SULFATED MUCOPOLYSACCHARIDE AND BASIC PROTEIN IN CERTAIN GRANULES OF CIRCULATING HETEROPHILS OF RABBITS DURING ENDOTOXIN-INDUCED LEUKOCYTOSIS

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The mechanism by which two appropriately spaced injections of bacterial endotoxin produce the fibrinoid glomerular occlusions of the generalized Shwartzman reaction has not been completely elucidated. Following their observations that nitrogen mustard-induced leukopenia inhibits the generalized Shwartzman reaction and that dextran sulfate and similar acid polymers "prepare" a rabbit for provocation of the reaction by a single injection of endotoxin, even in association with leukopenia, Thomas and colleagues [1,2] constructed an attractive hypothesis to account for this interesting pathologic reaction. They speculated that the necessary role of the leukocyte might be to make available to the test animal a substance with the properties of a large molecular weight acid polymer. The concurrence of this hypothetical substance, produced by the first injection of endotoxin, and heparin-precipitable fibrinogen, produced by the second endotoxin injection, might lead to precipitation of the fibrinoid occlusions, presumably as an acid polymer-fibrinogen complex. While this hypothesis was consistent with a variety of experimental observations, the origin of the tentative physiologic acid polymer was not accounted for, nor was the mechanism by which leukocytes made such a substance available. Biochemical analysis of leukocytes indicates that such cells may contain an acid mucopolysaccharide,[3-5] and Hedenius [8] has suggested that the isolated acid polysaccharide was derived from metachromatically staining granules.

In preliminary histochemical studies of leukocyte alterations accompanying the production of the generalized Shwartzman reaction, prominent granules with histochemical features of sulfated mucopolysaccharides were noted in circulating heterophils during the leukocytosis which follows injection of bacterial endotoxin. Significantly, these granules were similar histochemically to azurophil granules which are a normal constituent of immature granulocytes of the bone marrow. Sub-

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sequently, histochemical and autoradiographic techniques demonstrated that these azurophil granules of rabbit bone marrow contain a sulfated acid mucopolysaccharide together with a strongly basic protein.[6,7] This paper will report the occurrence of these constituents in circulating heterophils during the leukocytosis which follows the administration of bacterial endotoxin.

Material and Methods

NIH stock albino rabbits of both sexes, weighing approximately 1,000 gm., were used. The endotoxin (Difco *Salmonella enteritidis* lipopolysaccharide, control #123497) was suspended in normal saline at a concentration of 100 μg. per ml. shortly before its injection into the marginal ear vein. The generalized Shwartzman reaction was produced in approximately ⅔ of animals receiving 2 such injections at an 18 to 21 hour interval. Animals were sacrificed 12 or 24 hours following the second endotoxin injection, and gross evidence of cortical necrosis was taken as an indication of a positive reaction.

Blood smears were prepared on glass slides directly from a small puncture of the marginal ear vein. Smears were dried for several minutes and fixed by immersion for 1 to 2 hours in 95 per cent ethanol. White cell counts were performed by conventional methods.

Paraffin sections of kidney tissue, fixed 18 to 24 hours in 10 per cent formalin, 2 per cent calcium acetate solution, and bone marrow, fixed overnight in sodium acetate-mercuric chloride-formalin ("B-5"), were prepared in selected animals.

Histochemical procedures, detailed elsewhere,[7] included Gomori's aldehyde fuchsin, the low iron-diamine method, alcian blue, the Hale-Rinehart colloidal iron method, alcoholic PAS, azure A at graded acid pH levels, and Biebrich scarlet at varied alkaline pH values. A modification of the iron-diamine method used previously, designated as the high iron-diamine technique, was employed as a specific method for sulfated mucopolysaccharides. This procedure involved overnight staining in a mixture prepared by the sequential addition of 120 mg. of N,N-dimethyl-*meta*-phenylenediamine $(HCl)_2$, 20 mg. of the *para* isomer, 50 cc. of distilled water, and, finally, 1.4 cc. of official N.F. ferric chloride solution (containing 10 per cent iron). Nitrosation and acetylation (6 hours, 60° C.) procedures were performed as described by Lillie,[8] and fast green FCF staining as noted elsewhere.[9]

S^{35}-sulfate, obtained from Oak Ridge National Laboratories, was diluted to a convenient volume in a physiologic salt solution (1 mc. in 1 or 2 cc.). Animals received 1 mc. per injection intraperitoneally, according to the schedules given with the results.

Autoradiographs of blood smears were prepared with Kodak AR-10 stripping film and processed as described previously.[7] Staining of smears with aldehyde fuchsin prior to the application of stripping film allowed a direct comparison of localization of acid mucopolysaccharide and incorporated isotope.

Results

Changes in Heterophil Count and Granule Content After Endotoxin Injections

The typical biphasic response of the circulating leukocyte count following each of sequential endotoxin injections has been observed repeatedly in this laboratory. The magnitude of the post-endotoxin heterophilia in a typical experiment is indicated in Table I.

Cytologic study of circulating heterophils obtained during the periods

of leukocytosis after each of 2 endotoxin injections revealed striking alterations in the constituent granules of these cells. In Giemsa-stained smears numerous metachromatically-staining red-purple cytoplasmic granules were observed in many of the heterophils (Fig. 7). A similar distribution of such granules was noted following staining with 0.02 per

TABLE I

HETEROPHIL COUNTS IN 6 RABBITS BEFORE AND DURING PRODUCTION OF THE GENERALIZED SHWARTZMAN REACTION

Animal no.	Before endotoxin	12 hr. after first endotoxin	12 hr. after second endotoxin	Generalized Shwartzman reaction
1	2150	7200	9050	+
2	1475	14800	6500	+
3	2100	17100	7450	0
4	1725	13600	5450	+
5	2175	7900	8575	+
6	2950	8500	4800	+

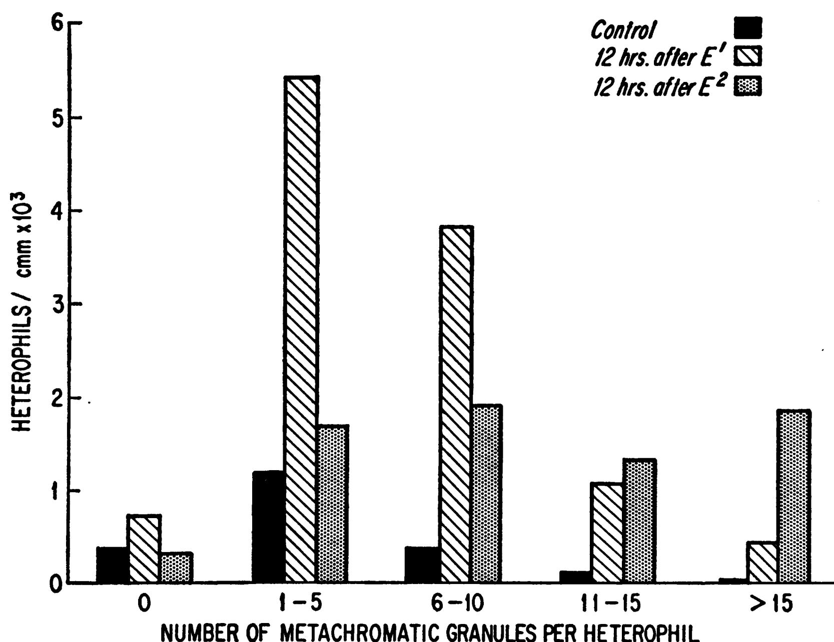
cent azure A at pH 5.0 (Figs. 3 to 6), and with other staining methods described below which are more specific for acid mucopolysaccharides. In normal animals, without leukocytosis, some of the circulating heterophils contained relatively few similarly staining granules (Figs. 1 and 2). However, during an endotoxin-stimulated heterophilia, both the percentage of cells containing the metachromatic granules and the number of granules per cell appeared greatly increased.

To quantitate the metachromatic granules of circulating heterophils during production of the generalized Shwartzman reaction, white blood cell counts and smears of blood were made on 6 rabbits prior to endotoxin administration and 12 hours after both the first and second injections of endotoxin. Differential counts of blood smears, stained with azure A at pH 5.0, were made, and the heterophils were categorized in 5 groups according to their content of distinctly metachromatic granules: 0, 1 to 5, 6 to 10, 11 to 15, or 16 or more granules. As presented graphically in Text-figure 1, following one endotoxin injection the number of cells containing moderate numbers of metachromatic granules increased greatly. Following a second such injection, given 21 hours after the first, the number of cells containing very numerous metachromatic granules was substantially increased, and there were fewer cells with moderate numbers of such granules.

The more heavily granulated heterophils appearing after endotoxin were usually larger than the sparsely granulated or ungranulated cells seen normally (compare Figs. 1 to 10), suggesting, as did the mere presence of numerous azurophil granules, that the cells with metachromatic

granules might be relatively immature. However, the great majority of these hypergranulated cells, in spite of their relatively large size, showed nuclear segmentation or deep nuclear folding (Fig. 7), and very few circulating myelocytes were evident.

In this experiment 5 of the 6 animals showed typical gross pathologic findings of the generalized Shwartzman reaction. No obvious correlation was found between the presence of or degree of cortical necrosis and the heterophil response in this experiment (Table I) or any of several similar experiments. The presence of marked leukocytosis following the second endotoxin injection in the minority of animals which did not develop renal necrosis indicated that the heterophilia was not necessarily a result of the renal injury.



TEXT-FIG. 1. Distribution of heterophils according to number of metachromatic granules present normally and 12 hours after each of sequential endotoxin injections (E^1 and E^2). Means of 6 rabbits.

Histochemical Observations

Several histochemical methods previously used to establish that azurophil granules of normal bone marrow contained a sulfated mucopolysaccharide and a basic protein were applied to smears of peripheral blood obtained from normal rabbits and rabbits with an endotoxin-induced leukocytosis. Those granules of circulating heterophils de-

scribed above which stained metachromatically with Giemsa or with azure A at pH 5.0 showed histochemical characteristics identical with the azurophil granules which are abundant in myelocytes of normal bone marrow. Granules in both myelocytes and post-endotoxin circulating heterophils stained metachromatically with dilute solutions of azure A at pH 4.5 and above (Figs. 3 to 6), but they were unstained at a lower pH. They showed strong affinity for aldehyde fuchsin (Fig. 8), a stain which, in unoxidized sections, is largely specific for acid polysaccharides, particularly sulfated mucosubstances.[10] The low iron-diamine method, which stains both sialo- and sulfo-mucins, and the high iron-diamine method, which is largely selective for sulfated mucins,[11] colored these granules (Fig. 9). These methods also stained the granules of basophil leukocytes (Fig. 9), structures which are known to contain a strongly sulfated polysaccharide.[7,12] However, heterophils and basophils were easily differentiated on a morphologic basis. The granules, in both marrow cells and post-endotoxin circulating cells showed affinity for alcian blue, another method which stains many acid mucopolysaccharides. The Hale-Rinehart colloidal iron method for acid polysaccharides has not proved effective in staining either the azurophil granules of bone marrow or the metachromatic granules of circulating heterophils.

The presence of abundant glycogen in circulating heterophils following endotoxin administration obscured any possible PAS-reactivity of the granules unless the smears were pre-incubated with diastase. Digestion with diastase for 5 minutes removed glycogen from heterophils on ethanol-fixed blood smears as from similarly-fixed paraffin sections of liver. Azurophil granules in normal bone marrow could not be demonstrated by the aqueous PAS method, apparently because of their solubility during periodate oxidation, but they could be visualized when the periodate oxidation step of the PAS procedure was performed in 90 per cent ethanol.[7] Similarly, the metachromatic granules in post-endotoxin circulating heterophils were unstained by the conventional PAS technique, but the alcoholic PAS method, applied to diastase-treated smears, revealed weak staining of granules in the distribution of those showing other histochemical characteristics of acid mucopolysaccharide.

Since azurophil granules in bone marrow stain with the acid dye, Biebrich scarlet, at highly alkaline pH, indicating the presence of a strongly basic protein,[13] this technique was applied to blood smears and to paraffin sections of formalin-fixed kidneys of rabbits with heavy polymorphonuclear infiltrate secondary to the generalized Shwartzman reaction. Again, the pH 9.5 Biebrich scarlet method demonstrated cytoplasmic granules with the same distribution as the acid mucopolysaccharide-containing granules. Relatively few were seen in normally

circulating heterophils, but following endotoxin administration greatly increased numbers were demonstrable in smears of peripheral blood (Fig. 10), and the leukocytes in kidneys showing the generalized Shwartzman reaction contained abundant Biebrich scarlet-positive granules (Figs. 15 and 16). It was especially clear in formalin-fixed tissue that the affinity of the leukocyte granules for Biebrich scarlet was present even at a pH as high as 10.5. This high extinction value for the acidophilia is characteristic of arginine-rich as distinguished from lysine-rich proteins.[9] Fast green FCF also visualized the granules in formalin-fixed tissue, another feature typical of proteins high in arginine content.[9,14]

The acidophilia of the granules was not impaired by 24-hour formalin fixation or 4-hour nitrosation, procedures which block the ε-amino groups of lysine and hydroxylysine without reacting with the basic guanidino group of arginine.[8,9] A 6-hour acetylation at 60° C., a technique which inhibits staining of arginine-rich protein,[8] abolished acidophilia of the granules. The affinity for Biebrich scarlet at high pH levels and for fast green FCF, and the results of these blocking reactions indicated that the acidophilia of these granules was probably due to a protein with a high arginine content.

Autoradiography

The histochemical properties of metachromasia, affinity for aldehyde fuchsin, iron-diamine, and alcian blue, and weak PAS-reactivity indicated that the acidic moiety in the granules in question was an acid mucopolysaccharide, and staining with the high iron-diamine method further suggested that the polysaccharide was sulfated. Therefore, additional experiments were performed to determine if circulating heterophils containing such granules incorporated S^{35}-sulfate, as would be expected if the constituent mucopolysaccharide were sulfated.[15]

Preliminary experiments indicated that 3 daily injections of 1 mc. of S^{35}-sulfate produced an apparently homogeneous labeling of azurophil granules in myeloid elements of normal rabbits. Following such a regimen of isotope administration, smears of peripheral blood in normal rabbits were prepared 1, 2 and 3 days after the last dose of isotope. Autoradiographs of smears taken on the second and the third days following the last injection of isotope revealed scanty but definite labeling of the heterophils which contained aldehyde fuchsin-reactive granules, while those lacking such granules appeared unlabeled. Two rabbits received endotoxin 1 day after, and 3 rabbits received it 2 days after the last isotope injection. During the leukocytosis, 12 hours after endotoxin, smears of peripheral blood were made and autoradiographs pre-

pared. Each of the 5 endotoxin-treated animals showed the anticipated heterophilia and marked increase in the number of acid mucopolysaccharide-containing granules, and the heterophils were correspondingly heavily labeled (Fig. 14). Thus it appeared that under these conditions S^{35}-sulfate was present in heterophils which contained granules with the histochemical features of sulfated mucopolysaccharide and, further, the amount of isotope corresponded to the degree of such granulation in both normal and endotoxin-treated rabbits.

In these experiments basophil leukocytes were heavily labeled in both normal and treated animals; this result was anticipated from abundant histochemical evidence that granules of basophils contain a heavily sulfated, heparin-like mucopolysaccharide.[7,12] Labeling of platelet clumps was also noted, confirming the prior observation in other species that platelets incorporate S^{35}-sulfate.[16] No evidence for labeling of circulating lymphocytes, monocytes or eosinophils was obtained, although the possibility of light uptake could not be excluded. Parenthetically it may be noted that immature eosinophil myelocytes in the marrow incorporate this isotope in morphologically distinct granules thought to be precursors of the specific granules of mature eosinophils.[7]

S^{35}-sulfate localization in heterophils following the second (provocative) dose of endotoxin was demonstrable on administration of a single 1 mc. tracer dose simultaneously with the first (preparative) injection of endotoxin. Under these conditions there was little S^{35} present in the heterophils during the primary leukocytosis; this suggested, as did the timing of isotope administration in the previous experiment, that the leukocytes released from the marrow by the first endotoxin injection contained sulfated polysaccharide synthesized prior to that endotoxin injection and that the cells which contributed to the primary leukocytosis did not synthesize significant amounts of sulfated polysaccharide subsequent to the time of endotoxin administration. Six and 12 hours following the second endotoxin injection, however, the circulating heterophils with acid polysaccharide-containing granules were heavily labeled (Figs. 11 to 13), indicating that a substantial proportion of the sulfated polysaccharide of these cells was synthesized after the first injection of endotoxin. Sections of bone marrow from 6 rabbits, taken 18 to 24 hours following a single injection of endotoxin, revealed myeloid hyperplasia. Accelerated proliferation of myeloid tissue during this interval following endotoxin injection presumably leads to heightened synthesis of sulfated mucopolysaccharide and greater uptake of available S^{35}-sulfate. It is noteworthy that, under the influence of sequential endotoxin injections, the interval from the time of isotope administration to appearance of circulating labeled heterophils was 28 hours or

less, but in the normal rabbit the interval is probably between 3 and 4 days.[7]

Discussion

Application of several histochemical methods to smears of peripheral blood indicated that certain distinctive cytoplasmic granules in heterophils contained a sulfated mucopolysaccharide and a strongly basic protein. Affinity for aldehyde fuchsin, alcian blue and iron diamine, together with weak PAS reactivity, indicated the presence of an acid mucopolysaccharide, and the high iron-diamine method, particularly, proved to be highly specific for sulfated mucins. That this acid mucopolysaccharide was sulfated was further supported by the autoradiographic demonstration that, following S^{35}-sulfate administration, cells containing these granules also contained incorporated isotope. The histochemical identity of these granules with the azurophil granules, a normal constituent of heterophil myelocytes, indicated that the sulfated mucopolysaccharide containing granules of circulating heterophils were the counterpart of marrow azurophil granules.

The autoradiographic demonstration that S^{35}-sulfate labeling of circulating heterophils following a single dose of endotoxin occurred when the isotope was given on a schedule which produced prelabeling of myelocytes, i.e., several days prior to endotoxin administration, suggested that the acid mucopolysaccharide-containing granules of heterophils were synthesized in the marrow compartment. Likewise, the lack of uptake of appreciable isotope by circulating heterophils during this primary leukocytosis, when the S^{35} tracer was given with the first injection of endotoxin, indicated that the acid mucopolysaccharide was probably not formed "peripherally" by the circulating heterophils. This observation supported the histochemical findings which suggested that the acid mucopolysaccharide-containing granules in both myelocytes and circulating heterophils were identical. Therefore, it seems reasonable to refer to both as azurophil granules, a term which is well established to designate the metachromatic granules where they occur in the marrow compartment.

It seems unlikely that the occurrence of azurophil granules in circulating heterophils is a specific response to endotoxin, although no attempt has as yet been made to study leukocytosis produced by other means. The presence of significant numbers of azurophil granules in normally circulating heterophils indicates that the granules are not a pathologic phenomenon, however, and the presence of increased granularity during heterophilia may be largely a function of the release of relatively immature forms.

The histochemical demonstration of sulfated mucopolysaccharide in circulating heterophils following both the first and second injections of endotoxin may have pertinence to the pathogenesis of the generalized Shwartzman reaction. The speculation by Thomas, Smith and von Korff [2] that the necessary role of the leukocyte in this reaction might be a function of its ability to supply in some way a substance with properties of a large molecular weight acid polymer seems to assume increased significance in view of the present observation that such an entity was, in fact, a conspicuous constituent of the circulating heterophil during the leukocytosis following administration of bacterial endotoxin. Current histochemical and autoradiographic studies indicating that a sulfated mucopolysaccharide is present in the fibrinoid occlusive glomerular lesions of the generalized Shwartzman reaction will be reported later.

Allison and Lancaster's observation [17] that heparin enhanced microthrombus formation in areas of acute inflammation, apparently an *in vivo* demonstration of heparin-precipitable fibrinogen, suggests that in sites of tissue injury fibrinogen is susceptible to conversion to an insoluble flocculate by acid polymers. The present demonstration that heterophils themselves may contain a sulfated polysaccharide bears consideration in relation to Allison and Lancaster's finding. It is tempting to speculate that some pathophysiologic mechanism, e.g., leukocyte sticking in sites of injury, might be related to the coincidence of an acid mucopolysaccharide constituent of the heterophil and locally engendered partially polymerized fibrinogen, leading to the production of an acid polymer-fibrinogen complex with adhesive characteristics.

A possible role of the azurophil granule in phagocytosis and bacterial inactivation is suggested by several observations that acid mucopolysaccharide and basic protein may be involved in these processes. Spicer [18] has suggested that the sulfated mucopolysaccharide or the basic protein of mast cells might contribute to the phagocytic ability of neighboring cells, and Selye, Gabbiani and Tuchweber [19] implicate mastocyte material in the fixation of foreign material prior to phagocytosis. Skarnes and Watson [20] isolated an arginine-rich protein with potent antibacterial properties from rabbit leukocytes, and Spitznagel and Chi have reported coating of phagocytosed bacteria by similar protein [14] which is apparently derived from granules of leukocytes.[21]

Preliminary cytochemical studies [22] in this laboratory suggested that azurophil granules were the site of nonspecific acid phosphatase activity, while smaller, less dense granules demonstrated alkaline phosphatase reactivity. Correlation of these ultrastructural studies with the present histochemical observations indicates that azurophil granules

contain sulfated mucopolysaccharide, arginine-rich basic protein, and nonspecific acid phosphatase.

Summary

Certain distinctive cytoplasmic granules, present in small numbers in circulating heterophils of rabbits in the absence of a leukocytosis, were greatly increased during endotoxin-induced leukocytosis. These granules, which were histochemically identical to azurophil granules of rabbit bone marrow, stained with azure A above pH 4.0, with aldehyde fuchsin, high iron-diamine, alcian blue, and alcoholic PAS, and were associated with the incorporation of S^{35}-sulfate, indicating the presence of a sulfated mucopolysaccharide. Affinity of the same granules for Biebrich scarlet at highly alkaline pH indicated the presence of a strongly basic protein; blocking reactions suggested that the basic nature of the protein was attributable to high arginine content.

The possible pertinence of these observations to the mechanism of the generalized Shwartzman reaction and to certain aspects of leukocyte function is discussed.

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[*Illustrations follow*]

Legends for Figures

Figures 1 through 14 are photographs of smears of peripheral blood of rabbits. All smears were fixed 1 to 2 hours in 95 per cent ethanol. Photographs were taken on Kodak Panatomic-X film, and the final magnification was constant at 1,600 times except where indicated otherwise. A blue-green filter was employed to enhance contrast in Figures 10, 15 and 16.

Figures 1 to 6 show heterophils stained for 25 minutes in 0.02 per cent azure A at pH 5.0.

FIGS. 1 and 2. Typical heterophils in normal rabbits. The absence of stained granules (Fig. 1) is characteristic of many normally circulating heterophils. In the absence of a leukocytosis, however, heterophils may contain a few metachromatically-staining red granules (Fig. 2).

FIGS. 3 and 4. Representative heterophils 12 hours after an initial injection of endotoxin. At this time the great majority of circulating heterophils contained moderate numbers of metachromatic granules.

FIGS. 5 and 6. Heterophils present 12 hours after the second endotoxin injection, during production of the generalized Shwartzman reaction. Heterophils with such large numbers of metachromatic granules were numerous under these conditions; they were less frequent following a single endotoxin injection and were extremely rare in the absence of a leukocytosis.

FIG. 7. Giemsa stain, pH 6.5. A large heterophil with numerous metachromatic granules, obtained 12 hours after a second endotoxin injection. Staining is similar to that obtained with azure A.

FIG. 8. Aldehyde fuchsin staining of a heterophil comparable to those in Figures 6 and 7. Obtained 12 hours following a second endotoxin injection.

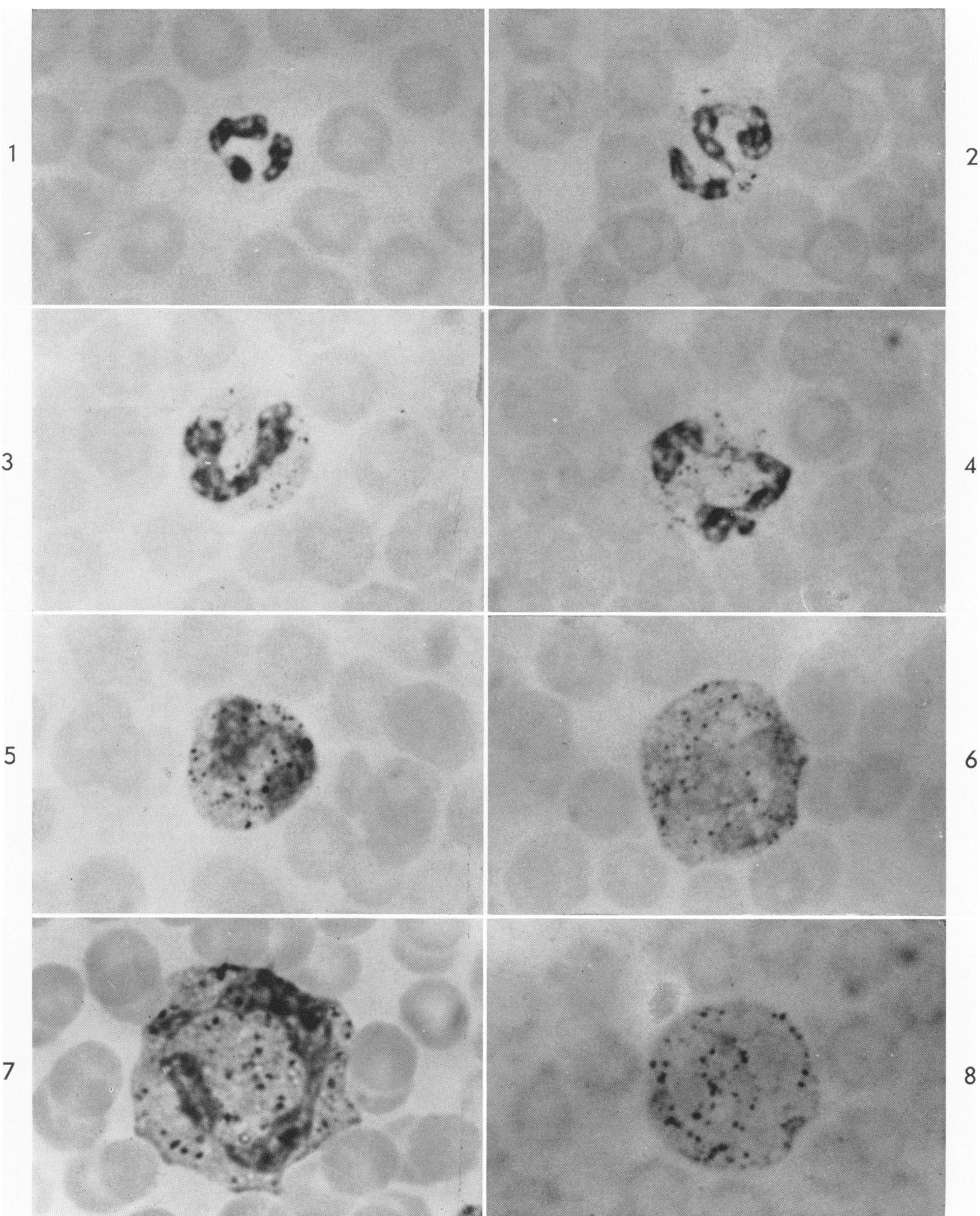
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FIG. 9. High iron-diamine staining of a heterophil (left) and a basophil (right). Obtained 12 hours after a single endotoxin injection. The granules of the heterophil stained by this method appear to correspond to those showing metachromasia with azure A and staining with aldehyde fuchsin. The morphologically distinct basophil shows granule staining with this method as with aldehyde fuchsin, presumably because of the heparin-like acid mucopolysaccharide constituent of its granules.

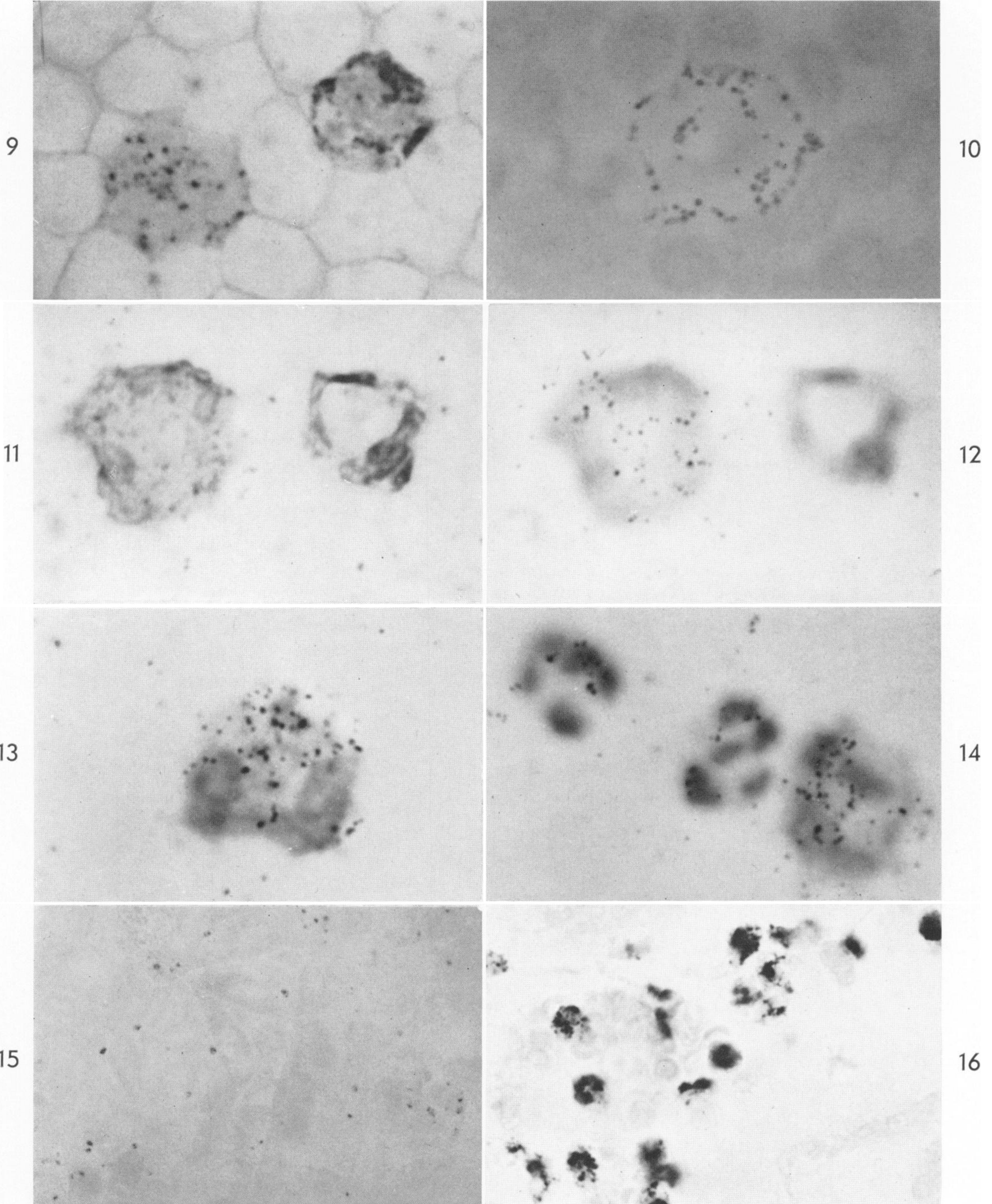
FIG. 10. Biebrich scarlet staining at pH 9.5 of a circulating heterophil comparable to those shown in Figures 6 to 8. Obtained 12 hours after a second endotoxin injection. Granules, stained by this method for coloring basic protein, occur in the same distribution as those stained by techniques for demonstrating acid mucopolysaccharide.

FIGS. 11 and 12. Autoradiographs of the same 2 cells, taken at slightly different planes of focus. One mc. S^{35}-sulfate was given with a first endotoxin injection. Twelve hours following a second endotoxin injection (given 21 hours after the first), circulating heterophils with acid mucopolysaccharide-containing granules are labeled. The somewhat larger heterophil on the left contains numerous aldehyde fuchsin-positive granules, but few or none are present in the cell on the right (Fig. 11). Correspondingly, the left heterophil is well labeled, and the right is unlabeled (Fig. 12). Aldehyde fuchsin-Giemsa stain, 62 days' exposure.

FIG. 13. A well-labeled circulating heterophil. Experimental conditions, staining, and exposure as in Figures 11 and 12.

FIG. 14. Several S^{35}-labeled circulating heterophils 12 hours after a single endotoxin injection. Three 1 mc. doses of S^{35}-sulfate given 96, 72 and 48 hours prior to endotoxin. The smaller and probably more mature heterophils (center and upper left) contain fewer stained granules (not visible in the plane of focus) than the larger heterophil (lower right) and correspondingly less label. Aldehyde fuchsin-Giemsa stain, 34 days' exposure.

FIGS. 15 and 16. Biebrich scarlet stain, pH 9.5, of formalin-fixed kidney obtained 6 hours after a second endotoxin injection. Heterophils are visible because of Biebrich scarlet staining of many of their granules, while all other tissue constituents are essentially unstained. Nuclear histone staining is blocked by formaldehyde. The persistent staining of these heterophil granules may be due to protein with high arginine content. Fig. 15. $\times$ 160. Fig. 16. $\times$ 720.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