The Combined Use of Typhoid Vaccine and P^{32} Labeling to Assess Myelopoiesis

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ALTHOUGH the function of the myelopoietic system is among the most vital of processes in the human body, relatively little is known regarding the source of cells contributing to leukocytosis, their rate of maturation or survival time. The problem remains complex largely because of the multiplicity of factors affecting the level of these cells in the peripheral blood. Physiologic alterations in different areas of the vascular tree may result in sudden and marked changes in the peripheral blood picture. These facts plus the ability of white blood cells to move freely outside the vascular system make it impossible to use the peripheral white blood count at any given moment as an indication of the total number of leukocytes in the body or their rate of growth.

It is well known that the extravascular pool of white blood cells is very great, far exceeding that within the vascular system. There are apparently many areas of storage including the lungs, liver, spleen, gastrointestinal tract, bone marrow, striated muscles, and kidney.[1] The spleen and lungs appear to play a prominent role particularly in acute changes. Since the work of Harvey in 1906,[2] the spleen has been considered one of the major sites of leukocyte reservoirs. This concept developed from the observation that leukocytosis occurred following contraction of spleen upon the intravenous administration of epinephrine. However, the identical response has been seen in splenectomized patients.[3] Bierman considers the lung the most important organ in the regulation of white cell levels in the peripheral blood.[3] He has suggested that this mechanism of sequestration may be disturbed in leukemia with the result that there is diminished destruction and removal of cells by the lungs. This work has not yet been confirmed.

Another concept, suggested by White[4] and mentioned by Vejlens,[5] has been that the huge extravascular tissue collections of white blood cells serve as reservoirs, releasing white blood cells upon stimulation. These white blood cells are thought to enter the blood stream by migrating through the blood vessel walls, and then are carried by the blood to areas of demand. However, previous work in this laboratory has produced evidence that the chief source of mature leukocytes taking part in an acute leukocytosis, such as that observed following leukopheresis, is the bone marrow.[6, 7]

Menkin has postulated[8] that a leukocyte-promoting factor is released from areas

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of inflammation. He believes that this factor resides in an inactive form in the euglobulin fraction of plasma. Upon activation by bacterial pyrogens this factor results in fever and leukopenia followed by leukocytosis.[9] The precise mechanism by which this substance acts has not been elucidated.

Leukocytosis also occurs following the administration of various macromolecular substances, killed typhoid and other organisms. It has been suggested[5] that the leukopenia occurring in this situation is the result of "sludging" or margination of the white blood cells in vessels with slow circulation. This is followed, in some unexplained manner, by an elevation of the white blood count. In these experiments, accumulation of leukocytes in various organs was marked in the leukopenic as well as in the leukocytosis stage, suggesting that the elevation in peripheral white blood count must be an absolute increase.

In an attempt to clarify some of these problems of myelopoiesis, the technic of in-vivo DNA incorporation of P^{32} in developing white blood cells was combined with leukopheresis. Results of these studies have previously been reported.[6, 7, 10] Evidence was presented that the majority of cells which contribute to the leukocytosis following leukopheresis are released from the marrow and do not come from extravascular and extramedullary sources. Heavily irradiated animals (LD 50) showed a response to leukopheresis that was qualitatively similar to that of normal animals but quantitatively less as the marrow stores became depleted of leukocytes. It was concluded, therefore, that the response to leukopheresis represents an accurate measure of the capacity of the marrow to supply the peripheral blood with leukocytes.

The question, however, remained whether other types of stimulation to leukocytosis could be used to gauge the ability of the marrow to replenish the circulating blood leukocytes. Accordingly, we have conducted an investigation of the source of leukocytes involved in the leukopenia and leukocytosis following the administration of killed typhoid organisms.

Method

Technics previously described[7] were used in this study. Dogs were the experimental animals ranging in weight from 30–45 pounds. Radioactive phosphorus (P^{32}) obtained from Abbott Laboratories was administered as disodium phosphate intravenously in a dose of 15 microcuries per pound. This was given at specified times prior to the administration of typhoid to insure P^{32} labeling of leukocyte DNA. The usual dose of formalin-killed S. typhosa was 4×10^8 organisms given intravenously in 5 minutes. This dosage range invariably resulted in a severe leukopenia and a subsequent leukocytosis.

Dogs were bled at appropriate intervals using siliconized equipment. White blood cell suspensions were prepared with the use of dextran,* 15 ml. of a 6% solution per 25 ml. of blood. After separation of the white cells, the red blood cells and plasma were reconstituted and returned to the animal at the time of the next bleeding. This technic, per se, caused no alteration in the white blood count.

The succeeding steps in the isolation of nucleii and the extraction of DNA have been described previously.[7] Simultaneous aliquots of DNA in distilled water were then taken for determination of phosphorus fractions and the specific activity of P^{32}.†

* Commercial Solvents, av. molecular weight 120,000.

† A Decade Scaler (Nuclear-Chicago, Model D181) with a TGC-2 Geiger-Muller Tube was used for P^{32} counting with statistical reproducibility of 1–2%. All values have been corrected for decay.

The Block modification[11] of the Maximow technic for preparation of marrow sections was used to evaluate bone marrow changes. Routine bone marrow smears were also studied.

Dogs exposed to x-irradiation were given 250r delivered from two heads which is approximately an LD 50 for dogs and compares with 350 to 400r delivered from a single head.

Results

Fifteen dogs were given one or two intravenous injections of killed typhoid after having received P^{32} two or three days earlier. On each occasion a severe leukopenia was induced within 1–3 hours concomitant with a drop in rectal temperature of 1–2 C. The animals showed no discernible ill effects from the typhoid injection. Respiration and blood pressure were unaffected. Shaking chills were not seen. This fall in peripheral white blood count was essentially a granulocytopenia. Platelet levels were uninvolved and the packed cell volume remained stable. After a delay of 2–3 hours during which the white blood count began to rise slowly, the rate of leukocyte recovery rapidly accelerated. The white blood count often rose to 200–300 per cent of baseline in 6–8 hours and was granulocytic in character. At this time the rectal temperature rose 2 to 3 degrees C.

It has been shown previously[7] that the peak of specific activity of DNA-P in dog leukocytes appears by the fourth day. At this time, cells coming from the marrow should show a great deal of specific activity while cells coming from other

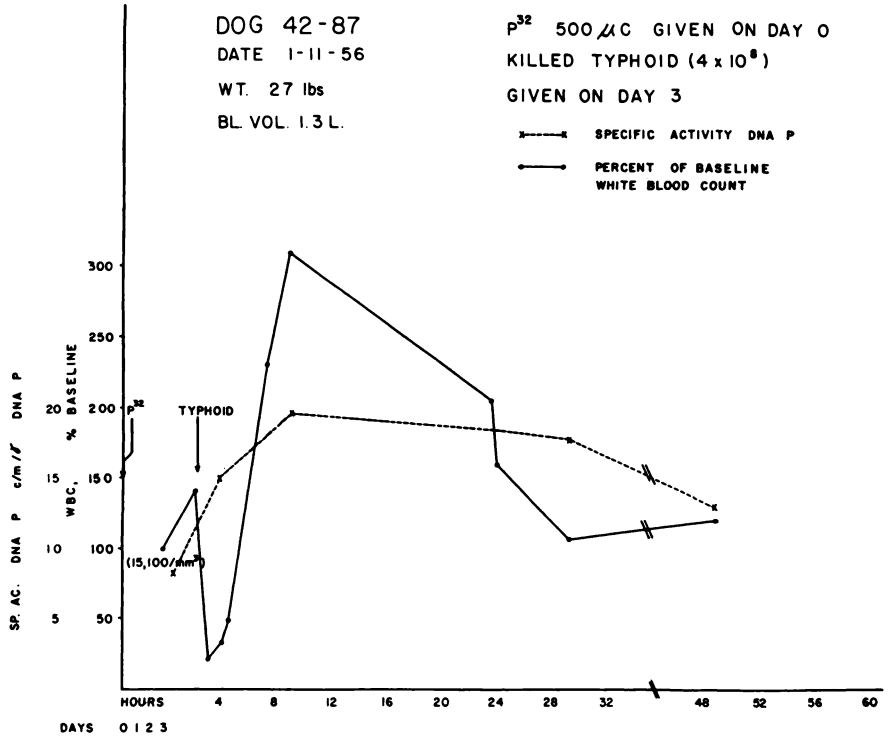

FIG. 1.—Typical response to the injection of killed typhoid organisms in a dog three days after having been given P^{32}. The leukopenic stage is followed by a period of marked leukocytosis. Leukocyte DNA-P^{32} specific activity begins to rise shortly after the administration of typhoid and reaches its maximum with the leukocytosis.

so-called "storage" areas should show little or no labeling. It is assumed, therefore that on the third day after P^{32} administration, the marrow should contain a large number of labeled cells. Figure 1 shows the typical response in a dog given P^{32} three days prior to the administration of typhoid.

The specific activity in leukocyte DNA-P began to rise shortly after the administration of the typhoid reaching its maximum concurrently with the height of leukocytosis. It is to be noted in figure 1 that even during leukopenia or lag phase, the specific activity was increasing.

In order to study the response of an animal to repeated typhoid stimulation, four dogs were given injections on three successive days. Figure 2 shows a typical response. The white blood cells contributing to the first leukocytosis contained a moderate degree of radioactivity. This increased following the second injection but no further increase was noted following the third injection.

Figure 3 shows the response in a dog given typhoid on five successive days. The maximum leukocytosis was attained following the first typhoid injection when a level 320 per cent of baseline was reached. The P^{32} was given the day prior to the first typhoid injection but the DNA-P specific activity did not begin to rise until the leukocytosis occurred in response to the second typhoid injection. The maximum specific activity appeared on the afternoon of the third day. Again, no appreciable change in specific activity following injection of typhoid on the fourth and fifth days was seen.

The following experiments were performed in an attempt to decide whether or not white blood cells released from the marrow re-enter the blood after once having left. Four dogs were irradiated with an LD 50 two days after having re-

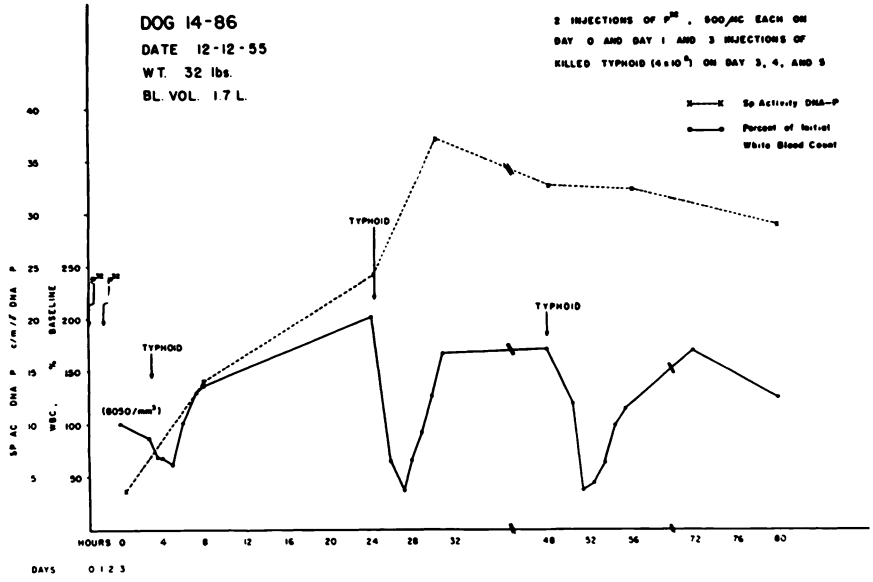

FIG. 2.—Typical response to the administration of killed typhoid organisms on three successive days. Leukocyte DNA-P^{32} specific activity showed an increase following the first injection and was marked after the second injection, but no change occurred with the final injection.

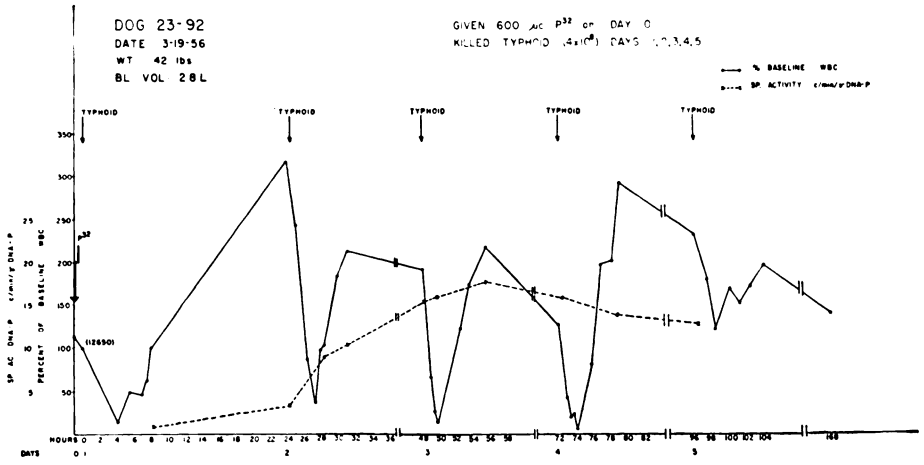

FIG. 3.—Alterations in peripheral white blood count induced by the administration of killed typhoid on five successive days. Note that the maximum leukocyte DNA-P^{32} specific activity occurred on the afternoon of the third day.

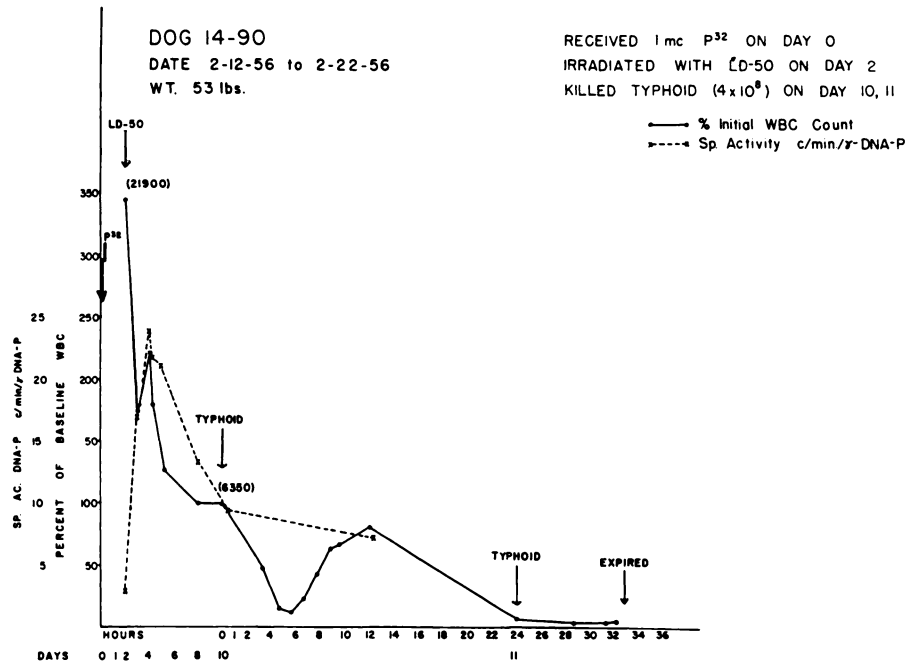

FIG. 4.—In dogs rendered leukopenic by irradiation, the administration of typhoid after most of the P^{32}-labeled leukocytes have left the peripheral blood does not result in an increase in leukocyte DNA-P^{32} activity.

ceived P^{32}. After it had been determined that most of the tagged cells had left the circulation (by the tenth day after P^{32}), the dogs were given typhoid intravenously. Figure 4 demonstrates that, following the administration of typhoid at this time, there is no increase in P^{32}-labeled granulocytes even though the

severe leukopenia obtained is followed by a return of the peripheral white count to the baseline value. This is evidence that if cells are being stored in extravascular reservoirs after being released from the marrow, they do not re-enter the circulation under the conditions of these experiments. It is also to be noted that in figure 4 another injection of typhoid on day 11 did not elicit a change in the white blood count. Marrow sections at this time revealed that cellularity was severely depleted.

If typhoid is administered on the same day as P^{32} or the next day, labeled cells do not contribute significantly to the leukocytosis. However, if typhoid is given on the second or third day following P^{32}, there is some acceleration in the time of appearance of cells containing DNA-P^{32}. This is shown in figure 5. The acceleration is compatible with our concept originally derived from leukopheresis data that a large marrow pool of mature leukocytes exists which enter the circulation prior to the release of tagged cells that have matured following incorporation of the P^{32} in the DNA development stage.

Observations of aspirated bone marrow section material revealed no obvious changes in cellularity or cellular distribution until the third day of typhoid administration. At this time no depletion was apparent, but a moderate increase

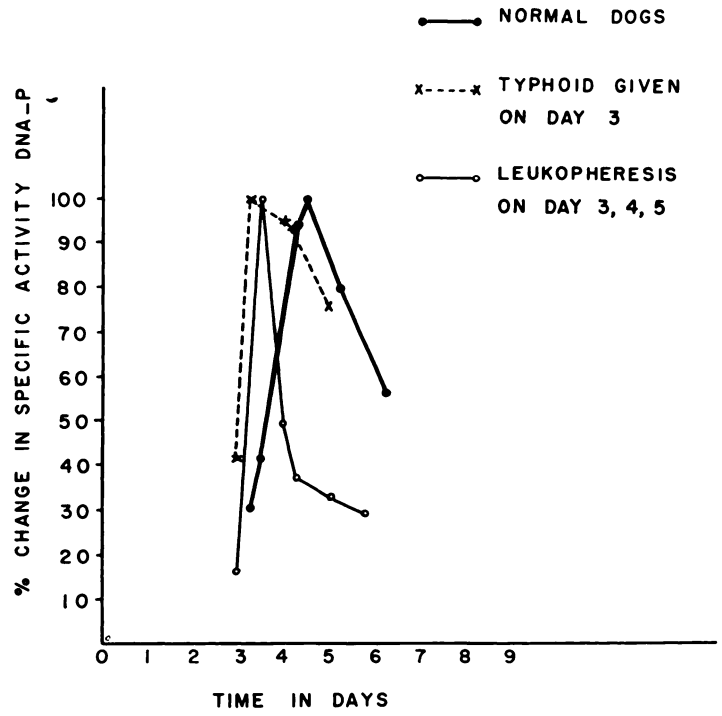

FIG. 5.—The administration of killed typhoid accelerates the rate of appearance of P^{32}-labeled leukocytes in the peripheral blood. If typhoid is given on the second or third day, the peak specific activity appears by the afternoon of the third day. This is similar to results obtained in dogs subjected to leukopheresis. The maximum DNA-P^{32} specific activity in normal dogs occurs on the afternoon of the fourth day.

in myelocytes and metamyelocytes with some over-all granulocytic hyperplasia had occurred. The day following the third typhoid injection, granulocytic hyperplasia and the shift to the left was even more marked but the erythroid elements and megakaryocytes were unchanged.

Discussion

In these experiments killed typhoid organisms have been used to elicit a leukocytosis and to place stress on the myelopoietic reserve capacity in dogs. By this method, it has been found possible to induce a severe leukopenia which, quite like the lag period observed after leukopheresis, may persist for two to three hours and is followed by a rapid linear rise in peripheral white blood count reaching a level 200–300 per cent of the baseline value.

The specific activity of the DNA-P obtained by this method shows a sharp rise coincident with the rise in the leukocyte count. Again, as in the leukopheresis experiments, the cells contributing to this phase following the induction of leukopenia are cells released from the labeled marrow pool. The close similarity between the response to leukopheresis and the response to repeated administration of typhoid is illustrated in figure 6 in which typical experiments with each technic are shown. The histologic changes in the bone marrow are similar in both types of experiments.

An interesting observation was the rise in specific activity of P^{32}-labeled DNA during the period of severe leukopenia or lag phase. This phase has been thought to be due to a redistribution of cells, with the neutrophils being sequestered in the capillaries of the internal organs, primarily the lungs. The main evidence supporting this concept has been autopsy data on animals given typhoid.[12] However, Gallagher in 1933 was of the opinion that the leukopenia was due to a destruction of the granulocytes.[13] An unequivocal cause of this neutropenic leukopenia cannot be stated definitely at this time, but our data indicate that labeled leukocytes enter the circulation at an accelerated rate shortly after the administration of typhoid. These cells may be redistributed to various tissue sites, and only when these demands are met does the peripheral white blood count begin to rise and the so-called "lag" phase terminates. Whatever happens to the originally circulating white cells, they contribute little to either the stage of leukopenia or leukocytosis. Cowie and Calhoun in 1912 may have been correct when, in referring to the leukocytosis following typhoid administration in the treatment of arthritis, noted that the "whole affair is one of genesis" and were impressed with the "newness" of the cells.

In the period after the peak of DNA-P^{32} specific activity has been passed, it may be presumed that the labeled leukocytes are disposed of in one of two ways: (1) destruction with disposal by the R.E. system, (2) migration from the vascular tree into the tissues from whence they can or cannot return. In this paper, experiments with irradiated dogs demonstrate that the leukocytes released into the circulation in response to a stimulus are not labeled cells. If leukocyte reentry were of any significance, there should have been a rise in specific activity in the peripheral blood.

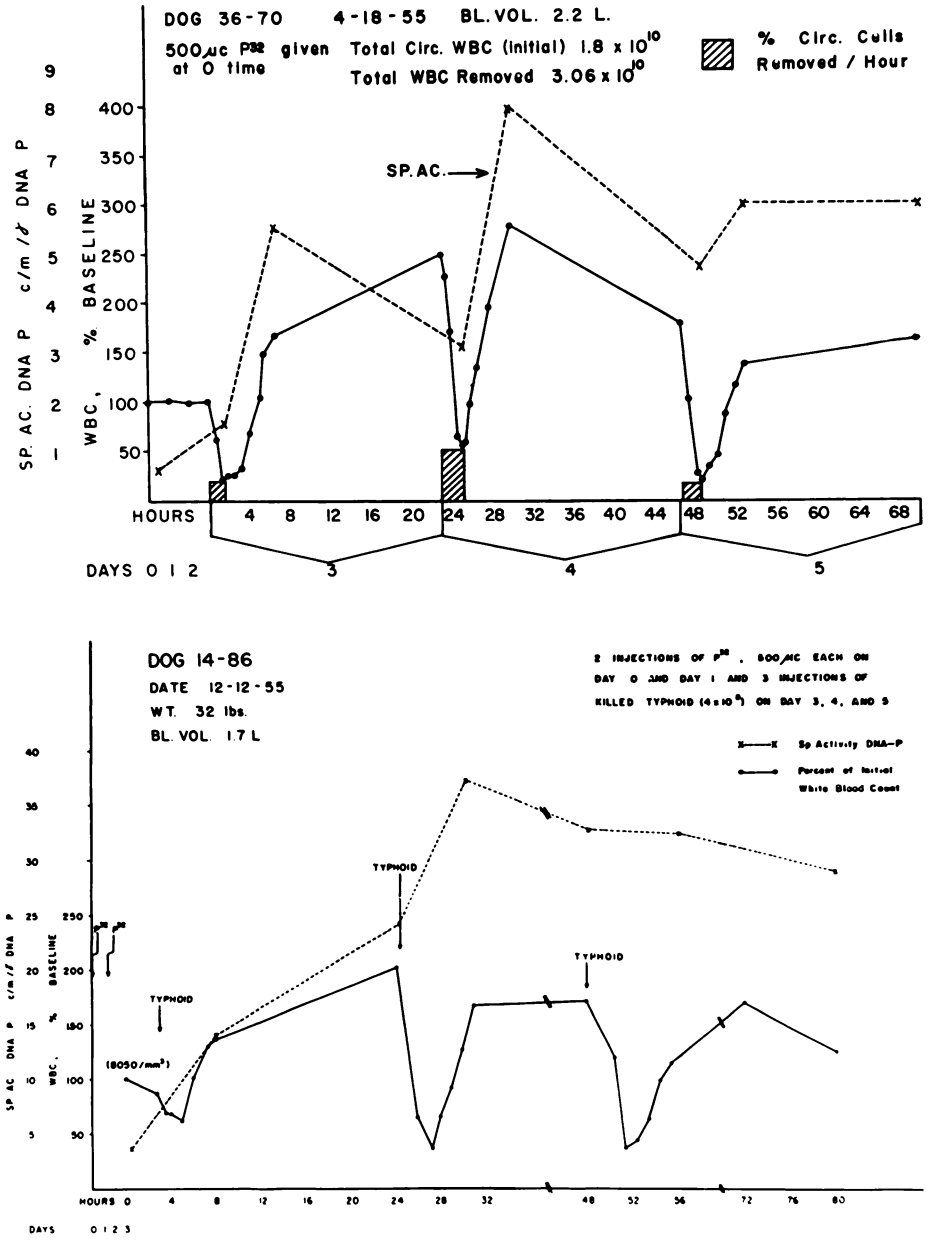

FIG. 6.—An illustration of the close similarity between the response in the dog to leukopheresis and the administration of killed typhoid.

These studies demonstrate the similarities between results following leukopheresis and typhoid administration in dogs and suggest the feasibility of using the stress of killed typhoid as a measure of assessing the capacity of the major storage site for leukocytes, the bone marrow.

Summary

1. The intravenous administration of killed typhoid organisms to dogs results in the development of a severe leukopenia which is followed by a marked leukocytosis.

2. The myelopoietic response of dogs to typhoid injections is quite similar to the response following leukopheresis.

3. Leukocyte DNA-P^{32} studies indicate that in response to typhoid stimulation, as with leukopheresis, the bone marrow constitutes the main reservoir for granulocytes contributing to peripheral blood leukocytosis.

4. Evidence is presented that, under these experimental conditions, leukocytes, after once having left the vascular tree, are unable to re-enter in any significant numbers.

5. The use of typhoid vaccine to stimulate leukocytosis combined with the use of P^{32} to measure the fate of cells released from the marrow is presented as an accurate and reasonably simple method to measure certain aspects of myelopoiesis in the experimental animal.

Summario in Interlingua

1. Le administration intravenose de morte organismos typhoide a canes resulta in le disveloppamento de sever leucopenia sequite per marcate leucocytosis.

2. Le responsa myelopoietic de canes a injectiones de typhoide es satis simile al responsa post leucopherese.

3. Studios con leucocytic acido disoxyribonucleic a P^{32} indica que in responsa a stimulation typhoide (come in leucopherese) le medulla ossee representa le principal reservoir de granulocytos contribuente al leucocytosis de sanguine peripheric.

4. Es presentate datos que indica que leucocytos que ha quitate le vasculatura sub le mentionate conditiones experimental es incapace a re-entrar in numeros significative.

5. Le uso de vaccino de typhoide como stimulante de leucocytosis in combination con le uso de P^{32} como adjuta in mesurar le destino del cellulas que ha quitate le medulla es presentate como un exacte e satis simple technica in le determination quantitative de certe aspectos de myelopoiese in animales experimental.

REFERENCES

[1] Bierman, H. R., Kelly, K. H., and Cordes, F. L.: The sequestration and visceral circulation of leukocytes in man. Ann. N. Y. Acad. Sc. *59:* 850, 1955.

[2] Cited by Bierman, H. R., Byron, R. L., and Kelly, K. H.: The role of the spleen in the leukocytosis following the intra-arterial administration of epinephrine. Blood *8:* 153, 1953.

[3] Bierman, H. R., Kelly, K. H., King, F. W., and Petrakis, N. L.: The pulmonary circulation as a source of leukocytes and platelets in man. Science *114:* 276, 1951.

[4] White, L. P.: The intravascular life span of transfused leukocytes tagged with atabrine. Blood *9:* 73, 1954.

[5] Vejlens, Gert: The distribution of leukocytes in the vascular system. Acta Path. et Microbiol. Scand. Supp. *33:* 1, 1938.

[6] Craddock, C. G., Jr., Adams, W. S., Perry, S., Skogg, W. A., and Lawrence, J. S.: Studies of leukopoiesis. J. Lab. & Clin. Med. *45:* 881, 1955.

[7] ——, PERRY, S., AND LAWRENCE, J. S.: The dynamics of leukopoiesis and leukocytosis, as studied by leukopheresis and isotopic techniques. J. Clin. Invest. *35:* 285, 1956.

[8] MENKIN, VALY: The determination of the level of leukocytes in the blood stream with inflammation. Blood *4:* 1323, 1949.

[9] ——: Pyrexin, the pyrogenic factor of inflammatory exudates and its relation to some bacterial pyrogens. J. Lab. & Clin. Med. *46:* 423, 1955.

[10] LAWRENCE, J. S.: Physiology and functions of the white blood cells. The Minot lecture. J. A. M. A. *157:* 1212, 1955.

[11] BLOCK, M., SMALLER, V., AND BROWN, J.: An adaptation of the Maximow technique for preparation of sections of hemopoietic tissues. J. Lab. & Clin. Med. *42:* 145, 1953.

[12] ROBERTSON, R. C , AND YU, H.: Leucopenia and the toxic substances of *B. Typhosus*. J. Hygiene *38:* 299, 1938.

[13] GALLAGHER, J. R.: The nonfilament polymorphonuclear neutrophil count in typhoid and undulant fever. Am. J. M. Sc. *185:* 391, 1933.

[14] COWIE, D. M., AND CALHOUN, H.: Non-specific therapy in arthritis and infections. Arch. Int. Med. *23:* 69, 1919.