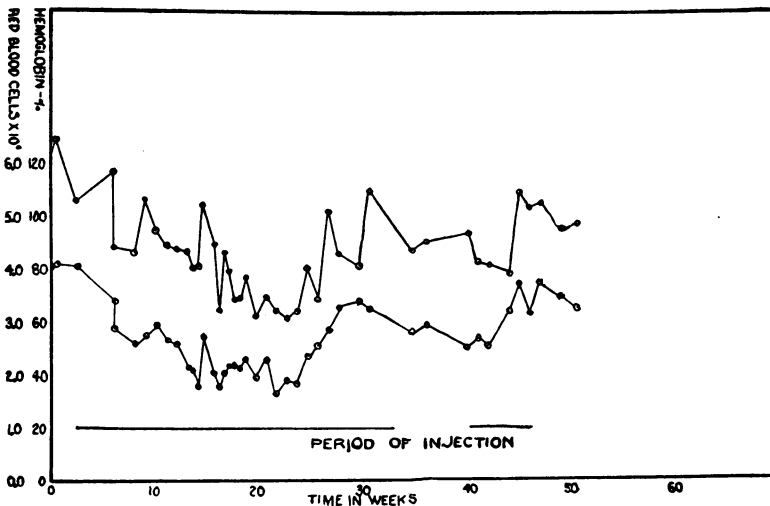


CHART 1. ANEMIA FOLLOWING SUBCUTANEOUS INJECTION OF A PERNICIOUS ANEMIA STOOL EXTRACT

Upper graph—red blood cells; lower graph—hemoglobin

was produced by long-continued subcutaneous injection. In 3 rabbits the subcutaneous injection of these extracts over a similar period of time did not produce a definite anemia.

Experiment 2 (chart 1). A saline extract was made from the stools of three pernicious anemia patients. It was slightly hemolytic in vitro, 0.5 cc. of extract producing hemolysis of a 5 per cent suspension of red blood cells. The rabbit received 4 cc. subcutaneously 6 days out of 7 for eight months. A severe anemia gradually developed, the

red blood cells eleven weeks after the first injection dropping from 5.30 million to 3.20, the hemoglobin from 86 to 36 per cent and the color index from 0.76 to 0.56. The blood smear at the height of the anemia showed marked anisocytosis, moderate polychromatophilia, slight poikilocytosis and numerous macrocytes. Nucleated red corpuscles were absent. Notwithstanding the continuance of injection, the count gradually improved, and six months after onset of experiment had reached the following, red blood cells 5.47 millions, hemoglobin 65 per cent, color index 0.60. After a free interval of seven weeks

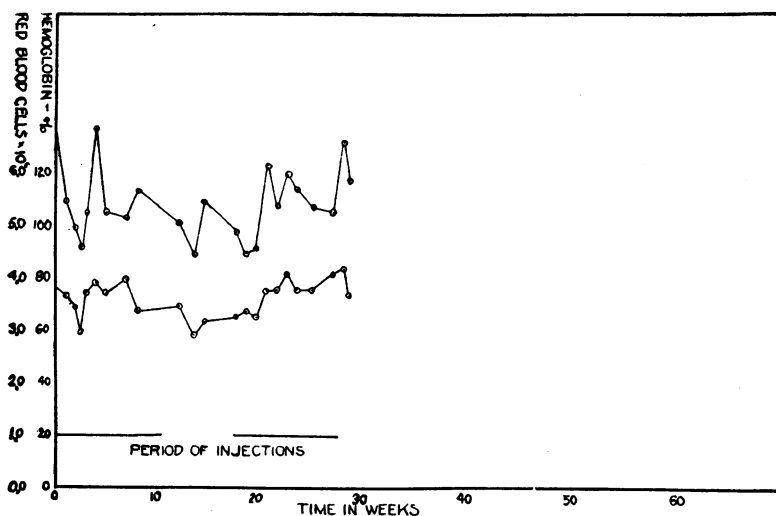


CHART 2. RED BLOOD CORPUSCLES AND HEMOGLOBIN FOLLOWING SUBCUTANEOUS INJECTION OF A PERNICIOUS ANEMIA STOOL EXTRACT

Upper graph—red blood cells; lower graph—hemoglobin

10 cc. of extract were administered subcutaneously 6 out of 7 days for five weeks. The blood count was lowered slightly and then returned to its normal value. After this course was completed, 20 cc. were administered intravenously, which was followed by the death of the animal.

In this experiment a severe chronic anemia was gradually produced by the long-continued subcutaneous administration of pernicious anemia stool extract. In a period of one year two remissions occurred

(see chart 1). The character of the anemia resembled that due to the Welch bacillus toxin, characterized by a low color index and a blood smear of secondary anemia type. In this experiment too, the blood count returned to normal during the period of injection, suggesting some immune response on the part of the organism.

Experiment 3 (chart 2). Four cubic centimeters of a pernicious anemia stool extract were injected subcutaneously 6 out of 7 days for ten and a half weeks. After a free interval of seven weeks, 5 cc. were

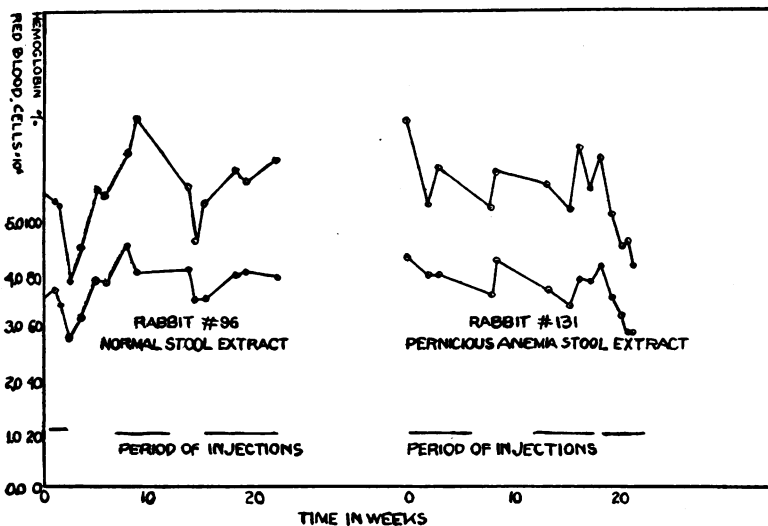


CHART 3. RED BLOOD CORPUSCLES AND HEMOGLOBIN FOLLOWING SUBCUTANEOUS AND INTRAVENOUS INJECTIONS OF STOOL EXTRACTS

Upper graphs—red blood cells; lower graphs—hemoglobin

similarly injected for ten weeks. One week later 10 cc. of extract were injected intravenously three times in one week, after the last of which the animal died. Variations in red count and in hemoglobin occurred but no definite anemia developed (see chart 2). (This extract caused slight hemolysis when 1 cc. of extract was mixed with 1 cc. of a 5 per cent suspension of red blood cells.)

In this instance the subcutaneous and later the intravenous injection of pernicious anemia stool extract resulted in no definite anemia.

Experiment 4 (rabbit no. 131) (chart 3). Five cubic centimeters

of pernicious anemia stool extract were administered subcutaneously to a rabbit 6 out of 7 days for six weeks. After a free interval of six weeks the same procedure was repeated for five and a half weeks. After a second free interval of one week, 10 cc. of extract were intravenously injected twice a week for four weeks. The day after the last injection the animal died. Sixteen weeks after the onset of the experiment the blood count had dropped slightly, red blood cells from 6.94 millions to 5.22, hemoglobin from 87 to 68 per cent, color index changed from 0.63 to 0.65. Three weeks later the count went up again, red blood cells to 6.20 millions, hemoglobin 83 per cent, color index 0.65. After three weeks of intravenous injection the blood count dropped, red blood cells 4.13 millions, hemoglobin 58 per cent, color index 0.70.

In this experiment a moderate reduction in red blood cells and hemoglobin occurred after subcutaneous and intravenous injection of pernicious anemia extract. The color index was variable. The blood smear showed anisocytosis. Death occurred in this animal as in the previous one without the development of severe anemia.

Results of administration of saline extracts of stools of normal individuals

Eight experiments were performed with saline extracts from the stools of normal individuals. In 5 instances anemia of varying degree was produced, in 3 no anemia. The extracts at times showed traces of hemolytic activity in vitro of the same degree and character as the extracts from pernicious anemia stools.

Experiment 5 (chart 4). Three cubic centimeters of normal stool extract was injected subcutaneously 6 out of 7 days for twelve weeks. After a free interval of eight and a half weeks 10 cc. were injected subcutaneously in the same manner for four weeks. During the three subsequent weeks 10 cc. were intravenously administered three times a week. Two days after the last injection the animal died. Three weeks after the onset of the experiment the red blood cells dropped from 6.72 millions to 4.42, the hemoglobin from 77 to 64 per cent, the color index rose from 0.57 to 0.72. The blood count rose during the period of injection reaching a maximum two months later of red blood cells 5.76 millions and hemoglobin 79 per cent, color index 0.68. As a result of the intravenous injection the red blood cells dropped from 6.62

millions to 3.90, and the hemoglobin from 81 to 50 per cent. The blood smear showed anisocytosis and polychromatophilia. Color index was 0.64.

This experiment with normal stool extract shows features similar to the results of Welch bacillus toxin and pernicious anemia stool extract. A reduction in blood count may be produced by subcutaneous injections and a more severe reduction by administration through the intravenous route. The anemia is temporary, notwithstanding the continuance of the injection.

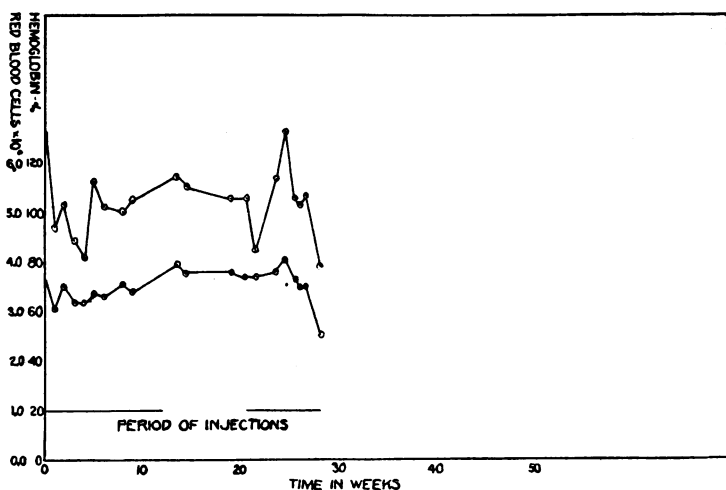


CHART 4. RED BLOOD CORPUSCLES AND HEMOGLOBIN FOLLOWING SUBCUTANEOUS INJECTION OF NORMAL STOOL EXTRACT

Upper graph—red blood cells; lower graph—hemoglobin

Experiment 6 (chart 5). Four cubic centimeters of normal stool extract were administered subcutaneously 6 out of 7 days for eleven weeks. At the end of three weeks a slight reduction of red blood cells and hemoglobin occurred followed by a prompt return to the normal count (see chart). After a free interval of eleven weeks, 10 cc. of extract were administered intravenously three times a week for eleven weeks without development of anemia. Four weeks later two intraperitoneal doses of Welch bacillus toxin, 10 cc. each, were administered in three days. The day after the last injection the animal died.

As a result of the Welch bacillus toxin the blood count promptly dropped, red blood cells from 5.31 to 2.65 millions, the hemoglobin from 87 to 48 per cent. The smear showed moderate anisocytosis and polychromatophilia. Polynuclear leukocytes were much increased.

In this experiment the temporary drop in blood count as a result of injection of normal stool extract, with increased resistance to subsequent injection, is again demonstrated. Furthermore, when Welch bacillus toxin was later injected a severe anemia resulted in three days, indicating that the earlier hemolytic factor in the extract had not been

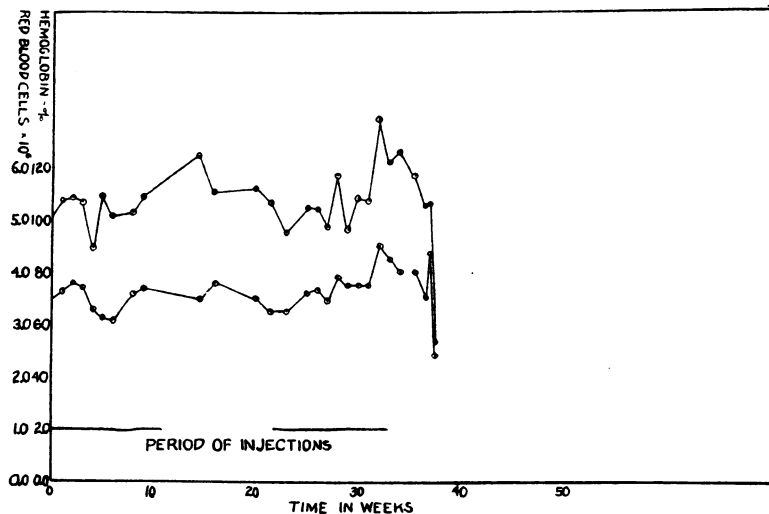


CHART 5. RED BLOOD CORPUSCLES AND HEMOGLOBIN FOLLOWING INJECTIONS OF
(1) NORMAL STOOL EXTRACT, (2) WELCH BACILLUS TOXIN
Upper graph—red blood cells; lower graph—hemoglobin

Welch bacillus toxin. The animal had a strong resistance to the hemolytic agent in the stool extract but none to the toxin. As above referred to the anti-toxin of the Welch bacillus, did not specifically prevent the *in vitro* hemolysis of the normal stool extract.

Experiment 7 (rabbit no. 96) (chart 3). Five cubic centimeters of normal stool extract were injected intravenously into a rabbit. Nine days later his blood count dropped; red blood cells from 5.36 millions to 3.81, hemoglobin from 74 to 56 per cent. Smear showed slight

anisocytosis and polychromatophilia. Eighteen days later the blood count had returned to its previous level, red blood cells 5.60 millions, hemoglobin 78 per cent. Smear was normal. Six weeks after the first injection 5 cc. of extract were administered subcutaneously 6 out of 7 days for five weeks. After a free interval of one month 10 cc. of extract were injected intravenously twice a week for seven weeks. The animal died without the development of anemia.

In this experiment a single injection of normal stool extract intravenously resulted in a temporary anemia from which the animal soon recovered. Attempts to produce anemia subsequently by subcutaneous and intravenous injection were unsuccessful. Thus, following a transient anemia, resistance to the hemolytic effects of the extract appeared.

Experiments 8 and 9. In these experiments mass aerobic cultures from stools of pernicious anemia patients were fed to one rabbit and mass anaerobic cultures to the second. They were fed three times a week on lettuce leaves. Blood counts were taken every week for six weeks. No anemia developed.

PATHOLOGY

The bone marrow in some cases was unaltered, in others moderate hyperplasia of the red cell elements was present. The liver showed occasional small deposits of hemosiderin. The spinal cord revealed no changes similar to those observed in combined sclerosis and pernicious anemia.

SUMMARY AND CONCLUSIONS

The administration of saline extracts from pernicious anemia stools to rabbits both by the subcutaneous and intravenous route sometimes results in an anemia of the secondary type. The blood count is diminished generally with a lowered or stationary color index. At times the color index is temporarily increased. The blood smear when anemia is present is characterized mainly by anisocytosis. Polychromatophilia is present, slight poikilocytosis, and slight macrocytosis but nucleated red blood cells are rare. The pathological study of the bone marrow, liver and spinal cord reveals no signs characteristic of pernicious anemia.

Saline extracts made from the stools of normal individuals induce the same changes as those from pernicious anemia patients.

We were unable to produce anemia in rabbits by the injection of stool extracts made according to Seyderhelm's technique.

The anemia caused in rabbits by extracts of pernicious anemia and normal stools shows a tendency to clear up notwithstanding the continuance of the injections. The anemia may be reinduced at a later period by larger doses of extract. In this respect, therefore, a similarity exists between the anemia produced by Welch bacillus toxin and that produced by stool extracts. That the Welch bacillus or its toxin was not responsible for the anemia produced by the stool extracts is indicated by the fact that a rabbit injected over long periods with a normal stool extract without the development of anemia was made anemic by two injections of Welch bacillus toxin. In both cases the animal develops a resistance which for the time being protects him from anemia, and in fact causes the disappearance of anemia. The nature of the hemolytic substance found in stool extracts is still unknown.

Our experiments show no relation between experimental anemia due to stool extracts and pernicious anemia. It must be emphasized again, however, that our failure to produce pernicious anemia in rabbits may be explained as well by the possible circumstance that these animals are by nature (i.e., constitutionally) unsuited to develop pernicious anemia, as by the failure to use an appropriate hemolysin.

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