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ERYTHROCYTIC INCLUSIONS IN CLINICAL AND EXPERIMENTAL TOXICOLOGY

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The author reviews the results of his studies of erythrocytic inclusions carried out at the Institute in the last twenty years. The importance of these inclusions and their role in clinical and experimental toxicology are pointed out. This year-long work has embraced principal non-parasitic inclusions, i. e. the structures within erythrocytes and their precursors cells that are morphological manifestations of intracellular biochemical processes: stippled cells, Heinz bodies, Schmauch bodies, and siderotic granules of Grüneberg and Pappenheimer. Evidence has been obtained of the myelogenic origin of stippled cells, of a morphologic resemblance and biological difference between Heinz and Schmauch bodies, and of the existence of the two variants of siderotic granulations.

Nearly twenty years have passed since the American biochemist *Granick,*(1) describing the chemistry and functioning of the mammalian erythrocyte, said that the red blood cell is a reagent of great sensitivity . . . Just as many years have elapsed since in this Institute we started studying various changes in erythrocytes. In the course of these 20 years it has become very clear that this apparently homogenous peripheral blood cell, without any perceptible structure and »almost lifeless«(2) but of an immense regenerative potential, may indeed be »a reagent of great sensitivity« on which to rely in the early detection of various chemical noxae

The erythrocyte can be studied by morphological and biochemical methods. However, these two approaches are no longer looked upon separately as it used to be in the past, because it has in the meantime become clear that morphological characteristics are but the manifestations of chemical processes and that some biochemical phenomena have become approachable and apprehensible just because of their morphological manifestations.

Non-parasitic cytoplasmatic structures appearing as morphological phenomena of disturbed biochemical processes in the erythrocyte are called by a common name erythrocyte inclusions. They comprise stippled cells, Heinz bodies, Schmauch bodies, and Gruneberg's and Pappenheimer's siderotic granules. The task we imposed on ourselves 20 years ago was to study erythrocytic inclusions as the morphological manifestations of

biochemical processes produced by the effect of various exogenous poisons. However, as erythrocytic inclusions are not only the result of the exogenous harmful agent but also a frequent concomitant of endogenous disturbances, their biological characteristics were to be included in our studies as well. Moreover, thought was to have been given also to the differential characteristics of erythrocytic inclusions, because some of them are very often mistaken for one another, especially when appearing concurrently.

The objective of this review is to set forth some of the results of our studies on the biological and differential characteristics of stippled cells. Heinz bodies, Schmauch bodies, Gruneberg's siderotic granules, and Pappenheimer's siderocytes.

1. STIPPLED CELLS

As the appearance of stippled cells is in the closest connection with the toxic effect of the poison, it was justifiable to start studying this kind of erythrocytic inclusions first. Although some humoral biochemical changes, owing to their specificity, have in the meantime become a more reliable symptom of poisoning, the simple testing of the presence of stippled cells has practically remained the most important diagnostic means.

At the beginning of the Institute's work opinions in the world on the origin of stippled cells were still divided. The occupational medicine of the time was still under the influence of the classic monograph on lead poisoning by *Aub, Fairhall, Minot,* and *Reznikoff* from 1926(3) who said that lead affects young erythrocytes in the peripheral circulation by coagulating their basophilic substance which gives it a granular appearance. *Hunter*(4), in 1944, still keeps up this opinion, while *Lloyd Davies,* in 1948, in his book »The Practice of Industrial Medicine«(5), states that lead has no effect on the bone marrow. On the other hand, even far earlier than that some basic hematological investigations had suggested a myelogenic origin of the stippled cell phenomenon. Our first task at that time was to bring into harmony the results of basic, mostly experimental hematological studies with the necessary clinical investigations in occupational medicine. Our first experience was published in 1958(6) when we pointed to our observation that in clinical lead poisoning a high percentage of normoblasts in the bone marrow contain stippled cells. Later(7) we have found that in a great number of exposed workers, not only in the poisoned ones, there are always erythroblasts with stippled cells in the bone marrow, their number depending, as a rule, on the degree of exposure. These own observations have provided an unquestionable evidence of the myelogenic origin of stippled cells. Furthermore, by continuing the study of the morphological characteristics of the bone marrow in lead poisoning we have found(8) not only a large number of punctated erythroblasts but also a pronounced hyperplasia of the erythro-

poietic bone marrow cells. The problems of qualitative changes in erythroblasts have specially attracted our attention. What was to be examined first was to find out if punctations in the specimens of the bone marrow of lead-poisoned persons are of cytoplasmatic or cariogenic origin, the latter possibility being advocated by the majority of older hematologists who thought that basophilic punctate were remains of the fragmentated erythroblast nucleus (9, 10, 11); they supported their opinion by the fact that erythroblasts and erythroblasts with the fragmentated (karyorrectic) nucleus simultaneously increase or decrease in number. Although this fact is beyond dispute, there were some authors that from the very beginning supported the old opinion of *Ehrlich's* and *Askanazy's* on the cytoplasmatic origin of stippled cells. Our observations in humans have justified the doubt expressed by *Stengel, White,* and *Pepper* (12) that the nucleus could simultaneously be in a state of both active mitosis and karyorrhexis. We have found stippled cells not only in orthochromatic normoblasts in the wholly intact nucleus but also in young polychromatic cells, and exactly in various stages of mitosis at that (6, 8). Moreover, we were able to show by microphotography that a careful analysis of the bone marrow film in lead poisoning reveals all transition stages from the basket-like and net-like cytoplasm to stippled cells (8). Cytochemical investigations have finally provided a definite evidence that stippled cells yield a negative Feulgen nuclear reaction, i. e. that they do not consist of desoxyribonucleic acid but that their affinity to basic dyes is due to the presence of ribonucleic acid and that they are an ingredient of the cytoplasma. *Fano* (13) has shown that stippled cells are brought about by the vital aggregation of ribonucleic acid round mitochondria which exactly in lead poisoning persist in erythrocytes but otherwise disappear from them (1). After all, most recent investigations by *Jensen, Moreno,* and *Nessis* (14) have also given evidence of stippled cells being a morphological variation of abnormal ribosomes.

2. HEINZ BODIES

The knowledge of Heinz bodies goes back as far as 1890, but only in the last three decades, thanks to *Moeschlin* (15), have hematologists and toxicologists been attracted by these formations. As a matter of fact, their discovery is historically connected with toxicological studies, occurring at the time when occupational and therapeutical contact with a series of chemical compounds producing these structures was becoming ever more frequent. Moreover, practice started in toxicological investigations to use the phenomenon of Heinz bodies as a test of toxic action not only in experimental studies in vivo but also in in vitro. This latter fact opened wide opportunities for experimenting with erythrocytes in vitro. After all, the possibility of such experimentation greatly facilitates also the morphological study of the structures produced. Carrying out such investigations in vitro we reported as early as 1951 (16) that the number

and size of Heinz bodies markedly depended on the chemical structure and concentration of the poison. By increasing, for instance, the concentration of phenylhydrasine we obtained ever more minute and ever more numerous structures, whereas by increasing the concentration of hydroxylamine ever larger structures were obtained. Phenyldiamine, however, in both low and high concentrations, always produced structures resembling those produced by hydroxylamine. By using human erythrocytes we repeated the experiments made by *Bratley, Burroughs, Hamilton,* and *Kern* with canine erythrocytes (17), producing Heinz bodies in the hanging drop and describing the spontaneous release of these structures from the erythrocyte. Somewhat earlier the morphological variations of Heinz bodies were known only from *Jung's* reports (18) who had studied them in great detail by means of electron microscopy. Observing morphological processes *Jung* concluded that Heinz bodies could not be some uniform structures, because they vary in their form and location a great deal, according to the kind and duration of poisoning. For this reason *Jung* avoided calling them »Heinz bodies« because sometimes they are no distinct, compact, solitary, or multiple »bodies« at all but only some minute, irregular granules, often visible only by electron microscope. Within the scope of optic microscopy our observations have proved in agreement with the electron microscope findings. Most recently *Borges* and *Desforges* (19) have ascribed variations in the size of Heinz bodies to a different speed rate of their production and different stages of their development.

Individual differences in the appearance of Heinz bodies in humans have been reported both in clinical poisoning and when studying the effect of the same poison in vitro. Attention to this fact was drawn by *Markoff* in 1943 (20) and *Reiner* in 1950 (21). Somewhat later (1956) we, too, reported on a clinical observation in which the same exposure to m-dinitrobenzene produced numerous Heinz bodies in one person and none in another, in spite of the fact that both persons proved to have pronounced methemoglobinemia. The fact that in some persons the same poisons may and in others may not produce Heinz bodies has been used by *Hochwald, Arnold, Clayman,* and *Alving* (23), *Dern, Weinstein, LeRoy, Talmage,* and *Alving* (24), and *Beutler, Dern,* and *Alving* (25) for the development of a simple laboratory test by which in vitro, by means of acethylphenylhydrazine, it is possible to predict whether a person will develop Heinz bodies and hemolytic anemia if coming into contact with a chemical compound that oxidizes and precipitates erythrocyte proteins.

3. SCHMAUCH BODIES

The experimental production of Heinz bodies depends on the species of animals. Many authors speak about the cat as the most sensitive in this respect. There are, however, very few authors who take into account the fact that in feline erythrocytes there is also the physiological presence of the structures that appear fully to correspond to Heinz bodies and

which in veterinary literature are called Schmauch bodies after the German pathologist *Schmauch* who in 1899 was the first to describe them in great detail (26). With regard to this spontaneous appearance of Schmauch bodies and the enormous interest arisen in Heinz bodies, one would expect that Schmauch bodies have been the subject of most detailed studies, the more so because recently ever more frequent cases of the spontaneous formation of Heinz bodies also in humans have been reported. However, surprisingly enough, there exist no published data on the comparative studies of Heinz and Schmauch bodies, and this is why in the last decade we have paid special attention to Schmauch bodies.

Since the time of the discovery Schmauch bodies have been mentioned in the world literature under this name or the name of Heinz bodies only in 14 papers, while the number of the cats examined has been put down only in 5 papers. However, it is only in one (unpublished at that) thesis that the number of the cats examined was more or less adequate (22 animals). *Schmauch* himself examined no more than 15 cats. We have, therefore, by a long, persistent random collection of cats, tried to examine the incidence of these formations in as many animals as possible. We have succeeded in examining 94 cats. Their age was determined in 63 cases and ranged from 2 days to 12 years.

We have studied the morphology of Schmauch bodies by phase contrast microscopy and supravital staining. It has been shown (27) that Schmauch bodies, just as well as Heinz bodies, are visible in the native, non-stained preparation. The affected erythrocyte proved to contain one round and rarely two or more irregular granules. By phase contrast microscopy it was possible to follow changes leading to the release of Schmauch bodies. These changes fully correspond to those described in the release of Heinz bodies induced by phenylhydrazine in human erythrocytes in vitro (16) or to changes described by *Bratley* and co-workers (17) in the formation of Heinz bodies in pyrodine-poisoned dogs. The technique of the native presentation of Schmauch bodies in phase contrast microscopy appears to have been used for the first time in such studies at this Institute. It is exactly toxicological studies where this technique is of particular value, because it excludes any possibility of supravital stains affecting the formation of Schmauch bodies but, at the same time, enables the erythrocyte to remain in the plasma. How important it is has been shown by *Webster, Liljegren,* and *Zimmer* (28) who very strongly pointed out the difficulty of finding a solution that would keep the erythrocytes of various species morphologically unchanged during supravital treatment. On the other hand, since the time of *Gutstein* and *Walbach'* studies (29) it has already been known that even Nile blue sulphate by itself may induce the formation of Heinz bodies. The technique of phase contrast microscopy has the advantage of a direct, adequate visualization of native erythrocytes floating in their natural environment.

Like Heinz bodies, Schmauch bodies stain supravitally with both methyl violet and Nile blue sulphate, but they become particularly apparent by their intense basophilia when stained with Nile blue sulphate. By

comparing the finding of Schmauch bodies in phase contrast microscopy with that in supravital staining, it can be seen that native inclusions in phase contrast microscopy are less strictly limited to the peripheral localization in the erythrocyte than is the case in the stained film. Besides, in the native preparation these inclusions appear to be smaller than they are in the stained one.

The incidence of Schmauch bodies in feline erythrocytes does not depend on the age of animals. We have proved it by the Kruskal-Wallis test (27), trying to find a correlation between incidence and age in 63 animals. In the literature, however, there are statements that old cats have Schmauch bodies. According to all our findings, it is obvious that Schmauch's spontaneous erythrocytic inclusions are morphologically identical with the Heinz inclusions induced by the effect of some poisons.

There remains, however, an essential, extremely important biological difference. It is a well-known fact that in humans the immediate result of the appearance of Heinz bodies is hemolytic anemia. In cats, however, we have shown (30) that even a high incidence of Schmauch bodies is not accompanied by the fall of erythrocytes. Moreover, when the cats already having Schmauch bodies in their erythocytes are poisoned by hydroxylamine and when in these cats Heinz bodies are also induced in this way, only half the animals develop hemolytic anemia, while all of them, even without any treatment, survive and do not succumb to the dose of the poison which otherwise, calculated per kilo body weight, would be highly lethal for man. In this way we have succeeded in proving a paradoxical resistance of feline erythrocytes to the Heinz bodies producing poisons.

Assuming that »credit« for this unexpected resistance of feline erythrocytes is to be given to cellular reduction mechanisms, we started, a few years ago, with the determination of glucose-6-phosphate dehydrogenase and catalase in feline erythrocytes (31, 32). With regard to the easy formation of Schmauch and even Heinz bodies we had expected to find decreased activities of G-6-PD in feline erythrocytes as compared with the activity of this enzyme in human erythrocytes. However, the finding was diametrically opposed: the activity of G-6-PD was increased and the catalase value normal or slightly increased. Of what importance is the increased activity of these enzymes, responsible for the anatomical and functional integrity of the erythrocyte, in the prevention of a quick, serious haemolysis in poisoned and non-poisoned cats remains to be elucidated.

4. SIDEROTIC GRANULATIONS OF GRÜNEBERG AND PAPPENHEIMER

Abnormal accumulation of iron in the mitochondria of erythroblasts, and sometimes also in the pathologically retarded mitochondria of erythrocytes, can be observed in thalassemia major, refractory »sideroblastic« anemia, congenital »sideroachrestic« anemia, in anemia due to pyrodixine deficiency (B_6 vitamin), and in a form of porphyria called por-

phyria cutanea tarda. However, it is interesting that also some chemical agents may produce the accumulation of iron in mitochondria. In view of this as early as 1954, we started studying this kind of erythrocyte inclusions.

The best known chemical noxa producing the accumation of iron in mitochondria is lead: it leads to the inhibition of hemoglobin biosynthesis at several levels. At a certain level, owing to the inhibition of haemsynthetase or the Goldberg enzyme, iron does not build in. A similar cause of iron accumulation is the effect of isoniazide hydrazide (INH). As is well known, pyridoxine – in the form of pyridoxal-5-phosphate – is an important enzyme helping the binding of glycine and succinyl-coenzyme A at the very beginning of hemoglobin synthesis. The lack of pyridoxine may, therefore, prevent the synthesis of porphyrin and, consequently, the synthesis of hemoglobin as well. Isoniazide hydrazine, as an antagonist of pyridoxal-5-phosphate, may lead to disturbances in the hemoglobin synthesis up to the time of the incorporation of iron into the porphyrin chain. Finally, as most recent literature data have shown, chloramphenicol – in a manner unexplained so far – also blocks the synthesis of hemoglobin, producing iron accumulation, which means the formation of siderotic granules.

Our interest in this kind of inclusions was, of course, primarily stimulated by the studies of the effect of lead, all the more so because one of authors (33, 34) emphasized the property of siderotic inclusions to stain not only with the Prussian blue reaction but also with Romanowsky dyes. This has led to much confusion and misunderstanding, even to the identification of stippled cells and siderotic granules (35). The relationship between siderotic granules and stippled cells has for a long time remained vague, mostly owing to a frequent co-existence of these two kinds of inclusions even in one and the same erythrocyte. Although as early as 1943 *Doniach, Grüneberg,* and *Pearson* (36) were certain that »granules of siderocytes are in no connection with basophilic stippling, later this fact seems to have been entirely ignored. Only in the studies carried out in this Institute (37) has a substantial body of evidence been obtained that these are entirely different intracellular structures, differing between themselves not only cytochemically but also in their localization in the cell: the simultaneous presentation of phase contrast stained granules and differential focusing coupled with photographs of the so-called optic sections have revealed (38) that even the level at which stippled cells and siderotic granules are localized in the cell is different. It is just this different localization observed in our studies that *Jensen, Moreno,* and *Bessis* (14) refer to, considering our work the corroboration of their results obtained by electron microscopy.

In the course of this work we have, however, succeeded in providing evidence not only that stippled cells and siderotic granules are two different kinds of inclusions but also that there exist two variants of siderotic granules. When *Grüneberg* in 1941 discovered »erythrocytes which contain a considerable amount of free iron«, he named them »sidero-

cytes«. A few years later *Pappenheimer, Thompson, Parker,* and *Smith* (39) described »erythrocytes which contain iron reacting bodies«. Since then red blood cells with stainable iron have been studied extensively but the fact seems to have been overlooked that the iron positive granules of Gruneberg's siderocytes are not identical with Pappenheimer's bodies although the latter, too, produce a positive reaction to iron. By carefully collecting and studying literature data on these two kinds of sidero--positive inclusions we have been able to discern as many as five different features of these inclusions: (1) While the siderotic granules of Grüneberg's siderocytes »are made visible *only* by means of Prussian blue reaction« (40, 41), the inclusion bodies of Pappenheimer and his collaborators are both *basophilic* and *siderotic*, i. e. they can be stained both with one of Romanowsky dyes and by means of Prussian blue reaction: (2) In unstained blood films Pappenheimer's inclusions are »readily seen as refractile and colourless structures« (34), while the presence of free iron granules in Grüneberg's siderocytes is not recognizable in unstained blood films (41); (3) All investigators agree that the siderotic granules of Grüneberg (40, 41) may always be revealed in the red cell precursors (»sideroblasts«, 42) even when not present in the mature red cells of the peripheral blood. In contrast, Pappenheimer's bodies stained with Romanowsky dyes have rarely, if ever, been demonstrated in the red cell precursors even when present in a great percentage of mature red cells in the peripheral blood; (4) There is no doubt that the free iron granules od Grüneberg as seen in »sideroblasts« and also in reticulocytes are subsequently incorporated into the haem (43, 44) »during the final ripening of the reticulocyte« (43), but it is highly improbable that Pappenheimer's siderotic granules could be used for haem synthesis. Accordingly, the free iron granules can be revealed in reticuloytes, but Pappenheimer's bodies seem to have never been observed in reticulocytes; (5) While siderotic granules of Grüneberg are found to occur in erythroblasts and erythrocytes regardless of the subject's possession of the spleen, Pappenheimer's bodies seem to have so far been reported mostly, if not only, in splenectomised patients.

In spite of these obvious differences the term »siderocyte« has by common usage become a blanket designation, applied to any iron-containing red blood cell, irrespective of its colour reaction in a Romanowsky-stained film. Recognition that there are two kinds of iron-containing erythrocytes has thus been neglected for many years.

The results of our year-long studies have shown that there are indeed two kinds of »siderocytes«. Since the decisive difference between them is the presence or absence of the *basophilia* of their granulation, there should be distinguished two variants of siderocytes: (1) siderocytes with basophilic sidero-positive granules and (2) siderocytes with non-basophilic sidero-positive granules. As long as their composition and significance is not fully elucidated, these two variants of siderocytes might, at least by their eponyms, be distinguished as Grüneberg's siderocytes and Pappenheimer's siderocytes. Or Grüneberg's initial definition of

siderocytes should be modified by adding that siderocytes are all those erythrocytes in which Prussian blue reaction reveals iron in whatever form it may be, regardless of whether the structures observed can or cannot be seen by another staining method (44). But even if defining siderocytes in this way, we consider it necessary to underline the principal difference between Grüneberg's and Pappenheimer's variants: Grüneberg's siderocytes may be found, though extremely rarely, also in healthy persons, whereas Pappenheimer's siderocytes appear to have never been reported in a healthy person.

CONCLUSION

In this review it was aimed at pointing out the share of our own investigations in the elucidation of the problem of erythrocytic inclusions. Wherever the methods of a simple conventional hematological approach have allowed it, efforts have been made to bring into concord the results of basic biochemical investigations and the results of our morphologico-hematological studies. In this endeavours we have succeded in providing evidence for some not yet reported phenomena, especially as regards qualitative changes in the bone marrow in lead poisoning, in the formation of Heinz bodies induced by the effect of various poisons, and in the attempt of their identification with Schmauch bodies in cats. Cytochemical investigations of siderotic inclusions, on the other hand, have resulted in the recognition of the two variants of these inclusions and their differentiation in relation to other erythrocytic inclusions.

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