Kinetics of the Clonal Proliferation of Granulocytes and Macrophages in Cultures of Mouse Bone Marrow Cells as Supported by Two Distinct Types of Colony-Stimulating Factors

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ABSTRACT. Two different types of colony-stimulating factors (CSF) were used to support the clonal growth of myeloid progenitor cells (CFUc) in semi-solid agar or viscous methylcellulose cultures of mouse bone marrow cells. The cultures stimulated for 5 days with RSP-2-P3 cell CSF (CSF<sub>RSP</sub>) contained mainly granulocyte colonies, whereas the cultures stimulated for 10 days with human urine CSF (CSF<sub>hu</sub>) contained mainly monocyte/macrophage colonies. Four lines of study were carried out: 1) a kinetic study using combinations of the two types of CSFs in the same culture; 2) a study of transferring CFUc from the initial 3-day cultures to recipient cultures containing the same or different types of CSF; 3) an examination of the morphology over time of colonies that were confined by glass capillaries plunged in agar; and 4) electron microscopic observations on disintegrating granulocytes. The results of all these lines of study suggest that about one third of the CFUc can be stimulated both by CSF<sub>RSP</sub> and CSF<sub>hu</sub> while the other two thirds react specifically either with CSF<sub>RSP</sub> or with CSF<sub>hu</sub>. The present study also suggests that granulocytes in the culture stop proliferation and disintegrate while macrophages are still growing there. Thus, mixed-type colonies containing both macrophages and granulocytes later become macrophage colonies.

Since the agar-plate method was developed about two decades ago to culture myeloid progenitor cells (CFUc) in vitro (1, 7), colony-stimulating factors (CSF) from various sources have been customarily used in the culture system to support clonal growth of these cells. In general, CSFs isolated from natural sources are sialoglycoproteins. Several distinct types of CSF have been hitherto discovered,
namely, macrophage CSF (M-CSF), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), interleukin 3 (multi CSF) etc. (5).

Although these CSFs act specifically at each stage of CFUc differentiation, it seems that the spectra of their action partially overlap each other so that no abrupt change might occur in the dependency of the differentiating CFUc upon a specified CSF.

In this paper, we report on the biological characteristics of two types of CSFs; one isolated from the culture medium of RSP-2-P3 cells (CSFRSP) and the other, from normal human urine (CSFhu). CSFRSP preferentially induces granulocyte colony formation (8) and belongs to a group of CSFs that are relatively small in their molecular weight (20,000-30,000 daltons), whereas CSFhu induces the formation of mainly macrophage colonies and belongs to a group of large molecular-weight CSFs (40,000-80,000 daltons) (10). Our present aim is to study the magnitude of the overlap of the action spectra of these two types of CSFs.

MATERIALS AND METHODS

Animals and cell lines. Inbred mice of the strain of LACA were used. All the animals were female and 10–12 week old.

The rat spleen cell line RSP-2-P3 was cultured in serum-free Iscove's medium (Boehringer Mannheim) supplemented with 2.5 g/L NaHCO₃ and 1 mg/L human transferrin. The production of CSF was induced by simultaneous administration of 2 mM Na butyrate and 1 µg/ml of E. coli lipopolysaccharide as reported previously (9).

Bone marrow cell culture. Femoral marrow cells were plated in 35-mm petri dishes, each with 1 ml of Fisher's medium (Nissui Seiyaku Co., Tokyo) containing 20% horse serum (Beijing Institute of Biological Products), 0.25% agar and adequate amounts of CSF. After incubation at 37°C in humidified 5% CO₂-air for various periods of time, the cultures were examined under a microscope. The number of colonies produced was proportional to the number of cells plated, within the range of 5 × 10⁴ to 2 × 10⁵ cells/plate. The assays reported below employed about 1 × 10⁵ cells/plate and were triplicated. The results were expressed in terms of the number of colonies per 10⁵ plated cells.

Cytologic examinations of the colonies produced. Individual colonies from the agar plate were randomly chosen and picked up with a pasteur pipette, and the cells composing the colony were microscopically examined after being stained with 0.6% orcein in 60% glacial acetic acid. As we reported previously (2, 8, 9), cell identification by this morphological method was equivalent to identification by a cytochemical method (esterase-staining) and by an immunological method (inhibition of colony formation by anti-CSF antibody). A granulocyte colony containing macrophages with a frequency of more than 2% or vice versa was scored as a mixed-type colony. A total of 30–100 colonies were thus examined for each group of three plates and classified into 3 types; i.e., granulocyte colonies (G), macrophage colonies (M) and mixed-type colonies (GM). The percentage of G, M and GM colonies in each plate was calculated for each colony type and the total number of colonies was scored for each plate.

Transfer of growing colonies into methylcellulose cultures. On Day 3 of incubation, CFUc growing in clusters in agar were transferred as whole individual clusters to plates which contained 1 ml of Fisher's medium containing 0.9% methylcellulose 20% horse serum and the appropriate CSF. Nine areas had been previously marked on the bottom of the plate and each area received an implantation of cells from one single cluster. After 4 days
of incubation at 37°C in 5% CO2-air, the cultures were examined and the number of G, GM
and M colonies scored as described above.

Colony-stimulating factor. The two types of CSFs were purified by modifications of
previously reported methods (2, 8).

Briefly, the CSFhu in fresh urine was absorbed to Tokusil UR (SiO2, Tokuyama Soda
Co., Tokyo), extracted from the adsorbent with 4% ammonium water, and precipitated by
ammonium sulfate at 65% saturation. Then it was subjected to a DEAE cellulose column
(6.5 x 48 cm) equilibrated with 20 mM Na phosphate buffer (pH 6.5) and containing 20 mM
NaCl. The CSF was eluted with 400 mM NaCl, dialyzed, and placed onto a second DEAE
cellulose column as described above. A linear concentration gradient of 20-500 mM NaCl
(a total volume of 7 liters) was applied to the column. The CSF eluted between 120 mM
and 170 mM NaCl. After concentration by ultrafiltration, it was chromatographed on
a Sephadex G-100 column (5 x 100 cm). The CSF activity was detected as a single peak at
1.44 Vv/Vo.

High pressure liquid chromatography (HPLC) (Walters Model ALC/GPC 440) was then
used. The HPLC instrument was equipped with a TSK 3000 SW column (7.5 mm x 30 cm)
which had been equilibrated with 150 mM NaCl containing 0.05% polyethyleneglycol (PEG)
and 5 mM Na phosphate (pH 7.4). The flow rate was 0.5 ml/min. The CSF activity was
found as a single symmetrical peak with a retention time of 25.6 min which corresponded
to a molecular weight of 53,000.

CSF_{RSP} was prepared from medium conditioned by RSP-2•P3 cells in the presence of
butyrate and LPS (9). The cell-conditioned medium was concentrated by ultrafiltration,
dialyzed against 10 mM Na phosphate buffer at pH 6.3, and applied to a Zeta-Prep DEAE-
100 cartridge (AMF, Yokohama). The bound proteins were eluted with the phosphate
buffer containing 150 mM NaCl. The eluate was concentrated and applied to a Toyo-Pearl
HW 55F column (1.6 x 84 cm) equilibrated with 5 mM Na phosphate (pH 7.4) containing
150 mM NaCl and 0.05% PEG. The CSF eluted at 1.6 void volumes.

The CSF was then subjected to HPLC in the same manner as described above for the
CSF_{hu}. The CSF activity was recovered as a sharp peak with a retention time of 20.4 min
which corresponded to a molecular weight of 17,000. There was no other peak of CSF
activity.

Electron microscopic observation. A piece of cover glass (2 x 2 cm) was placed at the
bottom of a 35-mm culture plate. Bone marrow cells were suspended in culture medium
containing 0.9% methylcellulose and either CSF_{hu} or CSF_{RSP} and added to the culture
plate. After the plate was incubated for 5-10 days as described above, the cover glass was
taken out. Methylcellulose was removed by rinsing the cover glass with a cold isotonic
sucrose solution (pH 7.4). The cells were fixed with glutaraldehyde (2-4 h) and OsO4 (2 h)
in the conventional manner. Samples were dehydrated with alcohol, dried by carbon dioxide,
coated with gold, and examined under a scanning electron microscope (Philips Model 505).

In addition, colonies of cells grown in agar were aspirated off with a fine plastic tube
under a binocular stereo-microscope. The cells were fixed with glutaraldehyde and OsO4
as described above, dehydrated and embedded in Epon 812. Sections were made on a LKB
Ultrotome IV and stained in the conventional manner with uranyl acetate/lead citrate. The
samples were examined with a Philips Model 400 transmission electron microscope.

RESULTS

Synergism between CSF_{hu} and CSF_{RSP}. In the experiment of which results are
shown in Table 1, the two different types of CSFs were used singly or in combination. Both CSFs were used either at a concentration giving about a half-maximal number of colonies (top three lines) or at a concentration which produces nearly maximal number of colonies (bottom three lines). The cultures were incubated for 5, 7 or 10 days and examined for the total number of colonies and for the morphological characteristics of the cells that composed the colonies.

The cultures stimulated by CSFRSP contained mainly G colonies in the early stages of incubation. However, the number of G colonies decreased with time, while the number of M colonies increased. CSFhu-stimulated cultures developed mainly M colonies, although it also induced a small number of G colonies and GM colonies to grow in the Day-5 cultures.

A positive synergistic effect was observed when the two CSFs were used at the half-maximally stimulating concentrations and the cultures were examined either on Day 5 or Day 7 of incubation. Thus, the combined use of CSFRSP and CSFhu produced 40 colonies in the Day-5 culture, which was 33% higher than the sum of the colony numbers produced by CSFRSP and CSFhu individually. Furthermore, 52 colonies were produced by the combination of the two CSFs in the Day-7 culture, which was 63% in excess of the expected 32 colonies.

However, M colonies decreased in number when CSFRSP was added to cultures that contained CSFhu at a maximally stimulating concentration. The suppressive effect of CSFRSP was most noticeable in Day-7 or Day-10 cultures. For example, 97 macrophage colonies were produced in the Day-10 culture that was stimulated with CSFhu alone, whereas only 45 macrophage colonies were found in the culture that contained both CSFhu and CSFRSP at maximally stimulating concentrations.

**Growth of cells after transferal from Day-3 culture to a new environment.** Colonies growing in agar usually contained less than 20 cells on Day 3 of incubation, whether CSFhu or CSFRSP was used for stimulation. A whole cluster of cells could be aspirated off with a fine glass capillary and transferred to a methylcellulose plate.

When the methylcellulose plate contained no CSF, nearly all of the implanted cells disintegrated and disappeared from the culture within 4 days after implantation.

### Table 1. Effect of CSFRSP and CSFhu alone or in combination, on the production of myeloid cell colonies in the agar cultures of mouse bone marrow cells

<table>
<thead>
<tr>
<th>CSF</th>
<th>Day-5 culture</th>
<th>Day-7 culture</th>
<th>Day-10 culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>GM</td>
<td>M</td>
</tr>
<tr>
<td>CSFRSP</td>
<td>23</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CSFhu</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Both CSFs</td>
<td>30</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>CSFRSP</td>
<td>59</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>CSFhu</td>
<td>3</td>
<td>7</td>
<td>34</td>
</tr>
<tr>
<td>Both CSFs</td>
<td>60</td>
<td>6</td>
<td>31</td>
</tr>
</tbody>
</table>

*a) The two CSFs were used at a concentration producing about a half-maximal (top three lines) or a nearly maximal (bottom three lines) number of colonies.

*b) Each figure in the table is the rounded off average of 3 plates. G—granulocyte colonies; GM—mixed-type colonies; M—macrophage colonies.*
Colony-Stimulating Factor

TABLE 2. GROWTH OF COLONIES AFTER IMPLANTATION OF GROWING COLONIES FROM DAY-3 CULTURES TO NEW PLATES CONTAINING THE SAME OR DIFFERENT TYPES OF CSF

<table>
<thead>
<tr>
<th>Stimulator&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Number of clones transferred</th>
<th>Number of colonies produced in the methylcellulose plates&lt;sup&gt;b)&lt;/sup&gt;</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Following</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF&lt;sub&gt;hu&lt;/sub&gt;</td>
<td>none</td>
<td>54</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>CSF&lt;sub&gt;hu&lt;/sub&gt;</td>
<td>CSF&lt;sub&gt;hu&lt;/sub&gt;</td>
<td>27</td>
<td>12 (44%)</td>
</tr>
<tr>
<td>CSF&lt;sub&gt;hu&lt;/sub&gt;</td>
<td>CSF&lt;sub&gt;RSP&lt;/sub&gt;</td>
<td>45</td>
<td>12 (27%)</td>
</tr>
<tr>
<td>CSF&lt;sub&gt;RSP&lt;/sub&gt;</td>
<td>none</td>
<td>20</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CSF&lt;sub&gt;RSP&lt;/sub&gt;</td>
<td>CSF&lt;sub&gt;RSP&lt;/sub&gt;</td>
<td>50</td>
<td>30 (60%)</td>
</tr>
<tr>
<td>CSF&lt;sub&gt;RSP&lt;/sub&gt;</td>
<td>CSF&lt;sub&gt;hu&lt;/sub&gt;</td>
<td>54</td>
<td>17 (31%)</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Concentrations of CSFs used were the same as in the previous experiments shown in Table 1 (bottom three lines) in which a nearly maximal number of colonies were produced, both for the initial stimulation (3 days) and following transferal to methylcellulose plates (4 days).

<sup>b)</sup> The number in the parentheses represents the percentage of clones which grew after being transferred.

(Table 2). However, when the cells were treated with CSF<sub>hu</sub> for the initial 3 days and then transferred to a plate containing CSF<sub>hu</sub>, 44% of the transferred clusters of cells grew into colonies during the following 4 days. An even higher survival rate was observed when the cells were initially stimulated with CSF<sub>RSP</sub> and transferred to a plate containing CSF<sub>RSP</sub>; i.e., 60% of the transferred clusters grew into colonies.

On the other hand, when the CSF used for the initial stimulation was of a different type than that used following transferal, the rate of survival was 27% or 31%, which was notably lower than the rates attained in the presence of the same kind of CSF throughout both the first and the second culture period. Although the cells that were treated with CSF<sub>RSP</sub> for the first 3 days were unable to develop G colonies later in the presence of CSF<sub>hu</sub>, cells primed with CSF<sub>hu</sub> produced G colonies when they were transferred to a plate containing CSF<sub>RSP</sub>.

Mixed-type colonies convert to macrophage colonies with incubation time. On Day 5 of incubation in agar, short glass capillaries were plunged into the agar in order to confine a colony within each capillary. The cells composing the individual colony should stay inside the capillary, while no cells should be able to migrate into the colony from the surrounding agar. For this purpose, compact-type colonies which represented 5–20% of all the colonies were selectively subjected to this treatment.

About a half of the compact colonies on Day 5 in the cultures stimulated by CSF<sub>RSP</sub> were G colonies, while the other half of the compact colonies contained both granulocytes and macrophages in various ratios. When the colonies confined as described above were examined after incubation for further 2 or 4 days, no G colonies were detected inside the capillaries (Table 3).

It was also observed that 27% of the confined colonies in the CSF<sub>RSP</sub>-stimulated cultures had disintegrated during incubation between Day 5 and Day 7. In contrast, the frequency of disintegration of the confined colonies was much less marked in the cultures that were stimulated by CSF<sub>hu</sub>.

In the later stages of culturing, the frequency of M colonies increased as the frequency of GM colonies decreased. Thus, 88% or 90% of the confined colonies were M colonies in the Day 9 cultures stimulated with CSF<sub>RSP</sub> or CSF<sub>hu</sub> respectively.
Observation of granulocyte colonies by electron microscopy. The upper panel of Fig. 1 shows a CSF<sub>RSP</sub> stimulated G colony from a Day-5 culture. Round cells with smooth surfaces were aggregated in the central part of the colony and also scattered around.

When observed by transmission electron microscopy, these immature granulocytes were seen to have a large nucleolar zone and sparse heterochromatin in their nucleus (lower panel of Fig. 1). Ribosomes were abundant in the cytoplasm but primary granules were seen only in limited numbers. Several mitochondria were observed, but vacuoles were not seen in the cytoplasm and the microvilli or ruffles of the cell surface membrane were not well developed.

As these cultured granulocytes matured, the nucleolus decreased in size and the nucleus became lobulated. The number of primary granules in the cytoplasm markedly increased (figure not shown).

In Fig. 2, the upper panel shows a mixed-type colony from a Day-7 culture of CSF<sub>RSP</sub>-stimulated cells. It appears that the granulocytes in this colony were degenerating. Examination of these dying granulocytes by transmission electron microscopy showed that a characteristic condensation of chromatin and extrusion of the nuclear membrane occur. It was also noted that disintegrating mitochondria and granules were retained in the cytoplasm of these cells (lower panel of Fig. 2).

**TABLE 3. DISINTEGRATION OF GRANULOCYTE COLONIES AND CONVERSION OF MIXED-TYPE COLONIES INTO MACROPHAGE COLONIES IN MOUSE BONE MARROW CELL CULTURES**

<table>
<thead>
<tr>
<th>Culture&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Number of plates examined</th>
<th>Number of colonies confined</th>
<th>Number of colonies disintegrated</th>
<th>Number of colonies persisting</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF&lt;sub&gt;RSP&lt;/sub&gt;&lt;sup&gt;b) &lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>Day 5</td>
<td>3</td>
<td>17</td>
<td>—</td>
<td>7</td>
</tr>
<tr>
<td>Day 7</td>
<td>2</td>
<td>15</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Day 9</td>
<td>4</td>
<td>31</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>CSF&lt;sub&gt;Sh&lt;/sub&gt;&lt;sup&gt;b) &lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Day 5</td>
<td>3</td>
<td>32</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Day 7</td>
<td>3</td>
<td>20</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Day 9</td>
<td>3</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a)</sup> On Day 5, short glass capillaries were plunged into the agar in order to confine compact-type colonies. The contents of some of the capillaries were checked immediately (i.e., on Day 5), whereas the contents of other capillaries were examined after incubation of the culture for further 2 or 4 days (i.e., on Day 7 or on Day 9).

<sup>b)</sup> These CSFs were used in the same concentrations as in the experiment shown in Tables 1 (bottom three lines) and 2 in which a nearly maximal number of colonies were produced.
Colony-Stimulating Factor

Fig. 1.
DISCUSSION

The small number of G colonies found in the Day 5, CSF_hu-stimulated cultures (Table 1) may be due to G-CSF that was produced endogenously by the CSF_hu-stimulated macrophages in the culture. Such a secondary effect of added M-CSF has been reported by other workers (6). However, we cannot eliminate the possibility that CSF_hu induced the production of G colonies directly.

Table 1 also shows a synergistic action of the two CSFs. A similar synergy observed with CSF-1 and interleukin 3 was interpreted to mean that the cells responding to interleukin 3 are more primitive than those responding to CSF-1 (3). In the present study, however, positive synergy was observed only when the two CSFs were both used at low concentrations. The two CSFs together at high concentrations induced a smaller number of M colonies to grow than the sum of the M colonies induced by each CSF alone. The result could be explained by the assumption that bipotential cells such as those demonstrated by Metcalf (4) were down-regulated by the over-supply of growth signals. It seems that the retardation of the growth rate was accompanied by the persistence of bipotentiality in this subset of CFUc. The frequency of mixed-type colonies increased and remained high in these cultures until Day 10.

When CSF_RSP was replaced with CSF_hu in the middle of culture period, the number of colonies decreased more profoundly than when CSF_hu was replaced with CSF_RSP (Table 2). The results suggest that 17% (i.e., 44−27=17) of the transferred CFUc were dependent specifically on CSF_hu, while 29% (60−31=29) of the CFUc were dependent on CSF_RSP. It appears that the remaining 27% (44−17=27) or 31% (60−29=31) of the transferred CFUc were reactive to both types of CSFs. If we assume that 25% of the transferred CFUc were lost due to non-specific damages caused by the transfer procedure and that the loss occurred equally among the three types of CFUc mentioned above, we could say that 39% and 23% of the total CFUc were dependent on CSF_RSP and CSF_hu respectively, while the remaining 38% could be stimulated both by CSF_RSP and by CSF_hu.

The results of the implantation experiments also suggest that a majority of CFUc had retained the capability of changing the direction of their differentiation after 3 days of stimulation with CSF_hu. In contrast, CSF_RSP caused an equal number of mixed-type colonies and M colonies to grow in Day-5 cultures while later, M colonies increased in number over mixed-type colonies (Table 1). Replacing CSF_RSP with CSF_hu did not notably accelerate this change (Table 2).

Disintegration of G colonies was demonstrated by confining the compact colonies in glass capillaries (Table 3). The percentage of disintegrated colonies (i.e., 27% or 23%) was smaller than the percentage of CSF_RSP-dependent CFUc obtained as shown above (i.e., 39%). The difference may be due to the fact that compact colonies do not represent all the colonies in the culture, as reported in a previous paper (2). The results shown in Table 3 also suggest that the mixed-type colonies converted into M colonies during incubation. Electron-microscopic examination (Figs. 1 and 2) also supported the assumption that granulocytes disintegrate much sooner than macrophages.

All of the observations reported above are in accord with the following concepts: (i) In addition to CFUc which respond only to CSF_RSP or to CSF_hu, there exist bipotential CFUc which can respond both to CSF_RSP and to CSF_hu. (ii) CSF_RSP can induce the differentiation of the above-mentioned bipotential CFUc into...
macrophages or granulocytes nearly equally, while CSF$_{hu}$ induces the differentiation of these cells into macrophages mainly. (iii) The action of CSF$_{RSP}$ and CSF$_{hu}$ on the bipotential cells is interdependent, while the actions of the two CSFs are independent of each other when they interact with unipotential cells on which only one of the CSFs acts. (iv) Granulocytes stop proliferating sooner than macrophages, and the granulocytes produced start to disintegrate sooner than the macrophages. Thus, G colonies disappear in older cultures, while GM colonies become M colonies as the culture ages.

It could be argued that some of the GM colonies originated from a couple of G and M progenitors that were placed closely together in the plate at the start of the culture. Powell et al. (11) reported evidence for the multicellular origin of such mixed-type colonies. However, the frequency of GM colonies observed in the present study was less than 22% of the total (Table 1). Even if some of these GM colonies were biclonal, it would not discredit the above-mentioned concept about the existence of CFUc which can respond both to CSF$_{RSP}$ and to CSF$_{hu}$. The whole concept mentioned above is illustrated in Fig. 3.

Fig. 3. Summary of the observations on the changes over time in the number of macrophage (M), granulocyte (G) and mixed-type (GM) colonies in cultures of mouse bone marrow cells maximally stimulated with CSF$_{RSP}$ and/or CSF$_{hu}$. Arrows 1–3 show the disintegration of the G colonies, the conversion of the GM colonies to M colonies, and the slow growth of M colonies, respectively. The length of each horizontal column is proportional to the number of colonies of each type in cultures incubated for from 5 to 10 days. CFU-G—granulocyte colony-forming cells; CFU-GM—granulocyte/macrophage colony-forming cells; CFU-M—macrophage colony-forming cells.
REFERENCES


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