A STUDY OF THE LEUCOCYTES IN RATS UNDER INSULIN STRESS

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Introduction

Previous studies have dealt with anatomical and physiological differences between normal and dwarf vertebrates (Downs et al, 1958; Downs and Benson 1958) and with the total and differential leucocyte count as a measure of response to non-specific physiological stress (Downs and Benson, 1959; Downs, Pennebaker and Benson, 1959). In these reports, it was noted that a number of stress-agents, i.e., insulin, ACTH, histamine and extreme cold, caused similar and seemingly predictable changes in the total and differential leucocyte count in cattle, mice and rats. These effects have been considered as a phase of the General Adaptation Syndrome (GAS) of Selye (1946). Such stress causes an initial rather rapid fall in the total count, lasting from one to three hours, succeeded usually by an increase, lasting from three to five or six hours, and gradually falling for a period of from four to as much as sixteen hours or longer, to return finally to about normal. In cases of severe stress which recover, the secondary rise may be minimized or absent, and the total count show a slow levelling-off to return to normal by a very slow increase. In cases of terminal shock from such stress, the count may continue to go down and may fall as low as 10% of the initial count and terminate in death. The intensity and duration of all of these changes in the total count are conditioned in part by the dosage or intensity of the stress-agent, by such intrinsic factors as age, sex and nutritional state, and to a very great degree by heredity (Downs, Pennebaker and Bowling, 1959). These changes in the total count are particularly evident in the contrast in response of normal and dwarf animals. Younger animals are more responsive than older ones, females more so than males, and obese ones respond less than do those in an optimum state of nutrition. Certain strains of animals are

markedly resistent to particular agents, as evidenced by strains of mice which can tolerate massive doses of insulin without harm (Beyer, 1952). In general, those animals which react more promptly and sharply to stress, tend to return to normal more quickly.

The response of the differential leucocyte count to such stress is even more marked and constant than is the total count. Almost from the instant that stress is applied, the lymphocyte count drops very sharply and the granulocyte count rises rapidly. These are both relative and absolute. The increase is almost entirely in heterophils (neutrophils). There is usually an eosinopenia which appears rapidly and basophils are very rarely seen at any stage. Many of these findings are in agreement with those of Dougherty and White (1944) in their studies in which ACTH was used. We, like they, found that even very small doses gave a characteristic response. They did not, however, subject their animals to shock.

Differentials	Myeloblasts & Promyelocytes	Myelocytes	Metamyelocytes or "juveniles"	Heterophils	Basophils	Eosinophils	Moncytes	Lymphocytes
1. Initial Count	0	0	3	33	0	2	2	60
2. At Shock								
Peripheral	9	12	40	26	0	0	3	10
Cardiac	58 (28-90)	13	8	11	0	0	6	4

Figure 1 — Average of differential leucocyte counts on seven rats before insulin injection and during shock (at from three to five hours after injection of from .8 to 1 unit of protamine zinc insulin per 100 gms. body-weight.

Observations

Beginning about two hours after the application of stress, fairly numerous immature heterophils begin to appear, and there is a consistent shift to the left in the peripheral blood (Fig. 1). At lower levels of stress this may extend no further than to cause a relatively slow increase in "juveniles". At a slightly higher level of stress, the peripheral blood contains fairly numerous earlier cells (promyelocytes and occasional myeloblasts). A generalized hyperplasia of all elements is characteristic of earlier stress. As the result of very high doses of the stress-agent, especially those resulting in severe shock, the blood contains large numbers (up to 90%) of myeloblasts and promyelocytes, with fewer later forms. Should the animal recover, there is a slow build-up of the successive stages of development of granulocytes, largely heterophils, until, perhaps several days later, the peripheral blood returns to normal. These changes

may occur rapidly or slowly depending in part on the dosage, and in part, on intrinsic factors affecting resistance to shock.

Studies of Latta and Henderson (1937) revealed similar results from repeated small and increasing doses of insulin, but did not consider changes resulting from shock. Other studies reported in the literature have apparently approached the problem from quite different angles.

Methods

The effects on the peripheral blood-picture indicated the need for an intensive study of the leucocytogenic organs of stressed animals. To accomplish this, it has been necessary to test many techniques for fixation, embedding and staining these organs, especially the extremely difficult bone-marrow. We believe that our present method is reasonably satisfactory, though still open to improvement. With this method, one can, with sufficient practice, identify each type of cell in the bone-marrow, as well as stages of cell-division and development with not more than about a 5% error.

Because of the numerous difficulties inherent in the material, our present technique is given in considerable detail in the hope that it may be helpful to others.

Young, adult, albino rats of a closely inbred strain of Wistar stock originally obtained from the Charles River Breeding Laboratories, but which we have bred toward homozygosity in blood-count, are used throughout. Beginning at about 8 weeks of age and over 100 gms. in weight, they are handled in pairs of litter-mates of the same sex, one experimental and one control animal. Initial total and differential leucocyte counts are done on peripheral blood drawn from the tail veins of both animals, by the methylene-blue and phloxine in propylene glycol method (Randolph, 1944). Smears are also made, using the May-Grunwald and Giemsa technique. These smears are then covered and labeled to become a part of the permanent record of each animal. Immediately after the blood for these studies is obtained, the experimental animal is injected intraperitoneally with the stress-agent. In the present series, insulin zinc protamine) is the agent used throughout. Dosage varies in a regular progression from .2 units per 100 gms. body-weight to 1.5 units per 100 gms. At regular periods after stress (2, 4, 6, 8 hours, and 5, 10, 15, 20 hours) total and differential leucocyte counts and smears are made. In some instances where the animals go into shock, blood is obtained by cardiac puncture for comparison with the peripheral count. Both experimental and control animals are sacrificed immediately after bleeding, by cervical fracture or decapitation. As previous studies had proven that the epiphysis of the femur was the best and most consistent area for the study of leucocytogenesis, both femurs are removed, cut below the epiphysis, the wall is split long-

itudinally down one side, and they are fixed in Zenker's fixative with 5% formalin added, as are spleen, thymus, pituitary and adrenals. Tissues are allowed to remain in the fixative for 8-16 hours, then rinsed in running water for 4-6 hours.

Following this, soft-tissues are run up through graded alcohols and embedded as usual. Femurs, however, are placed in 7% formic acid in 10% formalin, with a large amount (200 c.c.) of the decalcifying solution, for about 36-48 hours, following which the tissues are again washed in running water for 4-6 hours. They are then dehydrated and cleared through graded alcohols to absolute, then to absolute alcohol and ether, then embedded in thin celloidon for 2-3 days, to chloroform for 6 hours, then to toluene and cedar oil in equal parts, then to toluene-cedar oil and paraffin in equal parts, then through two changes of paraffin to be embedded in 55° paraffin (Fisher Tissue-Mat). Each step from absolute alcohol into paraffin should take an hour to an hour and a half, and the tissues should remain in the melted paraffin for not less than six hours nor more than twelve before embedding. The oven should be kept at not more than 58° C. After embedding, blocks are kept in 5% glycerine in 70% alcohol until ready to be sectioned.

Sections are cut at 3 micra and mounted with very thin albumin fixative, are dewaxed in two changes of xylol, passed through absolute and 95% alcohol, then, to remove the mercury crystals, into 95% alcohol to which tincture of iodine has been added (until a deep cherry-red) for ten minutes. This is washed out with 95% alcohol (5-10 mins.) and then the sections are passed through graded alcohols to water. Sections are stained for 75-90 seconds in Harris' Hematoxylin, rinsed in H_2O, then immersed in 0.5% lithium carbonate (aqueous solution) until distinctly blue, rinsed in tap-water, and counter-stained in Giemsa stain (50% standard stock solution, 50% distilled H_2O) for 15-20 minutes, rinsed, then into 0.2% acetic acid in distilled H_2O until a distinct pink color results, are rinsed and passed through graded alcohols and xylols to be covered. Due to the need for comparative studies of the same or similar cell-types, spleen, thymus and occasionally lymph-nodes are stained with the same techniques as bone-marrow.

Discussion

Although there is yet much work to be done, both in the accumulation of additional data, and on their interpretation, certain facts are beginning to emerge from our studies on the bone-marrow. These rest upon studies on more than one-hundred animals, handled as described above.

As the granulocytes increase in the peripheral blood, first there is a relative increase in the femoral myelocytes of both

earlier and later stages, especially later ones, and an increase in mitoses in these cells. However, as the peripheral granulocytosis reaches a peak, fewer late myelocytes are seen in the marrow, and mitoses except in the very early cells, are rare. There is evidence that this granulocytosis is correlated with motility of these cells and this, in turn, to increased contractibility on their part (Polak - 1958).

Coincident with the "shock" stages of massive stress, the marrow becomes depleted of myelocytes and older forms and there is a relative hypoplasia of all elements. It is at this point that large numbers of very early cells (myeloblasts, promyelocytes) are seen in the circulating blood. The appearance and number of these early cells is so striking that it seems

Figure 2   1. Myeloblast   4, 5. Metamyelocytes or Juveniles
                 2. Promyelocyte   6 to 9. Mature Heterophis
                 3. Myelocyte   (in order of maturity).

Normally, peripheral rat blood contains only types 6 to 9, with occasional Juveniles, usually "stab" forms. During stress the proportions shift toward the earlier forms. In animals under terminal shock, almost all cells in marrow are 1 to 3, primarily myeloblasts.

In rats there are certain differences in the morphology and staining qualities of the leucocytes which must be taken into consideration. Cells which belong to the neutrophil series in humans, in rats show in very early stages a slightly basophilic cytoplasm, changing gradually to eosinophilic in later stages of development. In the mature, lobulated heterophils, the sytoplasm shows both basophilic and eosinophilic granules in varying proportions. Also, the juveniles, the cells which in humans are classed as "stab" cells, and contain a horseshoe-shaped nucleus, in rats often have instead, a doughnut-shaped nucleus which not infrequently is completely peripheral to a faintly eosinophilic cytoplasm.

likely that this phenomenon might well be considered a measure of intensity of stress (Fig. 1). As this stage approaches, megakaryocytes in the marrow are now increased materially in numbers, and are apparently a part of the reticulum of the marrow. Littoral cells are rarely seen at this stage. Frequently megakaryocytes may be seen "fragmenting" or being broken up into tiny fragments. At this state, also, blood in the tail clots so rapidly that it is very difficult or impossible to obtain blood from this source. These changes in the large, multinucleated cells of the marrow deserve and will receive much further study, as will the exact behavior of the myeloblasts, promyelocytes and myelocytes under a variety of stress-conditions.

Although it is believed that this technique yields a satisfactory result, it is our hope that further refinements will continue to show improvements.

We would like to give due credit to the sources of the above technique but it consists of our own modifications of techniques developed by many previous workers, to such a point that this would be impossible. Methods of Sabin and her co-workers (1925, 1928), Maximow (1924), Kindred (1940), Krumbharr (1935), Latta & Henderson (1937), Wintrobe (1956) and many others are included, but have been modified according to our needs. We are greatly indebted to J. R. Neeley, Amna Williams Cornett and Faye Johnson, Graduate Biology students at Tennessee Polytechnic Institute, for much laborious technical assistance.

SUMMARY

In rats, under stress, using insulin as the stress agent, indications are that non-specific stress causes predictable and fairly regular changes in the peripheral total and differential leucocyte count. This is especially true regarding the relative behavior of lymphocytes which decrease, the heterophils, which increase, and the earlier cells of the myelocyte series, which increase markedly as a result of stress of increasing intensity, especially in deep shock. These changes are absolute as well as relative. Eosinopenia is a characteristic, as is a possible slight monocytosis. At the peak of stress, as reflected in shock immature cells dominate this picture and myeloblasts and pro-myelocytes are common, with numerous myelocytes and juveniles to be seen. Correlated with the peripheral changes are, first, an increase in the myelocytes and in mitoses of these cells in the marrow in earlier and lesser stress. There is a generalized hyperplasia of all elements, and, except for three earliest forms, a marked decrease in all of these cells in later and more intense stress. There is a generalized relative hypoplasia with terminal shocks. Typical changes in the large multinucleated cells of the marrow are also noted. A detailed technique for the preparation of tissues for the study of bone-marrow is included.

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