Table 3. **Relation of Duration of Leucocyte Cultures to Stimulatory Effect of Precipitate**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Length of Initial Cultures</th>
<th>Control cultures of mixed leucocytes</th>
<th>Homologous leucocyte cultures from single subject containing precipitate from mixed cell cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1 day</td>
<td>914</td>
<td>2,442</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>733</td>
<td>1,896</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>535</td>
<td>3,907</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>600</td>
<td>3,575</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>365</td>
<td>5,230</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>385</td>
<td>4,822</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>300</td>
<td>34,075</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>590</td>
<td>29,591</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>594</td>
<td>29,591</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>763</td>
<td>32,455</td>
</tr>
</tbody>
</table>

(*1) See legend for Table 1.

added was in no instance as great as that found in cultures of mixtures of intact leucocytes (Tables 1 and 2). The precipitate from mixed leucocyte cultures was more active than that from homologous unmixed leucocyte cultures. The precipitate from cultures of leucocytes from a single donor produced no blastogenesis when added to freshly prepared cultures from the same individual. The precipitate from 5-day cultures was more active than that from 3-day cultures, and the precipitate from a 24-hour culture medium showed slight but still significant activity (Table 3). The stimulatory activity increased by increasing the volume of the added medium. When the volume was doubled (2 ml.), a definite increase of stimulatory activity was observed in some, but not all, instances. When the volume was quadrupled (4 ml.), the activity almost uniformly was sharply increased.

These experiments have shown that when human blood leucocytes are cultured, a material is released into the culture medium which is concentrated by ultracentrifugation and which stimulates blastogenesis when added to cultures of homologous leucocytes. The amount of this material released into the culture medium seemed to be related to the numbers of large basophile cells which appeared in the culture medium when the cultures contained blastogenic activity.

The results presented herein permit no conclusions as to whether the presence of this factor precedes and stimulates the production of these blast cells or is produced by these cells. It is not known whether the stimulatory factor is secreted by these or other cells or is a sub-cellular fraction released into the medium as a result of damage or death of cells. At present, the nature of this factor is unknown. The absence of blastogenic effect when this material is added to cultures of autologous leucocytes demonstrates its antigenic specificity. It is possible that this factor may contain transplantation antigen or complexes between RNA and antigens.

In summary: (1) The medium from human leucocytes cultivated in vitro stimulated blastogenesis when added to cultures of homologous leucocytes. (2) Medium from mixed leucocyte cultures was more active than that from homologous unmixed leucocyte cultures, but was less active than mixtures of intact leucocytes from the same donor. (3) Medium from unmixed leucocyte cultures was inactive when added to cultures of autologous leucocytes. Some activity was found in the precipitate obtained after ultracentrifugation.

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**Shinpei Kasakura**

**Louis Lowenstein**

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A Lymphocyte-stimulating Factor produced **in vitro**

Bain, Van and Lowenstein have recently reported that when peripheral leucocytes of two individuals are cultured together, some of the cells enlarge and undergo mitosis. Bach and Hirschhorn have further shown that not only intact cells, but also extracts of leucocytes, disrupted by freezing and thawing, could stimulate cells to divide. Experiments reported in this communication demonstrate that cell-free media obtained from leucocytes in culture contain mitogenic factor(s), and suggest that these factors might be produced by the cells in vitro.

Cells of normal human volunteers were cultured according to the method of Bain et al., with the exception that cultures were initiated with 4 x 10^6 cells derived from one donor in a total volume of 4 ml. TC199 containing 10–20 per cent autologous plasma. Mixed cultures contained a total of 4 x 10^6 cells derived from two donors in the same volume of medium.

To test preparations for blastogenic activity the following procedures were used: Single or mixed cultures from cells of two donors were prepared; leucocytes in single, or in most experiments, in mixed cultures were treated after different periods of incubation by either one of the following procedures: centrifugation, three cycles of freezing and thawing, disruption by ultrasonic vibration, lysis in distilled water or heating at 50°C for 5 min. Cells, cell fragments, or cell cultures were derived from these cultures were transferred for testing to single cultures initiated at the beginning of the experiment. The cultures were examined for blastogenesis 5 days after transfer.

Cell-free culture media used for assays were collected 1–5 days after initiation of the cultures. The tubes containing the cultures were centrifuged at 540g for 10 min. The medium was removed using Pasteur pipettes and was either tested at once or was filtered on a column of Sephadex G-25. The material appearing in the effluent immediately after the void volume was collected and lyophilized. For testing, the powder was dissolved in fresh culture medium and passed through a Millipore filter (pore size 0.22μ). In some experiments the supernatant was frozen and was kept at –20°C until used.

Experiments were carried out to test the effect of 5-fluorodeoxyuridine (5FUdR) and puromycin on mixed leucocyte cultures. (5FUdR was a gift from Hoffman-La Roche, Ltd., Montreal, while puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp.). These substances were added at the beginning of the incubation period, in concentrations of 10 and 2-5 μg/ml, respectively. Media obtained from these cultures and from others to which 5FUdR or puromycin was added at the end of the culture period were filtered on a column of Sephadex G-25, lyophilized, and assayed as already described here.

The activity of any given fraction was assessed in terms of its capacity to induce cell transformation in single cultures. Smeads prepared from cultures after 5 days of incubation were graded using an arbitrary scale of 0 to +++. In each experiment every preparation was tested in duplicate or triplicate cultures and smears from some experiments were also scored independently. (We thank Dr. B. Bain, of the Department of Haematology of this Hospital, for examining these slides.)

In six experiments, the extracts of 2–40 x 10^6 freshly drawn leucocytes, disrupted by either of the three methods described, gave consistently negative results, whereas strong reactions were obtained using as few as 2 x 10^6 intact cells (Table 1). Cells heated at 50°C for 5 min also failed to stimulate cells to transform. In contrast to the negative results obtained with freshly drawn disrupted cells, leucocyte cultures (cells and medium) subjected to freezing and thawing after 24 h of incubation gave consistently positive results. Cell-free culture media...
removed from leukocyte cultures after 1–5 days of incubation also possessed stimulating capacity.

Media from single cultures were active but were less potent than those obtained from mixed cultures. Media from single cultures added to autologous cells displayed no activity.

Mitogenic activity appeared to accumulate in the culture media; the longer the incubation period, the more activity was recovered. Furthermore, aliquots of fresh culture media put in contact with the same cells on two or three consecutive days each contained activity, indicating that mitogenic activity is released to the medium continuously for at least 4 days.

The mitogenic activity could be due to a preformed factor present in cells and released either continuously or on cell death, or it could be synthesized by the cells during incubation. Failure to find activity after cell disruption by three different methods would make release during culture unlikely. Indeed, activity could not be recovered from cells disrupted when drawn or after 4 days of incubation. This latter is especially interesting in view of the high levels of activity found in the culture media derived from the same tubes (Table 2). The ability of the cells to elaborate mitogenic activity remained after 4 days of incubation since leukocytes mixed after having been kept in single cultures for 4 days exhibited strong blastogenesis. Finally, freezing and thawing itself would not be expected to deactivate the factor since whole cultures, comprising cells and media subjected to this treatment, yielded activity.

Thus, the experiments described do not support the view that preformed mitogenic factor is present in the cells in concentrations detectable by the methods used. The results presented can be more readily interpreted by assuming that the mitogenic factor is synthesized in vitro. This assumption is supported by the results obtained using inhibitors of protein and nucleic acid synthesis; both puromycin and 5FUdR inhibited blastogenesis and media obtained from these cultures were devoid of mitogenic activity. If mitogenic factors were preformed and released from cells during the culture period, then one would have expected to recover activity from these media.

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HISTOCHEMISTRY

Fluorochrome Stains for Histological Diagnosis of Visceral Mycoses

Fluorescence microscopy facilitates the demonstration of various fungi in tissues, because it is possible to examine larger parts of the tissue-sections in a short time. Simple stains with fluorochrome dyes—acridine orange for example—are inadequate for diagnostic use. Two methods which have hitherto been used for the demonstration of poly saccharides proved useful in staining fungi.

(1) Among the fluorescent Schiff-type reagent (Kasten) acridine yellow after oxidation with periodic acid gives the brightest fluorescence. The strength of the reaction in the different tissue-forms of fungi in Europe is registered in Table 1. A fluorescence specifically weakly appears in the tissue, unless the fraction of the dye is separated in glacial acetic acid. In practice