Humoral Factor(s) in Experimental Hypersplenism

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The syndrome of hypersplenism is characterized by splenomegaly, selective or total blood cytopenias, normal or hyperplastic bone marrow, and disappearance of the cytopenias after splenectomy. The mechanism of the various forms of hypersplenism (splenic thrombocytopenia, spenic anemia, splenic neutropenia, splenic pancytopenia) is still under discussion. Clinical and experimental evidence has been presented in favor of two different hypotheses, the destruction of blood elements by sequestration in the spleen, and the inhibition of maturation and/or liberation of bone marrow cells by humoral mechanisms.

Experimental hypersplenism has been produced by the injection of hydroxylamine, which is toxic for the reticuloendothelial system, by surgical ligation of the splenic and gastric coronary veins in the rabbit, by the administration of anti-bone marrow serum, and by injecting macromolecular inert polymers such as polyvinyl alcohol or methylcellulose. The last method was shown by Palmer et al. to give more consistent results than the others: their animals developed splenomegaly, anemia, hyperplastic bone marrow, reticulocytosis, leukopenia and moderate thrombocytopenia, and the blood picture returned to normal after splenectomy. Histologic examination of spleen, liver, kidney and bone marrow revealed large and foamy macrophages, presumably containing the polymer.

Using the methylcellulose type of experimental hypersplenism, Baldini was able to demonstrate that the anemia in the hypersplenic rat was not only due to increased hemolysis caused by engorgement of red cells in the enlarged spleen, but also to a defective bone marrow response to the anemia. Furthermore, by the lactating rat technic, the author could demonstrate the presence of humoral factor(s) in the milk of the hypersplenic animals. Rats born of normal mothers but fed from hypersplenic female rats developed anemia after 14 days of lactation, but normal baby rats fed from hypersplenic mothers splenectomized soon after parturition did not develop anemia. On the other hand, Giblett et al. working with methylcellulose-induced hypersplenism in adult rats studied the red cell survival, the phagocytic mass in the spleen and other indexes and concluded that the anemia was due to a combination of splenomegaly with resultant stasis and red cell destruction; bone marrow inhibition played no identifiable role in the pathogenesis of anemia. These two studies indicate that the mechanism of anemia in methylcellulose-induced hypersplenism in the rat is probably due to both humoral and sequestration factors. The present report describes a group of experiments dealing with the presence in the urine of methylcellulose-hypersplenic rats of a factor(s) capable of inducing anemia and thrombocytopenia when administered intraorally to the normal adult rat.

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Material and Methods

A total of 107 normal young Wistar rats were used in the experiments. The initial weights varied from 100 to 150 Gm. The animals were caged in groups of 10 and fed rat pellets, carrots and water ad libitum. For the experiments the animals were separated in six groups, the treatment and purpose of which appear in table 1.

Induction of Hypersplenism

Following the method of Palmer et al.,\textsuperscript{7} 2.5 Gm. of 400 centipoise methylcellulose were dissolved in 100 ml. of distilled water at 80 C. Group I (42 rats) was injected intraperitoneally with 2 cc. of the methylcellulose solution twice weekly for 14 to 22 weeks. The animals developed splenomegaly which was easily palpable and persisted for 320 days after the administration of methylcellulose was discontinued. These rats were splenectomized 430 days after the beginning of the experiment, 320 days after the last injection of methylcellulose. Throughout the experiment the rats continued to gain weight and appeared normal.

Intragastric Administration of Urine

The urine of Groups I, V and another group of normal rats not included in table 1 was collected by placing the animals in individual metabolic cages and giving them warm water to drink. Urine was collected during the twenty-fifth week of the experiment in Group I, and 4 days after total body radiation in Group V. Enough urine was obtained in 18 hours to secure the amount necessary for oral administration to Groups II, III, IV and VI. Urine was always given undiluted through a tube placed in the stomach in order to insure that no fraction was lost by regurgitation or vomiting. The animals receiving urine gained weight and appeared normal in every respect; this was also true of Group III, whose members showed some changes in red cells and platelets but continued to grow and were otherwise healthy.

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Radiation-Induced Anemia

Group V was given 500 r total body radiation in order to produce anemia by a method different from hypersplenism. Radiation was delivered through a closed cone with filtration of 0.5 Cu and 2 mm. Al (a total filtration equivalent to a HVL of 0.96 mm. Cu) when using 200 K, 20 Ma at a distance of 50 cm; the output at this distance was 38 r per minute (sessions of 14 minutes, 30 seconds). The anemia produced varied in severity and duration, but the urine of this group was collected and used only when the animals had less than 7 million red blood cells per 100 cc; at that time, white blood cells were usually below 3000 and platelets below 50,000 per 100 cc.

Hematologic Studies

Weekly blood counts were made in all animals using tail blood. Results were compared with averages obtained from 217 blood counts of normal rats. In addition, in every animal at least two bone marrow studies were made with a modification of Cameron and Watson's technic. Graphs have been construed by averaging all figures available at one specific date and determining the per cent deviation from the normal average mentioned above. Mortality was low, and there were always more than 5 determinations for each point. Differences in average values were analyzed for statistical significance by means of a t table (p = 0.01).

Morphologic Studies

At the end of the experiments the rats were sacrificed with ether, complete autopsies were performed and blocks removed for histologic study of bone marrow, spleen and liver; microscopic sections were also prepared from the spleens removed in Group I. Slides were stained with hematoxylin and eosin and with the PAS technic.

RESULTS

Group I

The animals in this group developed a thrombocytopenia of 80 per cent of normal values in 15 days, which returned to normal at 30 days and appeared again after this time, maintaining an average level of 70 per cent of normal values throughout the rest of the experiment. This was true also of the 320 days during which no methylcellulose was given to the animals; after splenectomy was performed, thrombocytopenia showed a tendency to disappear, but the animals were sacrificed before the platelets reached normal values. On the other hand, the leukocytes showed an increase that varied from 10 to 30 per cent above normal and remained elevated during the 320 days in which no polymer was given; after splenectomy they showed a tendency to increase. Finally, the red blood cells showed an initial fall which disappeared at 20 days, and from then on variations from the normal values were not statistically significant. (fig. 1).

At the time of splenectomy the average weight of the spleen was 1 600 mg. (1200 to 2200 mg). When this figure is compared with the average weight of the spleen of 127 normal adult rats of the same colony, splenectomized for other purposes, which was 854 mg. (462 to 1033 mg), it can be seen that the increase in weight of the spleen of Group I was 187 per cent. The microscopic aspect of the spleen and of the other tissues examined was similar to that previously described by other authors: the organs with a significant amount of reticuloendothelial cells showed groups of large macrophages with
abundant, finely vacuolated cytoplasm and small, rounded nucleus (fig. 2). The material in the cytoplasm was slightly basophilic and intensely PAS positive.

**Group II**

Every animal in this group received 2 cc. of undiluted urine from Group I through a gastric tube, daily for 4 weeks. The procedure was discontinued for 2 more weeks and repeated during the following 6 weeks. The results were a progressive decrease of the red blood cells which at the end of 30 days were 63 per cent of the normal value, an initial rise and then a fall of leukocytes, and an immediate and marked depression of the platelets which reached 60 per cent of the normal values. During the following two weeks in which no urine was given to these animals, there was a return to normal of the number of red blood cells, a rise of leukocytes and no changes in the thrombocytopenia. When the administration of urine was resumed the results were strikingly negative, since neither the red nor the white blood cells were significantly modified, and the low platelets were not further decreased or changed in any way (fig. 3).

**Group III**

The intragastric administration of 2 cc. of undiluted urine of normal rat, every day for 4 weeks to this group produced a leukocytosis after the second
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Fig. 2.—Photomicrograph of the spleen of methylcellulose-hypersplenic rats. There are many macrophages with foamy cytoplasm ($\times$ 380).

week which went up to 50 per cent above normal values. Red blood cells and platelets were not influenced by the procedure. (fig. 4).

**Group IV**

This group received 2 cc. of undiluted urine of Group I, 12 days after these animals had been splenectomized, daily for 4 weeks. There was a slight elevation of platelets and leukocytes; changes in red blood cells were not statistically significant (fig. 5).

**Group V**

Total body radiation induced anemia, leukopenia and thrombocytopenia of severe degree but variable duration in this group. At times the blood changes persisted for only 7 days before regeneration would tend to improve them, while at other times the blood picture remained unchanged for 20 days or more.

**Group VI**

This group received urine of the animals of Group V according to a schedule similar to that of Groups II, III and IV. The results were similar to those of Group III, i.e., fluctuating leukocytosis without significant variations in red cells or platelets (fig. 6).

The data obtained from differential counts of leukocytes were extremely variable, but in general there was no significant deviation for normal average
Fig. 3.—Effect of intragastric administration of urine of methylcellulose-hypersplenic rats to normal rats. Note that during the second period of administration there is no change in the blood counts.

Fig. 4.—Effect of intragastric administration of urine of normal rats to normal rats.

values. Reticulocyte counts showed them to increase slightly in Group II at the end of the first period of administration of urine of Group I (8 per cent); in all other groups reticulocyte counts were never above 1 per cent. Bone marrow studies revealed normal or slightly hyperplastic pictures regardless of group or time of experiment.
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Fig. 5.—Effect of intragastric administration of urine of methylcellulose-hypersplenic rats 12 days after splenectomy to normal rats.

DISCUSSION

The intraperitoneal administration of methylcellulose to normal rats (Group I) in these experiments resulted in splenomegaly and thrombocytopenia of moderate degree; anemia and/or leukopenia were not observed. This result is at variance with all other published reports on the experimental production of hypersplenism by the intraperitoneal administration of the inert polymer, in which anemia has been a prominent feature. No explanation can be offered for this difference, but it has been confirmed in a new group of rats treated in identical manner in this laboratory as a part of a further study of some of the findings to be discussed presently. Nevertheless, the association of splenomegaly and thrombocytopenia with a normal or hyperplastic bone marrow, and the increase of platelets after splenectomy, provide an adequate experimental model of one type of hypersplenism.

Intragastric administration of undiluted urine of methylcellulose hypersplenic rats to normal rats (Group II) resulted in prompt and marked thrombocytopenia, delayed but definite anemia with reticulocytosis and at first leukocytosis which then returned to normal. That these effects are not due to the urine itself was demonstrated by the absence of such changes when urine of normal rats was given to normal rats (Group III). Furthermore, radiation-anemic rats provided a control for the urine of animals with anemia due to a different mechanism, thus showing that at least in this other type of anemia no factor(s) were eliminated with the urine (Group VI). Therefore, the study of Baldini8 seems to be confirmed by our findings. It should be remembered that this author showed that in methylcellulose-hypersplenism the lactating rat eliminates a factor or factors with the milk which is respon-
sible for the anemia of the young weaning rats. The present study demonstrates that the hypersplenic rat eliminates similar or the same factor or factors with the urine.

The close relation of the spleen to such humoral factors(s) was demonstrated when the methylcellulose-hypersplenic animals were splenectomized and their urine given to a group of normal rats (Group IV). The result was the same as when the urine of normal rats was administered. Therefore, the absence of the enlarged spleen was accompanied by the disappearance of the humoral factors(s) from the urine of previously hypersplenic rats.

An interesting finding was that even when the methylcellulose-hypersplenic rats failed to show anemia of any degree throughout the 420 days of the experiment, their urine was able to produce definite anemia in the normal rats. The fall in the red blood cell count disappeared when the administration of urine was discontinued. The presence of a reticulocytosis of 8 per cent in this group seems to indicate that hemolysis is at least partially responsible for the anemia. However, the bone marrow response was inadequate to compensate for the loss of red cells, and it has been shown that a mild anemia induced by bleeding results in considerable increases in the erythropoietic rate.12

Furthermore, normal rat urine, radiation-anemic rat urine and splenectomized-hypersplenic rat urine failed to induce anemia in the normal rat. On the basis of these facts, it can be speculated that the methylcellulose-hypersplenic rat is producing a factor(s) capable of inhibiting the erythroblastic activity of the bone marrow but in quantities too small to show this effect in its own blood. The concentrating function of the kidney would tend to increase the
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amount of such factor(s), and when the urine is given to a normal rat it would contain sufficient quantities of it to produce anemia.

In spite of the fact that the anemia induced in Group II by the administration of urine of Group I regressed completely two weeks after such treatment was discontinued, the thrombocytopenia remained unmodified. An additional study deals with the effect of splenectomy on such animals, since it has been postulated that the thrombocytopenic factor(s) can induce the normal spleen or other organs to produce the same type of effect on the platelet count and that this effect is spontaneously irreversible. Furthermore, when methylcellulose-hypersplenic rat urine was again given intragastrically to the same Group II for 6 additional weeks, no change was observed in any of the blood elements; i.e., there was no anemia or leukopenia, and the thrombocytopenia was not modified. This could be due to either the development of some form of resistance against the humoral factor(s) or the disappearance of the factor(s) from the urine of Group I rats. The second possibility seems unlikely, since there was no modification in the blood picture of the hypersplenic animals throughout this period. The development of resistance is also under study at the present time.

SUMMARY

A type of experimental hypersplenism characterized by splenomegaly and thrombocytopenia has been produced in the rat by the repeated intraperitoneal injection of methylcellulose. The urine of these animals was collected and given through a gastric tube to another group of normal rats for a period of 4 weeks. The results were a marked and rapidly developing thrombocytopenia, a delayed but definite anemia with mild reticulocytosis and leukocytosis. When the administration of urine was discontinued, the anemia regressed, but the thrombocytopenia persisted unmodified for 2 weeks. When the urine of hypersplenic rats was again given to this group for 6 additional weeks, it failed to induce anemia or to change the persistent thrombocytopenia. Intragastric administration of urine from normal rats, from rats made anemic by total body radiation and from the hypersplenic group after splenectomy to other groups of normal rats, failed to produce the same changes and only induced moderate leukocytosis.

On the basis of these results, it is postulated that in experimental methylcellulose-hypersplenism in the rat there is a humoral factor(s) responsible for the thrombocytopenia, that this humoral factor(s) is eliminated in the urine and that when such urine is given to normal rats, it is responsible for the thrombocytopenia and partially for the anemia that are observed. Such factor(s) are in some important way related to the presence of the spleen.

SUMMARIO IN INTERLINGUA

Un genere de hypersplenismo experimental characterisate per splenomegalia e thrombocytopenia esseva producite in rattos per le repetite injection intraperitoneal de methylcellulosa. Le urina de iste animales esseva colligite e administrate via un tubo gastric a un altre gruppo de rattos normal durante un periodo de 4 septimanas. Le resultatos esseva un marcate e rapidemente
progressive thrombocytopenia, un tardive sed definite anemia con leve grados de reticulocytosis e leucocytosis. Quando le administration del urina esseva suspendite, le anemia regredieva, sed le thrombocytopenia persisteva sin modification durante 2 septimanas. Quando le urina ab rattos con hypersplenismo esseva de novo administrate a iste gruppo de animales durante un periodo de 6 septimanas additional, illo non induceva anemia e non alterava le persistente thrombocytopenia. Le administration intragastric de urina ab rattos normal, ab rattos rendite anemic per irradiation del corpore total, e ab animales in le gruppo hypersplenic post que illos habeva essite splenectomisate non produceva—in altere gruppus de rattos normal—ulle del supra-mentionate alterationes con le exception de leve grados de leucocytosis.

REFERENCES

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