On the Nature of Castle’s Hemopoietic Factor

By SHEILA T. CALLENDER, M.D., M.R.C.P. AND L. G. LAJTHA, M.D.

ALTHOUGH THE VALIDITY of Castle’s observations on the relationship of achylia gastrica to pernicious anemia has never been doubted, the interpretation of his findings has recently appeared to need modification. Folic acid seems to have no place in the classical scheme and vitamin B₁₂ has features in common with the concept both of extrinsic factor and hemopoietic factor. The recent tendency has been to regard intrinsic factor simply as an agent which aids absorption of B₁₂ from the gastro-intestinal tract.

Tissue culture work has, however, shown that crystalline vitamin B₁₂ is not identical with the hemopoietic factor present in normal serum (Lajtha et al). B₁₂ does not ripen megaloblasts into normoblasts in vitro whereas normal serum and serum from patients with pernicious anemia treated with B₁₂ will.

The object of the present investigation has been to extend the use of the marrow culture technic to investigate further the relationship between vitamin B₁₂ and the hemopoietic factor in normal serum. First, Castle’s original hypothesis has been tested in vitro by studying the effect of various combinations of B₁₂ and gastric juice on megaloblastic marrow. Secondly, the heat lability of the hemopoietic factor in normal serum has been studied.

The term “ripening” will be used to signify the transformation of megaloblasts to normoblasts and the term “maturation” for the development of later cell forms from younger forms in both normoblastic and megaloblastic series of cells.

METHODS

Cell suspensions prepared from sternal marrows from patients with untreated pernicious anemia were cultured in vitro as previously briefly described. A detailed account of the technic is in press. Differential counts were made on the nucleated red cells after 48 hours culture.

1. The following were tested for their effect on megaloblasts cultured in a medium of 80 per cent pernicious anemia serum plus 20 per cent Ringer solution: (1) Gastric juice alone (IF). (2) Gastric juice plus B₁₂ (HPF). (3) Heated gastric juice plus B₁₂ (heated IF plus B₁₂). (4) Gastric juice plus B₁₂, heated (heated HPF). One-tenth ml. of the substance to be tested was added to 3 ml. culture medium.

Gastric juice was obtained with sterile precautions from fasting normal subjects or patients without anemia and with free acid in the gastric secretion. It was filtered through Whatman paper and kept at 4 C. for at least forty-eight hours in order to help self sterilization. Bacteriologic control showed such preparations to be sterile. Immediately before use (or before addition of B₁₂) the gastric juice was brought to neutral pH with sodium hydroxide.

Crystalline vitamin B₁₂ was added in the proportion of 0.02 μg. per ml. gastric juice

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TABLE 1.—The Effect of Hemopoietic Factor (Normal Gastric Juice Plus Crystalline B₁₂) on Megaloblasts in Vitro

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Initial count</th>
<th>48 hour cultures in</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PA serum</td>
<td>+ HPF</td>
<td>+ heated HPF</td>
<td>+ IF</td>
<td>+ heated IF + B₁₂</td>
</tr>
<tr>
<td>81b</td>
<td>32.0</td>
<td></td>
<td>63.0</td>
<td>37.0*</td>
<td>55.0†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.0†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>36.0</td>
<td></td>
<td>31.5</td>
<td>18.5†</td>
<td>25.5‡</td>
<td>34.0</td>
</tr>
<tr>
<td>85</td>
<td>44.0</td>
<td></td>
<td>17.5</td>
<td>7.0†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>84b</td>
<td>42.0</td>
<td></td>
<td>29.5</td>
<td>14.5†</td>
<td>17.0‡</td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28.5§</td>
<td>21.5‡</td>
</tr>
<tr>
<td>86</td>
<td>44.0</td>
<td></td>
<td>19.0</td>
<td>12.0†</td>
<td>15.0†</td>
<td>20.7</td>
</tr>
</tbody>
</table>

IF = gastric juice 0.1 ml./3 ml. medium.
HPF = IF + 2.0 μg. B₁₂.
* Not incubated before adding to culture.
† Incubated at 37 C. for 2 hours before adding to culture.
‡ 100 C. for 5 minutes.
§ Autoclaved.

TABLE 2.—Effect of Heated Normal Serum (56 C. for 1 Hour) on Megaloblasts in Vitro

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Initial count</th>
<th>48 hr. cultures in</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal serum</td>
<td>Heated normal serum</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.0*</td>
<td>30.0*</td>
<td>9.0</td>
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<td>42</td>
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<tr>
<td>63</td>
<td>38.5</td>
<td>11.7*</td>
<td>20.7*</td>
<td>9.7</td>
</tr>
<tr>
<td>3</td>
<td>31.0</td>
<td>11.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>48.0</td>
<td>20.0</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>46.0</td>
<td>3.0</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>81a</td>
<td>32.0</td>
<td>12.6</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>36.0</td>
<td>6.0</td>
<td>9.0</td>
<td></td>
</tr>
</tbody>
</table>

* 24 hour cultures.

(based on the “binding capacity” of gastric juice for B₁₂ found by Ternberg and Eakin').
“Heated” gastric juice was boiled for 5 minutes or autoclaved at 20 lbs. pressure for half an hour. In some cases the mixtures were incubated before addition to the cultures.
2. In the second group of experiments megaloblastic marrows were cultured in parallel in a medium of 80 per cent (1) fresh normal serum, (2) the same serum previously heated to 56 to 57 C. for 1½ to 2 hours.

The experimental error of the technic has been found to approach very closely the binomial distribution. Significant ripening was judged to have occurred in cultures where the percentage of megaloblasts decreased by more than twice the standard error.

RESULTS

It was found that while normal gastric juice alone was inactive, normal gastric juice plus B12 mixture produced significant ripening of megaloblasts in vitro (see table 1). Incubation of the gastric juice plus B12 mixture before addition to the cultures was not necessary for this effect.

No ripening effect was observed in two cultures with the addition of heated gastric juice plus vitamin B12. With gastric juice plus B12 heated, i.e., heated HPF, the results suggest that there is partial destruction of the hemopoietic factor with boiling for 5 minutes and more complete destruction with autoclaving.

Table 2, illustrating the second group of experiments, indicates that there is a tendency for some normal sera heated to 56 C. for 1½ to 2 hours to show less ripening effect than the same sera unheated. The difference, though not constant, is significant.

DISCUSSION

Clinical experiments have shown that normal gastric juice enhances the effect of oral vitamin B12 in the treatment of pernicious anemia (Berk et al.,1 Bothell et al.,2 Hall et al.,3 Ungley,4). The work of Ternberg and Eakin5 has shown that vitamin B12 in the presence of normal gastric juice becomes “bound,” forming a complex in which it is no longer available for microbiologic assay. On the basis of these observations it has been suggested that the function of the normal gastric juice (intrinsic factor) might simply be to aid absorption of vitamin B12 possibly by protecting it from the intestinal bacteria.

Our experiments, however, indicate that the complex formed from B12 and intrinsic factor is an active hemopoietic factor which will produce ripening of megaloblasts into normoblasts in vitro. Crystalline B12 itself being incapable of this effect appears to fill the role of Castle’s extrinsic factor. Addition of 0.1 μg. of vitamin B12 per ml. culture medium has previously been found to be ineffective but as little as 0.7 mg. per ml. medium in combination with intrinsic factor produced ripening of megaloblasts into normoblasts. The B12-gastric juice complex is thermolabile, the intrinsic factor being the labile part. There is some evidence that intrinsic factor alone is more thermolabile than the hemopoietic factor (Hall et al.,4 Spray9) but sufficient heating will destroy the HPF completely, “liberating” vitamin B12 which may then be assayed microbiologically.

Gastric juice alone may have some B12 activity as measured microbiologically but it is evidently insufficient to produce ripening of megaloblasts in vitro. One of the samples used was assayed with L. Leichmannii. It showed 0.64 mg. of B12 activity per ml. One-tenth ml. of this juice per 3 ml. medium had no obvious effect on the megaloblasts but when the B12 content had been raised by addition
of 20.0 mg per ml. crystalline B\(_{12}\), 0.1 ml. of the same juice became active in vitro on megaloblasts.

Since the gastric juice (intrinsic factor) plus B\(_{12}\) complex proved able to ripen megaloblasts to normoblasts in vitro, as do normal sera or sera from patients with pernicious anemia treated with B\(_{12}\), a relationship between "gastric" hemopoietic factor and "serum" hemopoietic factor was sought. Ross's\(^6\) investigations into the B\(_{12}\) content of normal sera and sera from patients with pernicious anemia treated with B\(_{12}\), indicate that the vitamin is present in the serum in a thermolabile complex form. In this form it is not available for microbiologic assay but on heating (above 70 C.), free B\(_{12}\) is liberated from the complex. Unfortunately serum heated much above 60 C. becomes useless for tissue culture purposes, but our results show that even heating to 56 C. for 1½ to 2 hours diminishes the megaloblast ripening effect of normal serum, suggesting a further parallel behavior of "gastric" and "serum" hemopoietic factor. Table 3 indicates that, as far as biologic activity is concerned, gastric and serum hemopoietic factors are identical. Both sources of hemopoietic factor are thermolabile; heating results in diminution of megaloblast ripening activity in tissue culture but increase in free B\(_{12}\) as shown by microbiologic assay.

These results suggest that Castle's original scheme, intrinsic factor plus extrinsic factor equals hemopoietic factor, is still valid today. However, this being so an interesting problem arises. Crystalline vitamin B\(_{12}\) given parenterally to patients with pernicious anemia appears in the serum as the active hemopoietic factor, not as free B\(_{12}\). This suggests that in pernicious anemia the intrinsic factor deficiency is only in the gastric juice and there must be an extra-gastric source of intrinsic factor with which the parenterally given B\(_{12}\) can combine. This extra-gastric intrinsic factor cannot be present in a free state in the serum, since B\(_{12}\) added to pernicious anemia serum has no megaloblast ripening effect in vitro. Further experiments are planned to investigate the possible source and nature of this factor.

In such investigations the marrow culture technic has the advantage over microbiologic assay in that not every "bound" form of vitamin B\(_{12}\) is necessarily also hemopoietic factor. There may indeed be several aspecific adsorptions of the

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**Table 3.** Relationship between "Gastric" and "Serum" Hemopoietic Factor

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Microbiologic activity</th>
<th>Effect on megaloblasts in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(_{12}) crystalline</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B(_{12}) crystalline heated</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B(_{12}) + intrinsic factor</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B(_{12}) + intrinsic factor heated</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Normal serum</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Normal serum heated</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Treated PA serum</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Treated PA serum heated</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
B$_{12}$ molecule to other substances, which have no HPF activity but which the microbiologic assay will show as "bound" forms of B$_{12}$. It is also possible that intrinsic factor itself may suffer changes leading to loss of HPF activity, while retaining B$_{12}$ binding capacity.

It is now possible to complete further the tentative scheme previously suggested$^6$ for correlating the factors controlling normoblastic and megaloblastic erythropoiesis. According to this scheme folic acid, through folinic acid, acts directly on the marrow cells but an inhibitory factor in the serum prevents the action of the physiologic folinic acid level unless hemopoietic factor also is present. Hemopoietic factor counteracts the action of the inhibitor either directly, or indirectly by action on the nucleated red cells. There is evidence to suggest that both B$_{12}$ and folic acid are necessary for normal hemopoiesis and that one is ineffective in the total absence of the other. In addition, either hemopoietic factor or vitamin B$_{12}$ appears to be concerned in the normal function of the central nervous system. Hemopoietic factor may be formed either by interaction of B$_{12}$ (extrinsic factor) and normal gastric juice (gastric intrinsic factor) or, if B$_{12}$ is given parenterally, by B$_{12}$ and the extra-gastric intrinsic factor. Hemopoietic factor being thermolabile cannot, as was previously thought, be present in liver extracts; the in vitro effect of liver extracts appears to be due to their folinic acid content (Callender and Lajtha$^3$).

Vitamin B$_{12}$ alone appears to be poorly absorbed from the gastro-intestinal tract so that lack of gastric intrinsic factor, as in pernicious anemia, results in a

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**Diagram 1.**—Interrelationship of Factors Controlling Normoblastic and Megaloblastic Erythropoiesis

<table>
<thead>
<tr>
<th>Parenteral B$_{12}$ (Liver extracts)</th>
<th>B$_{12}$ in Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXTRA-GASTRIC Intrinsic Factor</td>
<td>GASTRIC Intrinsic Factor</td>
</tr>
</tbody>
</table>

GASTRO-INTESTINAL TRACT

Folic Acid

Folinic Acid (Citrovorum Factor)

Inhibitor in Serum

? directly

HEMOPOIETIC FACTOR (also C.N.S. Factor)

Counteracts the action of the inhibitor

? indirectly

NUCLEATED RED CELLS

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deficiency of hemopoietic factor. This may lead to the development of central nervous symptoms. The deficiency also leaves the inhibitor free to prevent the action of folic acid with consequent development of megaloblastic anemia. Excess of folic or folinic acid overcomes the inhibitor by mass action, folic acid being converted in the body to the reduced and more active form folinic acid. This will restore the hematologic picture to normal, but the central nervous symptoms will continue to deteriorate. Hemopoietic factor, on the other hand, will improve both hematologic and neurologic signs without the addition of folic or folinic acid.

Other forms of megaloblastic anemia may be directly due to folic or folinic acid deficiency. These can respond only to folic or folinic acid. No instances are known in which a megaloblastic marrow cannot be affected by sufficient amount of folic acid.

It should be emphasized that this concept is offered as a working hypothesis and is not all proven fact.

SUMMARY

1. Normal gastric juice (intrinsic factor) and vitamin B12 together form a thermolabile hemopoietic factor which ripens megaloblasts in vitro, both gastric juice and B12 alone being inactive.

2. The hemopoietic factor in normal serum which ripens megaloblasts in vitro also appears to be thermolabile, heating to 56 C. for 2 hours destroying some of its activity.

3. The relationship of these factors is discussed and an extra-gastric as well as a gastric source of intrinsic factor is postulated.

REFERENCES


9 Spray, G.: Personal communication, 1951.


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