THE PROGRESSIVE ANEMIA FOLLOWING A SINGLE INTRA-MARROW INJECTION OF *B. WELCHII* TOXINS *

JOHN C. TORREY AND MORTON C. KAHN

(From the Department of Public Health and Preventive Medicine, Cornell University Medical College, New York, N. Y.)

This study is in continuation of our investigation of the anemia produced in laboratory animals by a potent *B. welchii* toxin. In a previous communication attention has been directed to the consistently and abnormally high *B. welchii* counts in stools from pernicious anemia cases.¹ These findings have received confirmation in recent papers by Davidson,^{2, 3} although Nye⁴ believes that the difference from the normal as regards the number of *B. welchii* in the colon is more apparent than real. It was also shown⁵ that a transitory anemia with a blood picture strongly suggestive of the pernicious type in a number of features could be produced in monkeys by intravenous inoculations of such a toxin. Similar observations for rabbits have also been made subsequently by Patterson and Kast,^{6, 7} and Barach and Draper,^{8, 9} although these investigators consider the resulting anemia of a secondary rather than primary type. The anemia produced by actual infection of rabbits with whole cultures of *B. welchii* was earlier studied by Cornell^{10, 11} and by Reed, Orr and Burleigh,¹² and its pernicious-like features noted. It has been our experience, however, that the morphological and tinctorial blood changes in rabbits following intravenous inoculation of toxin, while somewhat suggestive of pernicious anemia, are not so striking as in monkeys. We have also reported¹³ evidence of the absorption of this toxin from the gastro-intestinal tract of monkeys, the mucous membrane of which had been treated with a weak solution of sodium fluoride to induce a catarrhal inflammation. A blood picture in every way similar to that produced by intravenous inoculation resulted, together with a severe toxic degeneration of the marrow tissue, affecting particularly the fat cells and erythrocytes. The

* Read at the joint meeting of the American Association of Pathologists and Bacteriologists, and the American Association of Immunologists, Washington, D. C., May 1, 1928.

This study has been aided by a fund contributed by Mrs. W. K. Vanderbilt.

Received for publication November 26, 1928.

fluoride treatment alone caused no such marrow injury and only a moderate degree of anemia. The marrows of the two toxin-treated animals, however, were examined following their death from the anemia and there were thus no observations on the intermediate stages of the degenerative process. To gain information in regard to the effect of this sterile toxin on bone marrow, a large number of experiments have since been carried out in which a single small dose has been introduced directly into the tibial marrow of a series of rabbits and of a monkey. Somewhat to our surprise, we found that such a single treatment had a progressively injurious effect causing a chronic anemia with very marked loss of weight and frequently a fatal issue. Aside from any consideration of the possibility of absorption of *B. welchii* toxin being the essential etiological factor in the production of human pernicious anemia, the results aroused our interest because of the finding that the single inoculation into the marrow of one bone caused degenerative changes in quite equal, and at times in greater, degree in marrows of bones far removed therefrom. In view of the fact that the intravenous inoculation of the same dosage or a series of increasing amounts of toxin would not cause marrow changes of this character or degree, it is difficult to explain the apparent whole involvement of the marrow system following toxin injury to a single bone marrow.

It has been found that the *B. welchii* is capable of producing two apparently distinct hemolysins, one of which is active both *in vitro* and *in vivo* and the other, the more potent, causes blood destruction *in vivo* but only to a slight degree or not at all *in vitro*. Comparative tests of the action of these two products on bone marrow have been carried out both when introduced directly into the marrow and into the blood.

TECHNIC

Toxin: The *B. welchii* toxin was prepared by the growth of the organism in casein-digest broth in sealed tubes as described heretofore.² We have found that 2 cc. of fresh rabbit blood per 30 cc. of medium may be substituted for fresh pigeon muscle. The toxin obtained is fully as potent and the *in vitro* hemolytic element is completely, or almost completely, absorbed during the growth period. This product will be designated as "toxin" in distinction from "hemolysin" which was obtained by growth of *B. welchii* in tubes

with the glass unsealed and in medium without addition of pigeon muscle or blood. Both products were rendered sterile by passage through Berkefeld filters. The toxin was tested for potency by injection in the breast muscle of pigeons. A good product caused death in 0.25 to 0.5 cc. dosage, in twenty-four hours.

Intramarrow Inoculation: The animal was placed under ether anesthesia, the hair shaved from a site over the outer posterior aspect of the left tibia about 2 cm. from the distal end and iodine applied. The skin was then stretched well over to one side and an incision, about 1 cm. in length, was made over the proposed point of inoculation. This was done so that when the skin was allowed to return to its normal position the puncture in the bone would be entirely covered and the danger of infection reduced to a minimum. The bone, almost directly under the skin, was laid bare and a small hole drilled of a diameter such that the hypodermic needle would fit snugly. This close fit is very important as otherwise the substance inoculated will not run into the marrow. The injection was made slowly, the usual dosage being 0.5 cc., and a few seconds allowed to elapse before the needle was removed. When properly carried out there was no leakage. With removal of the needle the skin was allowed to return to its normal position and a dressing applied. In no instance was there subsequently any evidence of infection and frequently when the animal was killed several weeks later the healing of the bone was so complete that the site of the puncture could not be found.

EXPERIMENTAL RESULTS

The blood and marrow changes following the above treatment will be given first in detail in the case of a rabbit and a monkey and following that confirmatory tests will be described including a comparison of the action of *B. welchii* "hemolysin" and "toxin" introduced intravenously and into the marrow.

Rabbit No. 254: Male, weight 2200 gm. Inoculated into the left tibia with a single injection of 0.5 cc. of sterile "toxin" on March 11, 1926. The animal showed no obvious signs of discomfort following the injection. He was found dead seventy-five days later with a loss of weight of 580 gm., as shown in Table I. Twenty-four hours after the injection, both the hemoglobin and red cell count was reduced over 50 per cent. There was apparently also a marked leucocytosis

but this may have been a technical error as it was not noted in other rabbits similarly treated. About ten days following the inoculation the color index rose above 1.0 and stayed there until about two weeks before the death of the animal. For sixty days the animal was in a state of pronounced anemia with hemoglobin ranging from 50 to 60 and red cell count from 1,700,000 to 2,600,000. About one week before death a marked drop in hemoglobin occurred reaching a low point of 30. There was some evidence of a leucopenia, the white cell count reaching a minimum of 3,200 on two occasions. Morphological and staining changes in the blood were on the whole not quite so pronounced as reported heretofore as following intravenous toxin inoculations, although about five weeks after the toxin treatment poikilocytosis became very marked, much more so than when the toxin was injected intravenously. Nucleated red cells, punctated basophiles or those containing Howell-Jolly bodies were seldom observed. Pronounced anisocytosis was noted twenty-four hours following the injection and by the fourth day the microscopic fields showed principally microcytes staining more bluish with the Wright stain than normally. At least three attempts at regeneration are discernible; on March 19, April 10 and May 10. At these times there was a slight rise in hemoglobin or erythrocyte count and the blood picture was more nearly normal. On the day before death there was very marked anisocytosis, poikilocytosis, achromia and polychromatophilia. Two nucleated erythrocytes, three myelocytes and one myeloblast were found per 100 leucocytes counted.

For all the reports on the microscopic findings in the marrows and other tissues in this and other animals covered in this study, we are one indebted to Dr. James Ewing.

The marrows of the left and right tibia, right femur and left humerus were a dark maroon color throughout in the case of the two latter, and of a lighter shade in the distal halves of the two tibias. Sections of marrows from the left and right tibias, the left femur and left humerus showed essentially the same changes. Part of this tissue had been fixed in 10 per cent formalin and part in Zenker's fluid.* Dr. Ewing's report was as follows:

"There is in all a replacement of fat and lymphoid cells by a peculiar homogeneous granular material which appears to replace

* Our appreciative thanks are due to Dr. W. B. Castle of Boston City Hospital, who had sections of some of the Zenker-fixed material prepared.

TABLE I
Single Inoculation of *B. Welchii* Toxin into the Bone Marrow of a Rabbit
Rabbit No 254. Mature, Male. Weight 2200 gm.

Date	Toxin inoculation	Hemo- glo- bin	Color index	Red blood count	White blood count	Differential white cell count							Aniso- cytosis	Poikilo- cytosis	Nucleated reds	Punctated basophiles	Howell Jolly bodies
						Poly- morpho- nuclears	Lympho- cytes	Basophiles	Large mono- nuclears	Eosinophiles	Transitional	Myelocytes					
1926		per cent															
Mar. 11	Normal	82		4,310,000	7,000	2	75	—	2	20	1	—	—	—	—	—	—
" 12	0.5 cc.	40	1.0	2,080,000	24,000	3	77	—	—	20	—	—	—	+++	—	+	—
" 15	<i>B. welchii</i>	50	0.7	3,080,000	5,800	1	89	—	2	8	—	—	—	+++	—	+	—
" 19	toxin into	62	1.1	2,690,000	6,900	3	86	—	—	11	—	—	—	+++	—	—	—
" 22	tibial	60	1.4	2,130,000	5,000	2	85	—	4	9	2	—	—	+++	—	—	—
" 25	marrow on	55	1.4	1,910,000	3,200	—	72	—	1	26	1	—	—	+++	—	—	—
" 30	Mar. 11	51	1.4	2,150,000	5,200	—	78	—	—	21	1	—	—	—	—	—	—
Apr. 3		59	1.1	2,630,000	3,200	—	84	—	1	13	2	—	—	+++	—	—	—
" 10		61	1.4	2,100,000	5,000	—	73	—	1	25	1	—	—	—	—	—	—
" 17		58	1.3	2,240,000	2,400	4	72	3	—	21	3	1	—	+++	+	—	—
" 26		57	1.7	1,770,000	4,200	—	72	—	2	25	1	—	—	+++	+	+	+
May 3		50	1.3	1,860,000	3,200	—	60	—	4	35	1	1	—	+++	+	+	+
" 10		56	1.0	2,770,000	4,600	—	78	—	3	19	—	2	—	+++	+	—	—
" 17		35	0.8	2,080,000	5,800	—	—	—	—	—	—	—	—	—	—	—	—
" 24		30	1.0	1,520,000	2,400	—	81	—	2	17	—	3	—	+++	+	2	—

May 25 Rabbit died. Weight 1620 gm. at death.

* Per 100 leucocytes.

these cells and compose four-fifths of the tissue. The few remaining cells are mostly rather large, hyperchromatic granular mononuclears of the myelocyte type. No definite traces of forming red cells could be found. The changes resemble the so-called myeloblastic degeneration of bone marrow in pernicious anemia in an advanced atrophic stage." These findings are illustrated in Figs. 1 and 2. It may be noted that the degree of degeneration is even more marked in the right tibial marrow than in the left, into which the toxin had been injected.

Monkey Q: Mature, Nicaraguan Bull Head, Cebus type, female. May 6, 1926, 0.5 cc., *B. welchii* toxin inoculated into right tibia. A progressive anemia developed. When killed seventy-seven days later the animal was very anemic. The erythrocyte count was 1,700,000, the hemoglobin, 40. The tongue was pale and smooth, the liver, a light yellow reddish brown, the spleen, not enlarged and a light pink. No obvious lesions in the kidneys. The intestine was practically free of parasitic worms. The bone marrows were a dark maroon throughout, with the exception of those of the tibias in which the distal ends were of a lighter color. In this animal and in all *B. welchii* inoculated rabbits the blood serum was either colorless or a light straw color and never showed the lemon yellow hue so characteristic of pernicious anemia. The details of the blood examinations are shown in Table II. It will be noted that the erythrocytes fluctuated around 2,000,000 with counts below that mark during the last three weeks and the hemoglobin between 40 and 60 with evidences now and then of attempts at regeneration. The color index was generally slightly above 1.0 and there was at times a moderate leucopenia. Marked anisocytosis with many macrocytes was noted at practically every examination with occasionally distinct poikilocytosis and polychromatophilia. Occasional nucleated reds and myeloblasts were seen and Howell-Jolly bodies were considerably more prominent than in the blood of the rabbits similarly treated. In general features, however, the findings were very similar to those for the rabbits.

Microscopic examination of marrows of the right and left tibias, a femur and humerus showed degenerative changes, perhaps more marked in the right tibia into which the toxin had been inoculated, but prominent in the other marrows. The right tibia showed very numerous fat cells with scanty marrow cells which lay in clusters

TABLE II
Single Inoculation of B. Welchii Toxin into the Bone Marrow of a Monkey
Monkey Q. Young Adult Female

Date	Toxin inoculation	Hemo- globin	Color index	Red blood count	White blood count	Differential white count							Aniso- cytosis	Poikilo- cytosis	Nucleated reds	Punctated basophils	Howell Jolly bodies
						Poly- morpho- nuclears	Lympho- cytes	Basophils	Large mono- nuclears	Eosinophils	Transitional	Myelocytes					
1926		per cent															
May 5	Normal	78		4,250,000	8,800	57	36	—	2	3	2	—	—	—	—	—	—
" 6	Normal	74		4,780,000	9,600	62	32	—	2	3	1	—	—	—	—	—	—
" 6	0.5 cc.																
" 10	B. welchii	42	1.05	2,030,000	5,000	26	69	—	—	3	2	++	+	—	1*	—	—
" 17	toxin in	45	0.9	2,320,000	5,000	37	59	—	1	1	2	—	++++	+++	—	—	—
" 24	tibia on	46	1.0	2,190,000	8,200	42	50	—	3	2	3	—	++++	—	—	—	+
June 2	May 6	41	1.0	2,090,000	11,200	49	39	—	1	8	3	—	++++	+++	2	—	—
" 9		57	1.4	2,070,000	6,600	36	51	—	2	8	3	—	++	++	—	—	—
" 16		53	1.2	2,200,000	8,400	24	67	—	2	5	2	—	+++	++	—	—	+
" 23		49	1.1	2,110,000	7,000	26	68	—	2	3	1	—	++	—	1	—	+
" 30		35	0.9	1,850,000	6,400	26	68	—	2	1	3	—	++++	—	—	—	+
July 8		42	1.2	1,710,000	7,000	22	72	—	1	3	2	—	++++	—	1	—	—
" 14		39	1.3	1,470,000	7,800	42	44	—	1	11	3	—	++++	—	—	—	—
" 21		40	1.1	1,700,000	5,600	25	72	—	2	1	—	—	++	—	—	—	—
July 22	Monkey very weak. Chloroformed in order to obtain organs in fresh condition.																

* Per 100 leucocytes.

between abundant fat cells. Giant cells were frequent. There was a great abundance of large phagocytic cells containing yellowish pigment. Many polymorphonuclear leucocytes were present throughout the tissue. No islands of normoblasts but many nucleated erythrocytes of about normal or slightly increased size were found. The general impression was of blood destruction with active regeneration of an irregular type. Local inflammation was indicated by the leucocytosis. The marrow of the left tibia was more cellular, fat cells less numerous and most of the cells lymphoid mononuclears. Comparatively few nucleated reds could be made out and there were few polymorphonuclear leucocytes (Fig. 8). The marrows of the right femur and right humerus were very cellular with practically no fat cells present and no islands of red cells. Most of the cells were lymphoid mononuclears. The findings in this monkey, accordingly, differed from those in the rabbit in that in the former the marrows did not show a replacement of the marrow and fat cells by the homogeneous granular material which was such a prominent feature in the rabbit marrows but these cellular elements were replaced by lymphoid cells. As with the rabbit, however, there occurred a very marked decrease in fat cells in marrows far removed from the site of inoculation. In both species there was evidence of marked degeneration of the blood-forming cells in the marrows *throughout* the body.

CONFIRMATORY TESTS

On January 13, 1927, three large rabbits, Nos. 272, 273 and 274, ranging in weight from 2000 to 2200 gm. were each inoculated into the left tibial marrow with 0.5 cc. of *B. welchii* toxin. This was a minimal lethal dose for a pigeon when injected into the breast muscle. One of the animals, No. 272, died seventy-four days later, repeating almost exactly the history of Rabbit 254 (death in seventy-five days). The erythrocyte count was 2,360,000 and the hemoglobin 76. Rabbit 273 was killed 102 days later in a very weak and anemic condition and without doubt would have died from its condition. The erythrocyte count was 1,830,000 and the hemoglobin 64. Rabbit 274 was in a marked to profound anemic condition for 112 days when he was killed in order to obtain the bone marrow. The erythrocyte count about that time was 1,600,000 and the hemoglobin 46. The morphological changes in the blood in all these three

rabbits were much the same as for Rabbit 254 (Table I) except that nucleated reds were not observed at any examination. Several inoculations of toxin, given intravenously in monkeys, cause after two weeks a flood of nucleated reds as described in a previous paper ² but not when a single injection is given into the marrow. Polychromatophilia and basophilia in a moderate to a marked degree was

CHART I

Blood counts on rabbits after a single intratibial (marrow) injection of B. welchii toxin

observed at nearly every examination of the three rabbits beginning with the day following the inoculation. Transitional leucocytes and myelocytes were observed only on one or two occasions in each of these rabbits.

Chart I shows in detail the changes and fluctuations in the weekly erythrocyte counts and color indices determinations for the three rabbits. These curves have been superimposed and bring out in a striking way the almost identical reaction of the three animals to the single intramarrow inoculation of the toxin, both in degree and in point of time. The erythrocyte count dropped to between 2,000,000 and 3,000,000 the day following the inoculation and remained at that level continuously for seven weeks. There then occurred in all three animals a strong attempt at blood regeneration, as in each

the erythrocyte count rose to nearly 4,000,000 and the color index dropped to below 1.0. This lasted only a few days at the most, for on the eighth week examination the red cell count had dropped to the former level and the color indices were considerably above 1.0, as had been the case heretofore. In the case of Rabbit 254 a similar major regenerative attempt occurred in the eighth week (Table I). Minor remissions may also be noted in the third to fourth weeks and in the thirteenth to fourteenth weeks in the two surviving that long.

Rabbit 272 at death was markedly emaciated, the loss in weight being about 41 per cent. Rabbit 273, however, showed a loss of only 3 per cent. The internal organs in all were anemic but showed nothing of particular note macroscopically except a shrunken condition of the spleens. In two normal rabbits of approximately the same size, the spleens averaged 8 by 60 mm., whereas in Rabbit 272 the measurement was 5 by 15; in No. 273, 4 by 46; and in No. 274, 5 by 45. Microscopic examination showed heavy pigmentation and large microphages in the spleen of No. 272. The liver of No. 272 exhibited marked congestion with a rich deposit of fine granular yellowish pigment within the liver cells but no fatty or granular degeneration, whereas the liver of No. 273 was extremely fatty with acute universal lipoid degeneration and occlusion of capillaries from swollen liver cells. The marrows of Nos. 272 and 273 showed, in general, similar changes to those already noted for Rabbit 254, both macroscopically and microscopically. In No. 272 the changes in the right and left tibial marrow (into which the toxin was injected) were the same in type and degree, consisting of a remarkable proliferation of large cells replacing fat and causing an extensive atrophy of the blood forming cells. Among the remaining cells there were many polymorphonuclear leucocytes, a few myelocytes and many large hemoglobin-holding cells with pyknotic nuclei. There were also many pigmented microphages. The total blood-producing capacity of these marrows was greatly reduced and the type of cell formation abnormal. The right and left femurs showed similar changes but less marked. In No. 273, which lived longer than No. 272, the marrow changes in the right tibia were the same as in the left but less marked as the marrow was much more cellular and the large clear cells were less numerous. The changes in the right and left femurs were of the same character as in the left tibia but less marked.

In an attempt to determine the rapidity of the toxin action on the

bone marrows, Rabbit 500 was inoculated with 0.5 cc. *B. welchii* toxin into the left tibial marrow and then killed after eighteen hours. Before inoculation the erythrocyte count was 4,600,000 and the hemoglobin 80. Eighteen hours following inoculation the erythrocytes were 2,250,000 with marked anisocytosis and polychromophilia and slight poikilocytosis, the hemoglobin 59, color index 1.3, leucocytes 8,800. There was some hemorrhage into the left marrow. Microscopically there were the same changes in the right and left tibial marrows but curiously more marked in the right than the left. The right tibia (on the other side of the body from the inoculation) showed the tissue considerably damaged with complete atrophy of marrow cells in the center of the marrow, and marrow cells throughout much diminished in number. A good many fragmented nuclei, a great many polymorphonuclear leucocytes and numerous eosinophiles were noted; also cells showing atypical mitoses and degenerating giant cells. The fat cells were still normal and abundant. The right femur showed irregular congestion with areas devoid of cells, considerable degeneration of giant cells and many polymorphonuclear leucocytes. The left humerus exhibited the same changes as the femur with the same type of fat cells showing granular degeneration. The changes in the right tibial marrow are illustrated in Fig. 2, which may be compared with the normal marrow as shown in Fig. 1. The photographs reproduced in the plates are all from the same general region of the tibial marrow, about 1 cm. from the proximal end.

The above observations indicate that within the first twenty-four hours following an intratibial inoculation of the toxin the damage is by no means confined to the marrow into which the inoculation was made but has caused marked blood destruction, to the same degree as if the inoculation had been made intravenously, and also degenerative changes in marrows far removed from that inoculated.

SERIES OF RABBITS KILLED AND EXAMINED TWELVE DAYS AFTER INOCULATIONS

A rabbit (No. 98) was inoculated into the left tibia with 0.5 cc. of toxin, killed after twelve days and the marrows examined to determine the changes occurring after that interval. Twenty-four hours after the injection, examination of the blood showed a severe condition of anemia. There was an extreme degree of anisocytosis with a strong predominance of dark microcytes, marked poikilocytosis,

many shadow erythrocytes with cell walls broken and also numerous nucleated forms. The animal was so anemic that it was only with difficulty that sufficient blood for a count was obtained from a peripheral vessel. Further details are shown in Table III. The blood destruction twenty-four hours following the intramarrow injection was far more marked than that shown after the same period by Rabbit 593, inoculated intravenously with the same dosage of toxin (See Table IV). Rabbit 98, when killed after twelve days, had decreased in weight from 1820 to 1170 gm., a loss of 650 gm. or somewhat over one-third. Autopsy did not show anything of note except a rather small spleen, 35 by 8 by 4 mm. The marrows were a bright red throughout. Microscopic examination of the right tibial marrow showed an extremely marked atrophy of marrow cells and a marked increase of fat cells, most of which had degenerated. Polymorphonuclears were extremely abundant. The same changes were observed in the other marrows examined, *viz.*, the left tibia and right and left femurs. Degenerative changes were thus well advanced at this time (Fig. 3). The blood examination of this rabbit after the twelve-day period is detailed in Table III and for comparison the findings for a rabbit (No. 593) which was given a single inoculation of the same amount, 0.5 cc., intravenously and killed after twelve days (Table IV). The erythrocyte count for this latter animal showed a drop from 4,840,000 to 3,230,000 but no change in hemoglobin. There was a moderate degree of anisocytosis, slight polychromatophilia but the blood injury was in no way comparable to that of No. 98, as is shown in Table IV. The report on the microscopic examination of the marrows of this rabbit (No. 593) is as follows:

Right Tibia: The marrow is rather cellular, with fat and cellular tissue about equal. The fat cells show mucous degeneration in moderate degree. Polymorphonuclear leucocytes are very numerous. There are a great many large mononuclear acidophile cells and many of these appear to be megaloblasts. The blood formation seems to be of megaloblastic type (Fig. 3).

Right Femur: The marrow cells are limited to a narrow ring about the periphery of the marrow. There is slight mucous degeneration of fat cells with considerable capillary congestion. The polymorphonuclear cells are rather numerous. There are an unusual number of very small pyknotic nuclei which seem to belong to nucleated reds.

There are many large acidophile cells and some of these are eosinophile and granular. There are some large nucleated reds.

Right Humerus: About half the marrow is cellular and half fat tissue. The cellular marrow is more cellular than in the other specimens. Polymorphonuclear and eosinophile cells are very numerous. There are also a great number of small pyknotic nuclei belonging to the series of normoblasts, but few large nucleated reds are seen. The fat cells show little change.

The changes were thus much more delicate than in Rabbit 98 and not of the same character, particularly as regards degeneration of the fat cells.

Rabbit 96 of this series received into the left tibial marrow 0.5 cc. of a growth product of *B. welchii* which was very strongly hemolytic but weakly toxic, as shown by inoculation into the breast muscle of a pigeon. As noted above, our "toxin" inoculated in 0.25 to 0.5 cc. dosage would kill the bird within twenty-four hours with the development of a local massive and hemorrhagic edema, whereas 1 cc. of this hemolysin caused only very slight local reaction and no toxic symptoms. This hemolysin was prepared by growing *B. welchii* in the same medium as for the toxin (3 per cent peptone, 0.5 per cent glucose, casein digest broth) except that no pigeon muscle or fresh rabbit blood was added and the tube was not sealed in the blow flame. Further details in regard to the nature and antigenic properties of these two products will be given in another publication.

This rabbit was apparently in good condition eleven days after the injection of the hemolysin into the marrow. It had increased in weight from 1780 to 1940 gm. (compare with No. 98). Ten cc. of blood was taken from the ear vein. On the next day, however, the animal was found dead. Necropsy did not disclose the cause. The degree of anemia was not nearly as great as for No. 98. The spleen measured 44 by 11 by 3 mm., a little larger than that of No. 98. The marrows differed markedly from those of No. 98 in being a much darker red throughout. Microscopic examination of the left tibial marrow showed thrombosis of the main central vein with the surrounding tissues infarcted. There was also a very marked increase of large pale proliferating fat cells and a corresponding diminution of marrow cells which were not very much altered. There were many large hemoglobin-holding cells and eosinophiles. The marrow cells were not very much altered and there was not nearly as marked

TABLE IV
Blood Changes in Rabbit Twelve Days after a Single Intravenous Inoculation of B. Welchii Toxin

Rabbit	Date	Material inoculated	Hemo- globin	Color index	Red blood count	White blood count	Aniso- cytosis	Poikilo- cytosis	Nucleated reds	Punctated basophiles	Poly- chromato- philia
No. 593 Wt. 2960 gm. Wt. 3240 gm.	1928 Jan. 26	0.5 cc. Toxin intravenously	<i>per cent</i> 85	0.88	4,840,000	9,200	tr.	-	-	-	tr.
	Mar. 1										
	" 13		84	1.4	3,230,000	6,400	++	-	-	-	+

atrophy as in the case of Rabbit 98, in which the marrows were far more damaged. The right femur, the side opposite the site of inoculation, showed similar changes with moderate atrophy of marrow cells and considerable proliferation of fat cells. In fact, the proliferation of large fat cells was a very striking feature in this animal, and the findings, in general, were markedly different from those in the rabbit (No. 98) inoculated with the toxin.

Rabbit 97 of this series was inoculated into the left tibia with 0.5 cc. of the sterile culture medium. Subsequent examination disclosed that the animal was not entirely normal as there was evidence of an old lesion in the right tibial marrow. The animal lost weight markedly during the twelve days, decreasing from 1800 to 1275 gm. There was also a marked decrease in the erythrocyte count and the hemoglobin, as shown in the table, but the color index remained almost exactly 1.0 and no abnormalities were observed in the stained blood films. At necropsy the spleen measured 39 by 7 by 4 mm. Other organs appeared normal. The marrows were a bright red throughout, except the distal ends of the tibias which are normally much lighter in color. The right tibia, the site of an old lesion, showed a marked proliferation of fat cells but the changes were much less marked than in No. 98. The right femur marrow showed no change and was normal in appearance.

By way of further control two rabbits were inoculated intramarrowly with the sterile culture medium and observed for a period of about nine weeks. Rabbit 579 was inoculated into the right tibial marrow with 0.5 cc. of the sterile broth medium in which pigeon muscle had not been soaked, and No. 587 with the broth to which the muscle was added and incubated over night. The details for these animals are given in Table V. It will be noted that at the end of the period both animals had gained in weight in contrast with the very marked loss generally observed in the animals inoculated in the same way with the toxin. The erythrocyte counts and the hemoglobin fluctuated with a slight downward tendency in No. 579, but remained quite constant with No. 587. The color indices remained close to 1.0 and the blood showed no morphological or staining abnormalities throughout the experiment. Both animals appeared in fine condition at the termination of the experiment. The report on the right and left tibia and the right femur marrows of these rabbits is as follows:

TABLE V
Control Rabbits. Effect of Intramarrow (Left Tibia) Inoculation of Sterile Culture Medium Without Pigeon Muscle (No. 579) and with Pigeon Muscle (No. 587)

Rabbit	Date	Material inoculated	Hemo- globin <i>per cent</i>	Color index	Red blood count	White blood count	Aniso- cytosis	Poikilo- cytosis	Nucleated reds	Punctated basophiles	Poly- chromato- philia
No. 579 Wt. 2972 gm.	1928 Feb. 6	None	90	0.9	4,960,000	11,000	—	—	—	—	—
	" 7	0.5 Sterile culture medium	85	1.0	4,010,000	7,000	—	—	—	—	—
	" 14		82	1.0	4,240,000	14,600	—	—	—	—	—
	" 28		84	1.0	4,320,000	5,600	—	—	—	—	—
	Mar. 6		82	1.0	4,270,000	7,000	—	—	—	—	—
	" 13		85	1.0	4,070,000	8,600	—	—	—	—	—
Wt. 3225 gm.	" 27		81	1.0	4,080,000	4,200	—	—	—	—	—
	Apr. 10										
No. 587 Wt. 2675 gm.	Jan. 26	None	85	0.9	4,850,000	6,200	—	—	—	—	—
	Feb. 2	0.5 cc. Sterile culture medium*	88	0.9	4,910,000	8,200	—	—	—	—	—
	" 9		87	1.0	4,690,000	6,000	—	—	—	—	—
	" 16		87	1.0	4,500,000	6,600	—	—	—	—	—
	" 23		82	1.0	3,950,000	7,200	—	—	—	—	—
	Mar. 1		84	0.87	4,800,000	7,200	—	—	—	—	tr.
	" 8		78	1.1	3,520,000	8,400	—	—	—	—	tr.
	" 13		82	0.9	4,620,000	9,400	—	—	—	—	—
	" 29		89	1.0	4,470,000	9,800	—	—	—	—	—
	Apr. 12										

* Sterile pigeon muscle was added to the medium and incubated overnight.

"There is a marked reduction of all types of cells. The fat cells are normal. The femur marrows are slightly more cellular than the tibial. There is no other change in the marrow except simple atrophy. The condition is probably physiological and preëxisting." The appearance of the right tibial marrow of Rabbit 579 is shown in Fig. 7. This cellular atrophy may possibly have been induced by the medium ingredients, particularly the peptone, but the change was not sufficient to affect the blood picture and was in no way comparable to that following the toxin.

It seems evident, then, that medium ingredients were not responsible for the progressive degeneration of the hematopoietic system following the intratibial inoculation of the *B. welchii* toxin.

In the case of one monkey it was determined that the immunity following a long series of intravenous inoculations of *B. welchii* toxin included also the marrow. This monkey (J) had received twenty-eight treatments between January 14, and March 12, 1926, in dosage ranging from 1 cc. to 20 cc. After a prolonged period of anemia the blood picture returned to approximately normal. The animal was allowed to rest for a month and was then given 1 cc. of *B. welchii* toxin into the tibial marrow. Blood examinations during the following two weeks indicated the treatment had no effect whatever and it was thus evident that the immunity produced by the intravenous inoculation extended to such a susceptible tissue as the bone marrow.

INTRAVENOUS INOCULATION OF *B. Welchii* TOXIN AND HEMOLYSIN

For purposes of comparison with the effects of the intramarrow inoculations, a description is given of blood and marrow changes following the intravenous inoculation in rabbits with the same sterile *B. welchii* growth products. Three rabbits, Nos. 112, 114 and 269, were given five injections of 1 cc. dosage at weekly intervals and finally an intraperitoneal inoculation of 10 cc. Rabbit 112 received the "hemolysin," Rabbit 114 the toxin and Rabbit 269 a mixture of equal parts of the two. The toxin was only feebly hemolytic when tested *in vitro*, 1 cc. causing at the most partial lysis of 1 cc. washed (5 per cent) sheep cells after one and a half hours at 37° C., whereas the "hemolysin" prepared by growing the *B. welchii* in the same broth medium but without pigeon muscle or blood and with the tube

unsealed, was very potent *in vitro* causing complete lysis of 1 cc. of the washed sheep cells in dosage of 0.05 to 0.025 cc. within one hour at 37° C. One week following the first inoculation the greatest amount of blood destruction was observed in No. 269, receiving the mixture. Comparing the toxin and the hemolysin, a somewhat greater degree of blood destruction was noted in one hour following the inoculations of the hemolysin (Rabbit 112) than the toxin (Rabbit 114); as shown particularly in the hemoglobin readings, Rabbit 112 hemoglobin decreasing from 92 to 66 while that of Rabbit 114 dropped only from 92 to 85. As shown in Table VI, however, this difference was more than made up after a lapse of a week, the toxin rabbit revealing then the greater degree of blood destruction but not so much as the toxin-hemolysin animal (No. 269). These differences were confirmed in another set of rabbits. In spite of repeated intravenous inoculations of these *B. welchii* products, a marked immunity developed in all within three to four weeks. This immunity was strong enough to protect the blood against the 10 cc. intraperitoneal inoculations in the toxin and hemolysin rabbits but not in the animal treated with the toxin-hemolysin mixture.

The marrows of Rabbits 114 and 269 were a dark maroon color, whereas those of No. 112 (hemolysin rabbit) were a lighter red. Microscopically the marrows of Nos. 112 and 114 did not show any definite changes from the normal except those of No. 112 which appeared somewhat more cellular.

The above animals had developed an immunity and it was quite possible that injury done to the marrows might have undergone repair. Accordingly another rabbit (No. 582) was given three intravenous inoculations of toxin within a period of twelve days and killed ten days after the last injection and while still in a markedly anemic condition, the erythrocyte count being 2,340,000 and the hemoglobin 60 with marked anisocytosis and polychromatophilia. The marrows were dark red. Sections of the right tibial marrow showed a very cellular and considerably congested condition, but there were no changes in the fat cells. All types of marrow cells appeared in normal numbers, with eosinophiles moderately numerous. There were no definite pathological changes. A description has already been given (page 131) of the findings in a rabbit (No. 593) after a single intravenous inoculation of toxin.

DISCUSSION

The preceding experiments show clearly that a single inoculation of *B. welchii* toxin in small dosage into one bone marrow of a rabbit or a monkey causes a toxic degeneration of the cellular elements of the marrow with a consequent anemic condition persisting until the death of the animal ten weeks or more later (about 50 per cent of our rabbits died) or as long as the animal was observed, up to seventeen weeks. This degenerative change affects particularly the fat cells which seem to increase in number at first, but later undergo mucous degeneration to such a degree that they are finally entirely replaced by a peculiar homogeneous granular material. At the same time there occurs a degeneration of the marrow cells and the erythrocyte-forming cells. In the intermediate stages unusual numbers of polymorphonuclear neutrophils and eosinophils accumulate in the marrow. These changes begin immediately after the inoculation of the toxin and are not restricted to the marrow into which the injection is made but are quite as prominent or more so in marrows far removed therefrom, even within twenty-four hours or less following the treatment. Sufficient marrows were examined to indicate that the whole system was probably involved. Control inoculations with the sterile medium itself showed that the injury was due to the toxic growth products of the *B. welchii* and not to the medium ingredients. Such extreme marrow changes further do not follow intravenous inoculation of the toxin regardless of the dosage used or the number of inoculations. The marrows become more cellular and congested after intravenous inoculation. Following the first inoculation, the fat cells may show mucous degeneration in moderate degree but an immunity soon develops which arrests the process and permits a return to normal even though very large doses of toxin are employed (Table VI). It is reasonable to suppose that the inoculum reaches the various marrows of the body in the same or greater strength when the same dosage is injected directly into the blood stream as when inoculated into a single marrow as it is the blood and lymph which transports it in each case. If the degenerative changes were confined to the marrow receiving the toxin or were much greater there than in the marrows far removed, the severer effect would be expected because of contact with the undiluted toxin. Such, however, is by no means the case, as our observations demonstrate.

What then causes the strikingly different effect on the marrow system when the toxin is injected intravenously and intramarrowly? We have as yet no experimental data which would serve to explain the difference but the suggestion may be made that the undiluted toxin in its destructive action on the marrow tissue with which it is brought into direct contact may give rise to secondary toxic products particularly injurious to marrow tissue, and which on absorption throughout the lymph and blood are brought in contact with all the body marrows, causing as grave an injury to those farthest removed as to that into which the toxin was directly inoculated.

It would seem probable that an experimental anemia produced in this way may prove helpful in the study of the treatment of pernicious anemia, particularly as regards the use of liver extracts and their standardization. At our suggestion the methods employed in this study are now being used at the Lederle Antitoxin Laboratories in an experimental investigation of their applicability to liver extract standardization. In a preliminary report by Beard, Clark and Moses,¹⁴ it is clearly demonstrated that liver extract feeding to rabbits with the chronic anemia produced in this way is beneficial, resulting in a prompt and marked rise in reticulocytes, hemoglobin and red cell counts. There seems also to be a proportionality between the amount of liver extract given and the increase in number of red cells and hemoglobin content. The benefit, in fact, seems to parallel that occurring in a pernicious anemia patient on liver extract. Their findings offer hope that a method for standardization of liver extracts may thus be established. Whipple¹⁵ has found that a secondary anemia produced in dogs by repeated bleedings is only slightly benefited by feeding liver extract, although marked improvement follows the use of whole liver. It would seem, then, that our method offers a much more delicate method for potency titration. It is also suggested that this experimental anemia may provide a method for evaluating the blood regenerating potentialities of various chemical fractions of liver and other organs.

SUMMARY

1. It has been found that the single inoculation of 0.5 cc. of a potent sterile *B. welchii* toxin into a tibial marrow of a rabbit or a monkey gives rise to a chronic, persistent and finally often fatal anemia characterized by low hemoglobin content of the blood, low

erythrocyte count and a color index generally above 1.0. Anisocytosis and at times poikilocytosis are pronounced.

2. A single intravenous inoculation of *B. welchii* toxin of the same dosage or a series of increasing doses gives rise to an anemia of the same type as that resulting from marrow inoculation but is followed within three to four weeks by an immunity and a return of the blood to normal or nearly normal condition. Such intravenous inoculation of the toxin causes some mucous degeneration of the fat cells and an increase in the cellular elements in the marrow, particularly the leucocytes, but results in nothing like the destructive action of inoculation directly into the marrow.

3. The single inoculation of 0.5 cc. of the toxin into a tibial marrow starts a degenerative process which apparently affects the whole marrow system. Definite evidence of beginning degenerative changes were noted in the marrows on the side of the body opposite the site of inoculation within eighteen hours. After twelve days the process was well advanced as shown by the marked mucous degeneration of the fat cells, great diminution in other normal marrow and blood-forming cells and a marked increase in polymorphonuclear leucocytes. In rabbits which had died eleven or more weeks after the inoculation the marrows were found to be in an advanced stage of degeneration with fat cells replaced by a granular material and an extreme atrophy of the cellular elements. Nearly all the animals showed, at whatever stage examined, a rather more pronounced degeneration in marrows far removed than in that into which the toxin was injected. As intravenous inoculation of like or larger amounts of toxin causes no more than a transitory marrow injury, the cause of the difference in effect is not clear.

4. It is suggested that the chronic experimental anemia, produced in this way may prove a useful means for evaluating the potency of extracts of liver and other organs as blood regenerating agents.

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DESCRIPTION OF PLATES

PLATE 29

- FIG. 1. Normal rabbit, right tibial marrow. $\times 160$.
- FIG. 2. Rabbit 500. Right tibial marrow. Inoculated into left tibial marrow with 0.5 cc. *B. welchii* toxin. Killed after eighteen hours. Atypical mitoses and degeneration of giant cells, many polymorphonuclear leucocytes. $\times 160$.
- FIG. 3. Rabbit 98. Right tibial marrow. Inoculated into left tibial marrow with 0.5 cc. *B. welchii* toxin. Killed after twelve days. Extremely marked atrophy and degeneration of fat cells, numerous polymorphonuclear leucocytes. $\times 160$.
- FIG. 4. Rabbit 593. Right tibial marrow. Inoculated intravenously with 0.5 cc. *B. welchii* toxin. Killed after twelve days. Marrow rather cellular. Fat cells show mucous degeneration in moderate degree. Polymorphonuclear leucocytes very numerous. $\times 160$.

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PLATE 30

- FIG. 5. Rabbit 254. Left tibial marrow. Inoculated into left tibial marrow with 0.5 cc. *B. welchii* toxin. Died seventy-five days later. Replacement of fat and lymphoid cells by a homogeneous granular material. The few cellular elements are mostly myelocytes and polymorphonuclear leucocytes.
- FIG. 6. Rabbit 254. Right tibial marrow. Degenerative changes even more pronounced than for left tibia. Very few cells.
- FIG. 7. Rabbit 579. Control for medium. Right tibial marrow. Inoculated into right tibial marrow with 0.5 cc. sterile broth medium. Killed after sixty-four days. No change except a simple atrophy with a marked reduction of all types of cells. Fat cells are normal.
- FIG. 8. Monkey Q. Left tibial marrow. Inoculated into right tibial marrow with 0.5 cc. *B. welchii* toxin. Killed after seventy-seven days. Marrow very cellular, comparatively few fat cells and very numerous lymphoid mononuclear cells.

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