**In Vitro Production of Monoclonal Human Rheumatoid Factors**

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**ABSTRACT**

Lymphocytes from rheumatoid arthritis patients have been fused with drug-marked human myeloma cell lines and hybrids selected which secrete rheumatoid factors. Limiting dilution and soft agarose plates have been used to clone these hybrids, but so far stable clones have not been obtained. Epstein-Barr virus has been used to transform lymphocytes, and clones which secrete rheumatoid factors have been obtained.

**Introduction**

Rheumatoid factors (RF) are antibodies to determinants on the FC fragment of IgG. They occur in most patients with rheumatoid arthritis (RA) and also in patients with certain infections, e.g., subacute bacterial endocarditis, schistosomiasis. Recently, it has become clear that RF synthesized in vitro have different properties than RF found in serum, particularly in terms of affinity and IgG subclass specificity.

These in vitro synthesized RF are felt to represent physiologically active RF in RA, and studies on their properties might provide important clues to the pathogenesis of RA. To provide sufficient amounts of in vitro synthesized RF for these studies, methods to prepare cell lines which secrete RF are being investigated in our laboratory.

Two approaches are being used to establish these cell lines. The best way is to use hybridoma technology and fuse lymphocytes from RA patients with drug marked human myeloma cell lines. An alternative technique is to transform the lymphocytes with Epstein-Barr virus (EBV). In both of these approaches, it is necessary to select cells for RF secretion, clone them, and then establish stable cell lines from the clones.

Three human cell lines are available for fusion with patient's lymphocytes: the GM 4672 cell line which secretes IgG, kappa; the U 266 cell line which secretes IgE, lambda; and the LICR LON HM-2 cell line which secretes IgG, kappa. These three cell lines are deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase and hence are unable to grow in medium of hypoxanthine, aminopterin and thymidine (HAT).
Methods

Human Mononuclear Cells

Mononuclear cells were obtained from rheumatoid arthritis patients by separation of peripheral blood on Ficoll-Hypaque. The cells were >95 percent viable.

Myeloma Cell Lines

Each of the cell lines used was maintained in culture and checked for HAT sensitivity. The GM 4672 cells were pre-cultured with 6-thioguanine for seven days prior to use to eliminate any revertants.

Hybridization

Mononuclear cells (2 × 10⁷) were mixed with myeloma cells (2 × 10⁷) and pelleted. The pelleted cells were resuspended gently in one ml of 35 percent polyethylene glycol 1500 in RPMI* 1640 at 37°C, diluted slowly to 30 ml with RPMI 1640 over about five minutes, and pelleted. The cells were then resuspended to 10 ml in RPMI 1640 containing 10 percent fetal calf serum (FCS) and HAT, and seeded into one ml wells for culture. The medium was enriched with either endothelial cell growth factor (ECGF), (50 μl per well for the U 266 cells) or with insulin (0.5 μ per ml) and oxalacetic acid (0.132 mg per ml) for the GM 4672 cells, to promote growth. The medium was changed at days 1 and 4, and thereafter as needed. After two weeks, the selection medium was changed to HT, and after a further week the cells were cultured in RPMI 1640 + 10 percent FCS and growth factors. The medium was replaced when necessary, and the harvested medium analyzed for RF and for total immunoglobulins by enzyme labeled immunosorbant assay (ELISA).

Epstein-Barr Virus Transformation

Mononuclear cells (2 × 10⁷) were cultured in EBV conditioned medium at 2 × 10⁶/ml for two weeks, then fed with RPMI 1640 + 10 percent FCS as necessary. The EBV conditioned medium consisted of RPMI 1640 + 10 percent FCS + 50 percent spent medium from B95-8 cells.†

Cloning

Cells secreting RF were cloned by limiting dilution, at 10, 3, and 0.3 cells per well, or on soft agarose plates.† Cells were suspended to a final concentration of 10⁸ to 10⁶ per ml in 0.35 percent agarose† containing RPMI 1640 + 20 percent FCS and layered on top of 0.45 percent agarose in the same medium, which had been layered on top of a feeder layer of confluent G 152 cells, which are hypoxanthine guanine-phosphoribosyltransferase (HGPRT) deficient human fibroblasts.‡

ELISAS

Flexible vinyl plates§ were coated with either rabbit IgG, the IgG fraction of goat anti-human immunoglobulins, or bovine serum albumin (BSA, negative control), at one μg per ml in carbonate/bicarbonate buffer, 0.05M, pH 9, and 4°C overnight. The plates were washed with phosphate buffered saline, pH 7.4, containing 0.1 percent Tween 20. Unbound sites were blocked with one percent BSA for two hours at 20°C, and the

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* Roswell Park Memorial Institute, Buffalo, NY.

† Received from Dr. W. Henle, Temple University Medical School, Philadelphia, PA.

‡ SeaPlaque, FMC Corp., Rockland, ME.

§ NIGMS Human Genetic Mutant Cell Repository, Camden, NJ.

§ Cooke Microtiter, Alexandria, VA.
plates were incubated with cell supernatants for two hours at 20°C. The plates were washed again, incubated with horseradish peroxidase labelled goat anti-human immunoglobulins for two hours at 20°C, washed and substrate added. The RF ELISA was sensitive to one ng per ml and the total immunoglobulins ELISA to 10 ng per ml.

RESULTS

Initial experiments were performed with the GM 4672 cell line. Mononuclear cells were fused with GM 4672 cells and seeded at $2 \times 10^5$ to $2 \times 10^6$ cells per well, selected in HAT medium and grown. Production of RF was detected in cells from 7 of 19 patients, with an average of 38 percent of the wells positive for RF (see table I). Twelve of these (11 percent) were strongly positive for RF on successive tests. All were negative when tested against BSA coated wells or if polyethylene glycol (PEG) was omitted from the fusion. However, attempts to clone these cells by limiting dilution were unsuccessful; the hybrids grew very slowly and appeared to be viable only when in large clumps. The use of conditioned medium and various feeder cells (human monocytes, mouse peritoneal exudate cells, G 152 cells) did not improve the growth rate or lead to successful cloning.

Because of the problems experienced with the growth rate and cloning of the hybrids obtained with the GM 4672 cells, the use of the U 266 cell line for the hybrids was investigated by the present authors. These are inherently better cells to use for this project since they secrete IgE, which will not react with the RF. The U 266 cells grow very well in culture, and hybrids between these cells and patients' lymphocytes have shown much more robust growth than hybrids formed with the GM 4672 cells. Results of five fusions performed with the U 266 cells are shown in table II. Good immunoglobulin production was obtained in 40 percent of the wells and RF secretion was obtained in 10 percent of the wells. Cells from the RF positive wells, together with cells from a negative well, were cloned on soft agarose plates. Discrete colonies were picked off and grown up. The results of the cloning are shown in table III. Colonies picked off from the agarose were chosen arbitrarily; they were not preselected for RF synthesis. Of 58 colonies grown up, four secreted RF but were polyclonal, not monoclonal, when the secreted immunoglobulins were analyzed by double diffusion using monospecific antisera to human immunoglobulins.

| TABLE I |
| Fusions Between Human Mononuclear Cells and GM 4672 Cells |
| Experiment | Seeding Density | Wells RF+ |
| H - 6 | $2 \times 10^5$ | 54/96 |
| H - 7 | " | 28/96 |
| H - 8 | $2 \times 10^6$ | 21/50 |
| H - 12 | " | 1/10 |
| H - 13 | " | 3/10 |
| H - 14 | " | 6/10 |
| H - 15 | " | 1/20 |
| H - 15P | " | 0/20 |
| Average | | 38% |

RF = Rheumatoid factors

| TABLE II |
| Fusions Between Human Mononuclear Cells and U 266 Cells |
| Experiment | Seeding Density | Wells* RF+ | Wells† Ig+ |
| H - 21 | $2 \times 10^6$ | 0/10 | 10/10 |
| H - 30 | " | 2/10 | 5/10 |
| H - 31 | " | 3/10 | 3/10 |
| H - 32 | " | 0/10 | 1/10 |
| H - 33 | " | 0/10 | 1/10 |

*Positive on three successive weekly tests
†Average Ig concentration 1 mg per ml
RF = Rheumatoid factors
TABLE III

Soft-Agarose Cloning of Hybrids of Human
Mononuclear Cells and U 266 Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cloning Density</th>
<th>Clones RF+</th>
</tr>
</thead>
<tbody>
<tr>
<td>H - 21</td>
<td>10^5/ml</td>
<td>1/3 *</td>
</tr>
<tr>
<td>H - 30 (a)</td>
<td>10^5/ml</td>
<td>0/5</td>
</tr>
<tr>
<td>H - 30 (b)</td>
<td>10^6/ml</td>
<td>1/24*</td>
</tr>
<tr>
<td>H - 31 (a)</td>
<td>&quot;</td>
<td>1/6 *</td>
</tr>
<tr>
<td>H - 31 (b)</td>
<td>&quot;</td>
<td>0/6</td>
</tr>
</tbody>
</table>

*Polyclonal immunoglobulin production
RF = Rheumatoid factors

One of these four RF positive colonies was obtained from fusion H-21 in which RF secretion initially had not been detected. These positive colonies have been subcloned, but so far we have not obtained any subclones which secrete RF.

As an alternative to the hybridoma procedure, Epstein-Barr virus was used to transform B cells from RA patients. Five transformation experiments are shown in table IV. In general, most of the cells grew well, with polyclonal production of immunoglobulins in 85 percent of the wells. Secretion of RF was detected in five of the wells (10 percent). Secretion of RF by H-19 cells was maintained for five months before the cells became exhausted and died out; the average amount of RF secreted into the medium was one μg per ml per 10^6 cells. Attempts to fuse the RF secreting cells with the myeloma lines always resulted in a shut-off of RF secretion. In experiment H-40, two wells grew very strongly, secreting up to 10 μg per ml of RF, representing approximately 50 percent of total immunoglobulins secreted. Cells from these two wells have been cloned by limiting dilution. Six clones have been obtained which secrete monoclonal RF, and these clones are being expanded for characterization of the secreted RF.

Discussion

These results demonstrate that cells which secrete RF can be obtained by either the hybridization or EBV transformation procedures. Results obtained with two cell lines, GM 4672 and U 266, indicate that the RF production by the hybrids persists for several weeks before being lost. This loss of RF production by the hybrids is most probably due to overgrowth by non-secreting cells, which can be avoided only by early cloning. However, cloning has presented many problems. The GM 4672 cells exhibited a strong tendency to clump. Most of the viable cells were in large aggregates and dispersion of these aggregates prior to cloning resulted in non-viable cells. The use of several different feeder cells, with conditioned medium, did not solve this problem. The U 266 cells did not exhibit this tendency towards clumping, and grew well from very small innocula. Again, transient RF production was observed, and several wells secreted RF for several weeks but then stopped. Attempts to clone these cells were unsuccessful. There was no difficulty in obtaining growth of discrete colonies on soft agarose. However, when these colonies were picked off the agarose and grown, they secreted polyclonal immunoglobulins, even when subcloned. Secretion of RF by these colonies persisted for sev-
eral weeks but then stopped before definite clones could be obtained.

Human B cells transformed by EB virus grow well in culture and secrete immunoglobulins, although not generally to the same extent as does a hybridoma cell line. However, an EBV transformed cell line secreting monoclonal RF might provide a cell line suitable for fusion with an appropriate myeloma cell line in order to increase the amount of RF synthesized, and, possibly, to give a more stable and durable cell line. The cells obtained from experiment H-40 may well serve this function. Cells in two of twelve wells secreted RF; we were able to clone these cells by limiting dilution, and obtained several strongly growing clones which secreted monoclonal RF.

Acknowledgments

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References