The Intercellular Substances of Bone Marrow. 
I. Preliminary Studies of the Physical and Chemical 
Character of the Intercellular Bone Marrow 
Substances in Normal Rabbits 

By Robert E. Carter, Barbara Fleishacker and William L. Jackson

Much of the knowledge of mammalian bone marrow has come from classic histologic study and is limited to the identification of the cell types present. By 1880, largely through the application of Ehrlich's staining techniques, numerous students recognized the bone marrow as the source of certain peripheral blood cells, and the problems of identity and classification of these elements have occupied hematologists since that time. Serious and perhaps insurmountable problems have delayed consideration of the marrow as an organ rather than a group of unrelated cells. The inaccessibility of the marrow, its evident heterologous composition, its wide distribution in the body, and the inability to isolate its blood supply are responsible in part for a relative lack of information on marrow physiology and composition.

Sabin and Doan were among the first investigators to study the bone marrow as an organ, and Nye carried out early experiments on bone marrow volume and cellularity in experimental animals. Huggins et al. studied the chemical composition of whole marrow in rabbits and, in an extensive series of experiments, Dietz and Steinberg determined variations in the chemical composition of whole rabbit marrow under abnormal physiologic conditions. In these studies, the differences found indicated wide variations in marrow composition but failed to show whether these variations occurred in the cellular or noncellular components or both.

The microcirculation of the bone marrow was studied in detail in the rabbit by Branemark and in the rat by Fliedner and his co-workers. These studies indicate that many marrow cells develop at distances of from one to five cell diameters from the nearest blood capillary or sinusoid. While histologic sections of bone marrow prepared by conventional fixation and dehydration techniques rarely show intercellular substance other than fat, an intercellular matrix is almost certainly present, permitting the rapid proliferation of cells at some distance from the circulation and their ultimate release to the vascular compartment. Histochemical studies indicate that this intercellular ground substance contains mucopolysaccharides among other components.

In the attempt to learn more of the chemical and physical nature of the intercellular substances in bone marrow, we have carried out a series of studies on the rabbit. In the initial experiments covered in this report, following separa-
tion of the cellular elements from noncellular elements one physical measurement—the viscosity—and two chemical determinations—the total mucopolysaccharide content and the ether-extractable fat content—were made on the noncellular marrow component. Viscosity was chosen as the physical property most likely to correlate with the integrity of a highly organized intercellular gel, and the mucopolysaccharides were selected as one group of substances apt to be involved in the structure of such an intercellular gel. In the later portions of these experiments, the mucopolysaccharide content of the cellular marrow component was also determined in certain animals.

**METHODS**

Male and female New Zealand rabbits were used. One group ranged in age from 2 to 6 months. The second group were 12 to 18 months of age. The animals were maintained under identical conditions and were fed a standard laboratory diet. Sacrifice was done by the rapid intravenous injection of a concentrated barbiturate solution with no attempt at exsanguination. Both femora and one or both tibiae and humeri were removed. The femurs were opened and we removed as much shaft marrow free from bone spicules as possible. In general, a cylindrical core of firm marrow could be shelled from the bone without difficulty. All femoral marrow was grossly red. When the amount of femoral marrow was less than 2.5 Gm., marrow from the upper third of the tibia or humerus was also used.

The marrow was placed in a measured volume of normal saline and its weight and volume were determined by displacement. Samples were saved for sectioning and histochemical study. Following density determination, the concentration of marrow was adjusted to 0.25 Gm./ml. The entire material was transferred to a glass tissue mill of loose fit, and the plunger was gently passed through the material 10 to 20 times. The marrow could be broken up with ease, and serial counts of nucleated cells showed that the number of free cells increased in number up to the tenth pass of the plunger and then remained relatively constant through as many as thirty passes. After this mechanical separation of cells from stroma and the enumeration of nucleated cells, the marrow was centrifuged in a graduated tube at 400 g for 10 minutes. Cellular elements were well packed in the bottom of the tube, and the upper layer of the centrifugate was a well-delineated layer consisting of fat, an amorphous material faintly eosinophilic in its staining properties, some collagen-like fibers, and scattered cells trapped in their mesh. The layer of packed cells was separated from the upper layer by a column of slightly turbid saline (fig. 1). After measuring the volume of each layer, the upper fatty layer and the saline were decanted and rehomogenized. Cell counts on this component indicated that an average of 90 per cent of the cells had been removed by the centrifugation. This portion of the marrow will be referred to as the noncellular component in this report, but it must be emphasized that it was not cell-free. The relative volumes of the cellular and noncellular marrow components given in table 1 are based on the volumes of packed cells and upper fat-containing layer, as determined by the centrifugation and averaged 8.8 per cent greater than the total marrow volume determined by displacement immediately after removal from the animal. No correction for this difference was made.

The viscosity of the noncellular portion of the centrifugate was determined in a MacMichael viscosimeter. Thirty-four-gage torsion wires were used in all experiments and were calibrated periodically against oils supplied by the National Bureau of Standards. Two viscosity measurements were made, the first of the force required to produce shearing of the noncellular marrow against the plunger, and the second of the drag on the plunger with continued rotation of the sample cup. There was close correlation between these measurements in the majority of marrows studied, but occasional variations occurred, possibly related to the fat content of the marrow. On each marrow, repeated measurements were made at temperatures ranging from 20° to 40° C. The values reported were
Fig. 1.—Separation of cellular and noncellular marrow components after centrifugation.

Those determined for 39°C. In Fig. 2, the critical portions of the viscosimeter are diagrammed. The 3-mm. separation of plunger and cup wall was the minimum distance in all instances.

Following viscosimetric measurements, fat was extracted from the noncellular marrow homogenate with ether. In several animals, subsequent viscosity measurements on the defatted homogenate showed no significant increase or decrease compared with the value for the fat-containing material. All viscosity measurements reported were the values obtained on fat-containing homogenates. After fat extraction, the total mucopolysaccharide content of the noncellular component was determined by the method of Dyrybe and Kirk, with final estimation of the purified polysaccharide material by the turbidimetric method of Di Ferranti. Two modifications were made in the method reported by Dyrybe and Kirk. First, concentration of the material after digestion and filtration was accomplished by evaporation under partial vacuum at temperatures not exceeding 37°C. Second, following enzymatic digestion the samples were dialyzed against water for 48 hours before the subsequent steps of precipitation and purification were carried out. The addition of chondroitin sulfate to appropriate protein solutions, followed by analysis using the methods described, indicated a recovery of 75 per cent. In these initial experiments, uronic acid and hexosamine determinations were not made in our laboratory, but samples were analyzed through the kindness of Dr. A. J. Cifonelli of the La Rabida-University of Chicago Institute, Chicago, Illinois. Hexosamine and uronic acid analyses confirmed the validity of the turbidimetric estimation of total mucopolysaccharide content in this experiment. In later portions of these experiments, the total mucopolysaccharide content of the cellular marrow component was also determined, after washing of the cells, using the method outlined above.

Experimental Results

A total of 20 young and 16 older rabbits were studied. The various data are given in Table 1 for each group. The total count of nucleated cells per gram
INTERCELLULAR SUBSTANCES OF BONE MARROW

Table 1

<table>
<thead>
<tr>
<th>Animal age</th>
<th>2 to 6 months</th>
<th>12 to 18 months</th>
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</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Body weight (Kg.)</td>
<td>2.68 ± 0.12*</td>
<td>3.58 ± 0.13</td>
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<tr>
<td>Marrow cell count</td>
<td></td>
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<tr>
<td>(Nucleated cells/Gm. marrow × 10⁶)</td>
<td>1.49 ± 0.10</td>
<td>1.62 ± 0.09</td>
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<tr>
<td>Marrow density (Gm./cm.³)</td>
<td>0.983 ± 0.007</td>
<td>1.045 ± 0.016</td>
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<tr>
<td>Relative volume of marrow components</td>
<td></td>
<td></td>
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<tr>
<td>Noncellular component</td>
<td>0.725 ± 0.090</td>
<td>0.588 ± 0.045</td>
</tr>
<tr>
<td>Cellular component</td>
<td>0.275 ± 0.005</td>
<td>0.412 ± 0.044</td>
</tr>
<tr>
<td>Marrow fat content (Gm./Gm. total marrow weight)</td>
<td>0.311 ± 0.020</td>
<td>0.166 ± 0.022</td>
</tr>
<tr>
<td>Viscosity of noncellular marrow component</td>
<td></td>
<td></td>
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<tr>
<td>Shearing (entiposes)</td>
<td>86.7 ± 5.8</td>
<td>43.8 ± 6.7</td>
</tr>
<tr>
<td>Drag (entiposes)</td>
<td>54.1 ± 3.7</td>
<td>28.8 ± 3.6</td>
</tr>
<tr>
<td>Av. mucopolysaccharide content of noncellular component (µg./Gm.)</td>
<td>204</td>
<td>200</td>
</tr>
<tr>
<td>Av. mucopolysaccharide content of cellular component (µg./Gm.)</td>
<td>82</td>
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*Denotes standard error of estimate of the mean.

of marrow was not significantly different between the two groups of animals, whereas the older animals were found to have a significantly higher marrow density and lower average fat content than the younger group. The slightly higher average marrow cell count in the older animals and the relatively lower marrow fat content were consistent with the differences noted in the cellular and noncellular components, the older animals having a relatively but not significantly higher cellular component volume than the younger animals.

The difference in viscosity of the noncellular marrow component between the two groups was significant, the older animals having half the values found in the younger group. Both viscosity measurements were well within the range of good accuracy of the viscosimeter, and the variance found was less than might have been anticipated.

No difference was found between the two groups of animals in the average mucopolysaccharide content of the noncellular component of the marrow. The variation in each group was large: the range in the young animals was 29 to 337 µg./Gm, and that in the older animals was 62 to 408 µg./Gm. In seven young animals whose mucopolysaccharide content of cellular component was determined, the values found ranged from 6.5 to 170 µg./Gm. One animal in this group appeared singular, and accounted for the lowest value of mucopolysaccharide content in both the cellular and noncellular components, each value being less than one fourth that of the next highest specimen. These data were not excluded from the tabulation, since none of the other measurements made on this animal was unusual. Each marrow specimen had been analyzed for total mucopolysaccharide content at a different time and had been included in
DiscUssioN

The mechanical method used for separation of cellular and noncellular portions of the marrow seemed satisfactory for the purposes of these experiments. It is doubtful that greater than 90 per cent separation of cells from noncellular stroma could be accomplished without partial digestion or other chemical alteration of the noncellular stroma, since a certain number of cells are trapped in this stroma and carried to the top of the tube during centrifugation. It is unlikely that the presence of cells in the stromal portion of the marrow contributed significantly to its viscosity. In five of the animals in this and later experiments, viscosimetric measurements on the homogenized marrow before centrifugation did not differ from the values obtained on the noncellular component alone.

The reproducibility of the viscosity measurements on the noncellular component was surprising in view of the heterogeneous nature of the material. None of the components analyzed in these experiments accounted for the
measured viscosity or for the variations in the viscosity observed. The muco-
polysaccharide material itself could account only for an insignificant fraction.
The fat present did not account for the total viscosity, as shown by the similar
values obtained before and after fat removal. In later experiments, to be re-
ported, a doubling of the noncellular viscosity was seen under abnormal
physiologic conditions in animals whose noncellular fat content was one half
that of control groups. It is likely that a complex organization of fat, protein,
and polysaccharide exists in the intercellular substance of bone marrow and
that the viscosity is dependent on the chemical and physical integrity of this
material.

The amount of polysaccharide material found in the noncellular component
of rabbit bone marrow is within the limits of accurate chemical determination.
The polysaccharide content of the cellular component was sufficiently low to
establish that no more than 10 per cent of the measured amount in the non-
cellular component could result from cells trapped in this portion of the mar-
row stroma during centrifugation. These experiments appear to confirm the
presence of mucopolysaccharide material in the intercellular substance of bone
marrow; further analysis of the material obtained is in progress.

Summary

Separation of cellular and noncellular fractions of rabbit bone marrow was
accomplished by mechanical disruption of the marrow stroma and centrifuga-
tion. Viscosity studies performed on the intercellular substances showed a de-
creasing viscosity with increasing age of the animals. Chemical analyses con-
firmed the presence of mucopolysaccharide material in the intercellular subst-
ance of the marrow, but neither this material nor the fat present in the stroma
could account for the viscosity measured or the changes in viscosity observed.

Summary in Interlingua

Separation de fractiones cellular e non-cellular de medulla ossee de conilio
esseva compleite per medio de disruption mechanic del stroma medullari e
centrifugation. Studios de viscositate interprendite con le substantias inter-
cellular monstrava un viscositate que decresceva in animales durante que lor
etate se augmentava. Analyse chimic verificava le presentia de materia muco-
polysaccharidic in le substantia intercellular del medulla, sed ni iste materia ni
le grassia presente in le stroma poteva explicar le viscositate mesurate o le
alterationes in le viscositate observate.

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