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A pernicious anaemia-like blood condition produced in monkeys with B. welchii toxin.

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In a recent article by Moench, Kahn and Torrey[1] a report was given of an analysis of the fecal flora in 33 cases of pernicious anæmia. The most striking feature revealed by these examinations was the constant presence of unusually large numbers of *B. welchii* in the stool specimens and presumably throughout the large intestine. These findings were a confirmation and extension of the earlier observations of Herter[2] and also of Simond.[3] It was suggested that the absorption of *B. welchii* toxin from the intestinal tract might conceivably be the essential etiological factor in the production of the clinical syndrome which characterizes pernicious anæmia, and that our findings justified further inquiry by experimental methods. In the present communication we wish to report in a preliminary way our observations on the effect of a potent *B. welchii* toxin on the blood of monkeys when injected intravenously.

Several strains of *B. welchii,* isolated from different cases of pernicious anæmia, were compared as regards toxin production, employing the Bull and Pritchett[4] method of production, and injecting graded amounts of the filtered toxin into the wing vein of pigeons. Our strain "Navital" proved the best suited for our purposes but it was deemed advisable to enhance its virulence by animal passages. Accordingly it was passed through six successive rabbits by intravenous inoculation, and then through four pigeons by injections into the breast muscles and recovery from the lesions on the death of the bird. The virulence of the whole

[1] Moench, Kahn and Torrey, *J. Infect. Dis.,* 1925, xxxvii, 161.

[2] Herter, The Common Bacterial Infections of the Intestinal Tract, New York, 1907.

[3] Simond, Monograph 5, Rockefeller Inst., 1915.

[4] Bull and Pritchett, *J. Exper. M.,* 1917, xxvi, 119.

culture was finally such that 0.02 cc. caused death within 18 hours. Sterile filtrates of the culture were found to be, however, only slightly more toxic than the unpassed strain.

An attempt was then made to obtain a more potent sterile toxin by a modification of the Bull and Pritchett technic, and after considerable experimentation the following method was found to yield the best results:

Medium, beef heart infusion (boiled 15 minutes), peptone (Difco) 3 per cent, casein digest fluid 10 per cent, glucose 0.2 per cent, reaction, pH 7.2. Tubed in about 20 cc. amounts in large (potato) tubes and autoclaved. Fragments of fresh pigeon breast muscle, removed aseptically, were added to each tube of medium. Before adding the tissue, the medium was heated for 15 minutes at 100° C. in the Arnold, cooled to 45°. The muscle was then added, the medium seeded and a cap of sterile vaseline applied. To prevent blowing out of this cap and hence loss of anærobiosis, the tube was sealed in the blow flame. Controls for sterility were run.

Toxin test—The culture was incubated 18 hours at 37° C., then centrifuged at low speed and finally passed through a Berkefeld N candle. The filtrate was then tested for sterility and potency. It was found that 0.3 cc. of this filtrate injected into a pigeon's breast muscle would produce massive œdema (4×6 cm. area) with characteristic blistering and terra cotta coloration, but not death, whereas 0.75 to 1 cc. was uniformly lethal within 18 hours. Within 1 to 2 hours of injection even with 0.3 cc. dosage, symptoms of profound intoxication (drooping, lethargy) became apparent. This toxin maintained its full potency for about 10 days at ice box temperature. The same dosage of the sterile medium when injected into a pigeon's breast muscle produced no reaction.

The monkeys used were the ring-tail variety *(Cebus capucinus)*. It was found that young monkeys were more susceptible to this toxin. The animals were kept on the same diet during the course of the experiment (bread soaked in milk, chopped cooked meat, bananas and occasional peanuts). After examination of the normal blood the series of toxin intravenous injections were started, using the external saphenous vein on the posterior aspect of the calf of the leg. Blood for examination was

Monkey F. Young Adult Male.

Dates	Amount Toxin Inoculated and Dates	Hemoglobin		Red Blood Cells	White Blood Cells	Differential White Cells						Anisocytosis	Poikilocytosis	Nucleated Red Cells	Punctated Basophiles	Howell-Jolly Bodies
		Per cent	Index			Polymorph.	Lymphoc. (small)	Basophiles	Large Mono.	Eosinoph.	Myelocytes					
May 29	Normal	91	1.2	3,820,000	—	25	71	0	3	0	1	—	—	—	—	—
June 1	1 cc. 5/29	70	1.13	3,120,000	—	15	81	0	3	0	1	++	—	—	—	+
June 5	2 cc. 6/1	54	1.5	1,880,000	—	5	84	0	4	3	4	+++	—	+++	+	+
June 6	3 cc. 6/5	50	1.6	1,670,000	—	20	78	0	2	0	0	+++	+	8* ++	++	+
June 8	3 cc. 6/6	45	2.5	950,000	—	30	60	0	4	0	0	++++	+++	3 ++	+++	++
June 10	None	50	1.14	2,230,000	—	50	39	0	8	0	3	+++	+++	4 ++	+++	+++
June 12	2 cc. 6/10	52	0.93	2,730,000	5,800	61	22	0	0	3	9	++	+	+	—	+++
June 15	3 cc. 6/12	56	2.0	1,440,000	1,800	51	41	0	5	0	3	++++	+++	1 ++++	—	+
June 18	1.5 cc. 6/15	65	1.19	2,940,000	5,800	48	47	0	4	1	0	+	+	28 +	+	+
June 22	5 cc. 6/18 2 wks. old	68	1.21	2,840,000	5,000	28	65	0	3	0	3	++	— ++	1 —	—	++
June 26	3 cc. 6/22	52	1.3	2,000,000	2,600	41	50	0	7	1	1	++++	+++	++	+	+++
June 30	7 cc. 6/26 New toxin	73	1.2	3,070,000	2,000	45	39	1	8	2	5	++	++	2 ++	— +++	+
July 6	11 cc. 6/30 Intrap.	75	0.99	3,870,000	4,100	77	5	0	8	1	9	+++	++	2 + 0	+++	+ —

* Indicates number of nucleated red blood cells per 100 leucocytes counted.

obtained by puncture of the sole of the foot. The hemoglobin estimations were made with the Sahli apparatus and technic.

Three monkeys were treated, but the most striking results were obtained with two young adults, F and G. One of these, F, was given 1 to 3 cc. or more of toxin at 3 to 4 day intervals, and the other, G, 0.5 cc. doses at similar intervals. The control monkey, D, was given intravenous injections of the sterile medium. The results with monkey F are tabulated.

In all three monkeys treated with the *B. welchii* toxin changes in the blood developed which were strongly suggestive of pernicious anæmia. These included a marked drop in the erythrocyte count and in the hemoglobin percentage, rise in the index above 1.0, development of a marked condition of anisocytosis with many macrocytes and a moderate degree of poikilocytosis. These changes were noted in monkeys F and G very shortly after the first injection, and as treatments were continued became very marked. Nucleated reds also appeared, some with the morphology and staining properties of megaloblasts, together with punctated basophiles and polychromatophilia. At the stage of greatest anæmia these animals showed some desquamation of the tongue epithelium and also symptoms of nervous disturbance, irritability followed by periods of lethargy. In all the monkeys after about 20 days of treatment an immunity to the toxin began to appear and could not be broken down even with a much increased dosage as shown in the table for monkey F. For a time, however, the blood picture remained completely typical for pernicious anæmia, exhibiting every feature which is considered characteristic for that disease.

Erythrocyte count—In monkey G, which received the smaller dosage, the erythrocyte count dropped steadily from 4,150,000 to 2,060,000 during 6 days, and stayed around this level for about 20 days. With monkey F the lowest count, 950,000, was reached after 10 days. The animal became very weak and the injections were omitted for four days with a resultant rise in the count to 2,230,000, and this approximate level was maintained for 20 days, when the count began to rise in spite of increased toxin dosage.

Hemoglobin estimation—In these animals the hemoglobin reading started to drop shortly after the first inoculation. The lowest percentage attained for F was 45, and for G, 50. Both

these levels were reached in a week to 10 days after the first toxin treatment and simultaneously with the lowest erythrocyte count. The hemoglobin indices were much more often above than below 1.0. In monkey F an index of 2.5 was reached at the time of the lowest erythrocyte count.

Pathological changes in erythrocytes—Anisocytosis was a very prominent feature throughout the course of the experiments. This variation in the size of the erythrocytes became noticeable within 24 hours of the first inoculation (G), and progressed to such an extreme degree that erythrocytes of normal diameter were greatly outnumbered by the abnormal types. Microcytes were at first much more numerous than macrocytes, but as inoculations were continued the macrocytes increased in number until, when the erythrocyte count was lowest, they equalled or exceeded the microcytes. The development of poikilocytosis lagged behind anisocytosis, becoming fairly marked in monkey F about 10 days after the first inoculation. In no instance was it noted in extreme degree. Nucleated reds were in evidence three days after the first toxin inoculation in monkey F. From that time to the end of the experiment they were found at nearly every examination ranging in numbers from one or two after rather prolonged search to 28 per 100 leucocytes. This last count was obtained with monkey F about two weeks after the inoculations were started, and the blood picture resembled closely that seen in pernicious anæmia during a crisis. The majority of the nucleated reds observed were normoblasts, but occasionally forms resembling young megaloblasts were noted. Fragments of extruded nuclei were encountered at times, and frequently red cells containing single light or dark blue staining dots of varying size, the so-called Howell-Jolly bodies. Punctuated basophiles were numerous at times, particularly when the anæmia was most marked.

It has been our experience with this species of monkey that the small lymphocytes are generally about as numerous as, or outnumber the polymorphonuclear neutrophiles. The toxin injections tended to increase the latter until they exceeded the former at times. Myelocytes were noted in most of the examinations and tended to increase in relative numbers toward the end of the toxin inoculations, as did also the large mononuclear lymphocytes. The leucocyte count in the normal control monkey ranged from 7,600

to 9,100, but in the toxin treated animals the count ranged from 1,460 to 5,800, with an average for monkey G of 3,000. There developed, accordingly, a definite leucopenia as a result of this treatment.

As a control on the effect of intravenous injections of the medium itself, a normal monkey was given a series of four inoculations of 5 to 8 cc. each of the sterile casein digest broth during a period of ten days. This treatment had no effect whatever on the erythrocyte count (4,520,000 to 4,750,000), the hemoglobin percentage (97 to 101), or on the morphology of the erythrocytes.

While these experiments were nearing completion an article by B. S. Cornell[5] was published in which was described the blood changes occurring in rabbits in which a chronic infection by *B. welchii* had been effected by intra-splenic inoculations. The resulting anæmia was "usually chronic and mild, but in exceptional cases, acute and profound," the resulting blood picture bearing a close resemblance to that of pernicious anæmia. The most striking feature was the anisocytosis which appeared within a few hours, at times, after the inoculation, and continued until the death of the animal. Especially interesting to us is his observation that this anisocytosis is a specific effect of *B. welchii* infection and does not follow intrasplenic injections of a variety of other bacteria including hemolytic streptococci, staphylococci and *B. coli.*

In view of the fact that we have been able to produce in monkeys all of the blood changes which are considered typical for pernicious anæmia by the injection of our *B. welchii* toxin, and Cornell obtained similar results in rabbits by instituting a chronic infection with this organism and, further, that we have demonstrated that the presence of unusual numbers of *B. welchii* is a characteristic of the fecal flora of pernicious anæmia, it would seem that this organism is deserving of further serious consideration as the etiological agent of primary importance in pernicious anæmia, as, in fact, was advocated by Herter nearly two decades ago.

In view of the above findings we believe that a test of the possible therapeutic value of *B. welchii* antitoxin in pernicious anæmia is justified.

[5] Cornell, B. S., *J. Infect. Dis.*, 1925, xxxvi, 425.