

## LEUCOPENIA AND THE TOXIC SUBSTANCES OF *B. TYPHOSUS*

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In the leucopenia, which is such a characteristic feature of typhoid fever, we find the polynuclear cells markedly diminished, thus lowering the leucocyte count while the large mononuclear cells are relatively increased.

In the first few days of the illness we may find a moderate neutrophil leucocytosis, and it is usually regarded as being the case that this phase is associated with a typhoid septicaemia, the supervening leucopenia being a characteristic of typhoid lesions limited to the intestinal canal.

During the actual febrile course a marked diminution or even absence of eosinophils is noticed. These cells may increase in relative proportions during convalescence. The actual cause of the leucopenia of typhoid fever is not yet clear. Various suggestions have been advanced. Naegeli *et al.* (see Seitz 1929) seem to favour a functional paralysis of the haematopoetic organs as being the main causative factor. They found they could induce leucopenia in normal rabbits by injecting them intravenously with graduated amounts of typhoid toxic substances. Other workers give a different explanation: Goldscheider & Jacob (see Seitz, 1929) claim that the leucopenia is brought about by a different distribution of the white blood cells and that the neutrophils are attracted to the capillaries of the internal organs, especially the lungs. Gallagher (1933) recorded filament and non-filament neutrophil counts of seventeen cases of typhoid fever and found that in practically every instance there had been a striking increase in the number of non-filamentous forms. He suggested that the leucopenia of typhoid fever was due to a destruction of neutrophils, as there was insufficient evidence of the suppression of blood-forming organs. Antileucocytic serum has been prepared by Ledingham & Bedson (1915), Bedson (1921), Matsumo (1932) and others. Recently Chew *et al.* (1936) prepared antileucocytic serum relatively free of haemolytic antibodies. They found that when the antiserum was injected intracardially into guinea-pigs, there was an immediate drop in the number of white blood cells in the peripheral blood, the neutrophils being affected more than any of the other cells. These cells almost completely disappeared from the peripheral blood during the first 5 min.

Animals rendered leucopenic by benzol (Camp & Baumgartner, 1915; Rich & McKee, 1934) failed to show characteristic inflammatory reactions to local infectious agents. Guinea-pigs rendered leucopenic by antileucocytic

serum (Chew *et al.* 1936) showed no characteristic response to subcutaneous injection of *Staphylococcus aureus*. Bauer (1928) could produce no abscess by injecting turpentine subcutaneously during the height of typhoid fever, although when the fever disappeared in convalescence, the belated abscess appeared at the site of the injection.

Though the nature of the toxic substances of *B. typhosus* is not known, they undoubtedly play an important role in the process of typhoid infection. Stolyhwo (1936) demonstrated the toxic substances of *B. typhosus* in the urine and sweat of human cases of typhoid fever.

As the toxic filtrates of *B. typhosus* which we had at our disposal appeared to be very potent and rabbits are very susceptible to typhoid toxic substances, it seemed to us to be desirable to reinvestigate the effect of typhoid toxic substances on the leucocytes of these animals.

#### EXPERIMENTAL WORK

##### *Preparation of B. typhosus toxic substances*

The typhoid toxic substances may be obtained by a variety of methods. We adopted the simplest one, namely to cultivate the organisms in meat infusion broth at 37° C. for 6–7 days. The growth was then centrifuged and the supernatant fluid filtered through a Berkefeld filter.

##### *Toxicity test*

The minimal lethal doses of the toxic filtrates which were obtained from strains of *B. typhosus* of various degrees of virulence were worked out on rabbits. Rabbits were selected approximating the standard weight of 2000 g. The preliminary injections for each toxic filtrate was 0.5 c.c. Then decreasing amounts of filtrate such as 0.4, 0.3, 0.2 and 0.1 c.c. were employed. The M.L.D. was taken as the smallest amount of each toxic filtrate which will kill a rabbit weighing 2000 g. within a period of 4 hr., after intravenous injection.

The non-virulent culture "O" 901 and our Rawlings strains failed to kill rabbits with the 0.5 c.c. amounts of their respective filtrates. 1 c.c. amounts were then inoculated and tolerated by the animals. For the estimation of these minimal lethal doses about sixty-five rabbits were utilized. The rabbits became ill shortly after injection of the toxic filtrates, showing profuse diarrhoea, increased respiration, frequently involuntary muscular contractions; death often occurred within 2 hr. Table I shows the toxicity of culture filtrates of virulent, less virulent and non-virulent strains of *B. typhosus*.

This table indicates that the extremely virulent strains produce definitely more toxic substances than the non-virulent strains. The toxicity of the intermediate strains varies. For instance, culture 2842 is less virulent but produced potent toxic filtrates, whereas culture 2513 is more virulent but is less toxic. Culture "O" 901 produced very little or no toxic substances since 1 c.c. of the culture filtrate failed to kill the rabbit.

Table I. *Toxicity test of Bacillus typhosus*

Cultures	Type of organisms	M.L.D. of organisms for mice in millions	M.L.D. of toxic filtrates for rabbits c.c.
1421 "H"	Virulent	80	0.3
1677		80	0.2
2162		40	0.2
2200		40	0.2
2513		80	0.5
2399		Less virulent	600
2573	600		0.5
2842	600		0.2
3184	200		0.5
901 "O"	Non-virulent	2000	1.0 (survived)
Rawlings		2000	1.0

*Effects of B. typhosus toxic substances on leucocytes*

Our experiments consisted of the injection of a sublethal dose of the typhoid toxic filtrates intravenously into rabbits. Total white cell and differential counts were made at intervals. Toxic filtrates of *Staphylococcus*, scarlatinal *Streptococcus* and diphtheria *Bacillus* were used as controls. In all, twelve rabbits were tested with *B. typhosus* toxic filtrates and three rabbits each were tested with other toxins as control. The general blood picture in rabbits after the injection of the various toxic filtrates is illustrated in Table II.

The results tabulated above demonstrate the fact that the administration of a small amount of *B. typhosus* culture filtrate intravenously in rabbits produced a marked leucopenia within 1 hr. An increase in the number of neutrophils was usually present by the end of 24 hr. The leucocytosis persisted for about 2 days and returned to normal. From the differential count we see that in every case the leucopenia is due to a sharp drop in the proportion of neutrophils. We also injected a small amount of *B. typhosus* toxic filtrates intraperitoneally into rabbits, and the results were essentially the same as in those receiving intravenous injections. It may be mentioned that as a control, rabbits were given intravenous injections of plain sterile meat infusion broth. When this was done an increase in leucocytes resulted.

The blood picture of rabbits inoculated with typhoid bacillary toxic substances is similar to that of the same animals when they receive *Staphylococcus* filtrates. Rabbits injected with diphtheria and *Streptococcus* toxin also showed a moderate drop of leucocytes, but not to such an extent as in the case of *Staphylococcus* and *B. typhosus* culture filtrates; again there is not much alteration in the production of neutrophils and lymphocytes.

It is well known that with *Staphylococcus*, death and partial solution of the leucocytes is due to a substance called leucocidin, formed by the organism. With the *Streptococcus*, the leucocidin has been studied by a number of workers; the more recent studies of Channon & McLeod (1929) and Evans (1931, 1932) on the cytolytic effect of streptococcal filtrates, showed the presence of a thermolabile toxin which has a marked lytic action on the leucocytes. The

leucocidin of *Staphylococcus* and *Streptococcus* is thermolabile, and is easily destroyed by heat at 58–60° C. Leucocidin is therefore different from the corresponding toxin which is heat-stable. In the case of *B. typhosus*, experiment shows that culture filtrates of this organism, heated at 60° C. for 1 hr., still produced marked leucopenia in the same way as unheated filtrates when injected intravenously into a series of four rabbits. Although we have failed to find substances similar to leucocidin in *B. typhosus*, there still remains the possibility of an actual destruction of the leucocytes by the toxic substances of *B. typhosus*.

Table II. *Effects of B. typhosus toxic filtrates on leucocytes*

Material	Time of the counts	Total white cell count	Differential count		
			N	L	Others
Rabbit injected with 0.01 c.c. of toxic filtrates of <i>B. typhosus</i> (culture 2162)	Before injection	10400	46	54	—
	1 hr. after inj.	2400	9	90	1
	3½ hr. " "	2800	56	43	1
	24 hr. " "	28300	70	27	3
	48 hr. " "	29100	38	52	10
	96 hr. " "	11800	32	64	4
	5 days " "	11200	42	53	5
Rabbit injected with 0.01 c.c. of toxic filtrates of <i>B. typhosus</i> (culture 2200)	Before injection	8200	32	66	2
	1 hr. after inj.	2000	6	94	—
	4½ hr. " "	3000	74	24	2
	5½ hr. " "	4200	83	15	—
	24 hr. " "	20400	68	27	5
	48 hr. " "	12800	71	23	6
	96 hr. " "	7250	42	58	—
Rabbit injected with 1 c.c. of toxic filtrates of <i>B. typhosus</i> (culture "O" 901)	Before injection	7600	35	65	—
	1 hr. after inj.	1800	15	85	—
	4½ hr. " "	9300	61	37	2
	6½ hr. " "	24000	71	27	2
	29 hr. " "	14800	83	15	2
	49 hr. " "	9600	46	50	4
	96 hr. " "	8200	36	62	2
Rabbit injected with 50 s.t.d. of scarlatinal streptococcal toxin	Before injection	8000	61	31	2
	1 hr. after inj.	5200	57	42	1
	3½ hr. " "	5600	70	30	—
	5½ hr. " "	8400	72	27	1
Rabbit injected with diphtheria toxin 0.2 c.c.	Before injection	12200	60	37	3
	¼ hr. after inj.	8400	62	32	6
	3 hr. " "	13300	54	44	2
	22 hr. " "	10300	49	50	1
Rabbit injected with 0.5 c.c. <i>Staphylococcus</i> toxin	Before injection	15700	55	40	5
	¼ hr. after inj.	2800	40	55	5
	2½ hr. " "	400	30	64	6
	21½ hr. " "	9500	72	27	1
	48 hr. " "	24200	71	26	3
	96 hr. " "	14000	62	36	2

N, neutrophil. L, lymphocyte. Others, eosinophil, basophil, etc.

#### In vitro experiments

Experiments were first made by mixing the rabbit blood with *B. typhosus* culture filtrate *in vitro* in order that the cells might be brought in contact with a greater concentration of toxic filtrates than was possible in experiments on living animals. We desired to see whether there was a reduction in the number

or an actual destruction of the leucocytes. Accordingly, 10 c.c. of blood were taken from the heart of a normal rabbit and placed in 40 c.c. of 1% sodium citrate saline. 3 c.c. of the citrated blood were put up in a series of four tubes and each mixed with 1 c.c. of culture filtrate. The mixture was incubated at 37° C. for 5, 10, 20, and 60 min. As control a similar series was made with toxic filtrates heated at 60° C. for an hour. At the end of each incubating period the mixtures were centrifuged. Smears were made from the white-cell layer and stained with Wright's stain. A total leucocyte count was made before and 60 min. after mixing with *B. typhosus* toxic substances. The results showed that there was no alteration either in the total number or the morphology of the leucocytes. The citrated blood was then washed with saline, 1 c.c. of the packed cells was suspended in 4 c.c. of toxic filtrates and incubated at 37° C. for 4 hr. Heated toxic filtrates and plain broth were used as controls. No appreciable differences in the total number of leucocytes counted were noticed, and smears showed no alteration of the neutrophils and lymphocytes. From the above experiments, it would seem evident that the leucopenia produced by the toxic substances of *B. typhosus* is not due to the actual destruction of the leucocytes.

From the Schillings haemogram for rabbits injected with typhoid toxic substances in comparison with that of normal controls, we found that the percentage of segmenters in relation to juveniles is altered. There is an increase in the number of juveniles in the typhoid bloods. This suggested to us that the leucopenia is due to certain factors other than the suppression of the blood forming organs.

### *Pathology*

Histological examinations of the tissues were conducted with the object of observing whether the neutrophils had been immobilized in the organs or whether they were absent from the tissues as well as from the peripheral blood. Sections were taken from the various organs of the rabbits which had received typhoid toxic substances intravenously. The general histological findings noted in all of the experimental animals were as follows:

*Lungs.* The lungs showed focal areas of congestion and oedema. The capillaries were dilated, and neutrophils and lymphocytes were found lying near the walls of the vessels. In sections from the lung of a normal rabbit, the neutrophils were usually absent from the capillaries.

*Liver.* The liver cell columns showed necrosis, and sometimes foci of haemorrhages were noted. In the sinuses and capillaries some neutrophils and lymphocytes were noticed here and there. In the normal controls there was neither oedema nor necrosis, neutrophils were not found in the sinuses.

*Spleen.* The splenic pulp showed congestion and oedema. The sinuses were dilated, and in the spaces were neutrophils and lymphocytes. Sections from spleen of normal rabbit also showed a few neutrophils, but more macrophages in the sinuses.

*Mesentery.* Mesenteric lymph nodes differed from normal. There were many neutrophils, lymphocytes and myelocytes in the dilated sinuses. Occasionally some normoblasts were noticed here and there.

*Bone-marrow.* Bone-marrow from the femur showed congestion and hyperplasia of the marrow tissue. There was an increase in the white-cell elements with the myelocytes predominating. In the sinuses, many neutrophils, lymphocytes and some normoblasts were seen. In the normal rabbit the bone-marrow of the femur showed no hyperplasia of the white-cell element. There were few megakaryocytes, some myelocytes, nucleated red blood cells and very few neutrophils.

From the examinations of tissues, it seems that the leucopenia in rabbits following the injection of *B. typhosus* toxic filtrates is due to the mobilization of neutrophils from the peripheral blood in the internal organs.

#### DISCUSSION

The disappearance of the neutrophils from the peripheral blood is very rapid when *B. typhosus* toxic filtrate is introduced either directly into the blood stream or injected intraperitoneally. Opinion is divided as to how this rapid disappearance is brought about. We consider the phenomenon of leucopenia in rabbits following the injection of *B. typhosus* toxic filtrates is not due to the suppression of blood-forming organs, and there is no actual destruction of the leucocytes. There are no substances similar to leucocidin in *B. typhosus* culture filtrates which will destroy the leucocytes such as have been found in staphylococci and streptococci. We believe the transitory leucopenia in the peripheral blood of rabbits following injection of *B. typhosus* toxic substances is due to the temporary accumulation of neutrophils in the reticulo-endothelial organs, the lung, spleen, liver and bone-marrow. This temporary departure of the leucocytes from the circulation following injection of *B. typhosus* toxic substances is of course not quite comparable with the leucopenia in typhoid fever in man. In the latter the constant liberation of the toxic substances in the circulation will naturally cause the leucopenia to persist longer. Owing to the impossibility of producing the clinical course of typhoid fever in laboratory animals, all that we can say from our experiments is that the toxic substances of *B. typhosus* when introduced into the circulation will cause a transitory peripheral leucopenia. This is brought about by the accumulation of the leucocytes in the viscera.

#### SUMMARY

The transitory leucopenia in the peripheral blood of rabbits following injection of *B. typhosus* toxic substances is due to the temporary accumulation of neutrophils in the reticulo-endothelial organs. We deduce from this that the phenomenon of leucopenia in the typhoid fever of man is due to the constant liberation of *B. typhosus* toxic substances in the circulation during the course of the disease.

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