Evidence for the Presence of Anti-Burkitt Tumor Globulins in Pooled Human Immune Globulins

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SUMMARY

Each of 20 commercially prepared lots of human immune globulins tested produced specific immunofluorescence reactions with EB3 cells, a continuous cell strain derived from Burkitt's lymphoma. The immunofluorescence titers ranged from 1:80 to 1:640. Each of these 20 lots also suppressed growth of EB3 cell cultures. Complement was not required for these reactions. There was good correlation between the immunofluorescence and growth suppression titers. Suppression of cellular growth was related to the extent of EB virus infection in 6 human lymphoma and leukemia cell culture lines tested. The globulins responsible for the growth suppression and immunofluorescence reactions were partially 2-mercaptoethanol sensitive and were absorbed by EB3 cells but not by human amnion cells.

INTRODUCTION

Recent reports suggest that a specific immunological factor is present in the sera of patients suffering from Burkitt's lymphoma. Klein et al. (11) observed positive immunofluorescent reactions at the surface of freshly excised Burkitt tumor cells with sera from patients with Burkitt tumors and with sera from non-Burkitt tumor patients. These findings were supported by Burkitt (1) and Ngu (13), who observed regression of tumors in Burkitt tumor patients following administration of plasma from Burkitt tumor patients in chemotherapeutic remission. Henle and Henle (7) found that sera from Burkitt tumor patients and from some normal donors gave positive immunofluorescent reactions, in both direct and indirect tests, with 5 cell culture lines derived from Burkitt tumors.

Herberman and Fahey (10) have described a complement-dependent cytotoxic antibody in Burkitt tumor patient and normal human serum and, recently, Herberman (9) has presented results of absorption tests which indicated that cellular antigenic activity was not confined to tissues derived from cancers but was detectable in small quantities in normal human tissues.

In the present study, 20 lots of commercially prepared IHG2 were examined both for immunofluorescence and for noncomplement-dependent growth-suppression activity with 6 cell culture lines derived from Burkitt's lymphomas and leukemias.

MATERIALS AND METHODS

Established Cell Lines. The source and origin of the continuous cell lines used are listed in Table 1. These cell lines were examined and found to be free from Mycoplasma (M. F. Barile, NIH, personal communication). Except for the HA, these lines were maintained for periods of 2 months to 1 year in EMEM-10 FCS.

IHG. Twenty unselected lots of IHG produced from plasma by 5 different manufacturers were examined. Each lot represents the globulin fraction from at least 1000 donors and was commercially prepared in compliance with the USPHS Regulations for Immune Serum Globulin (Human) (USPHS Publication 437). Absorption of IHG was accomplished by suspending 16 X 10^6 test cells/ml of a 1:5 dilution and incubating at 37° for 3 hr and at 4° overnight. The suspension was centrifuged to remove the cells (20 min, 2000 X g) and the absorbed IHG was stored at 4° until use.

Immunofluorescence. The indirect fluorescent antibody staining procedure used was described by Henle and Henle (7). Serum from a Burkitt lymphoma patient was obtained from Dr. H. Orr, NIH. Fluorescein isothiocyanate-conjugated horse anti-human globulin was purchased from Roboz Surgical Instrument Company, Washington, D. C. Negative controls consisting of EB3 cells

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The abbreviations used are: IHG, human immune globulin; HA, human amnion cells; EMEM-10 FCS, Eagle's minimal essential medium containing 10% unincactivated fetal calf serum, glutamine (4.0 μM), penicillin (200 units/ml), and streptomycin (200 μg/ml); PBS, phosphate-buffered saline (Dulbecco's); GSI, growth suppression index; IHG-2, IHG Preparation 2.
Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>References</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB3</td>
<td>Burkitt lymphoma (Africa)</td>
<td>Epstein et al. (5)</td>
<td>Flow Laboratories, Rockville, Md.</td>
</tr>
<tr>
<td>AL3</td>
<td>Burkitt lymphoma (Africa)</td>
<td>Rabson, personal communication</td>
<td>Flow Laboratories, Rockville, Md.</td>
</tr>
<tr>
<td>SK-L2</td>
<td>Lymphoblastic leukemia</td>
<td>Clarkson et al. (2)</td>
<td>Flow Laboratories, Rockville, Md.</td>
</tr>
<tr>
<td>SK-L3</td>
<td>Myelomonocytic leukemia</td>
<td>Clarkson et al. (2)</td>
<td>Flow Laboratories, Rockville, Md.</td>
</tr>
<tr>
<td>HA</td>
<td>HA (FL)</td>
<td>Fogg and Lund (6)</td>
<td>DBS Tissue Culture Unit</td>
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</table>

Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>IHG preparation no.</th>
<th>Viable cell titer/ml (X 10^5) after incubation time (hr) of</th>
<th>Adjusted GSI</th>
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<td>5.5 11.2 0.68</td>
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</tr>
<tr>
<td>4</td>
<td>5.3 8.8 0.53</td>
<td>4.8 7.2 0.60</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.8 14.3</td>
<td>5.5 4.8</td>
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<td>6</td>
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<td></td>
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<td>3.8 6.2 0.83</td>
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</tr>
<tr>
<td>12</td>
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<td>5.6 2.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.9 21.3</td>
<td>3.6 3.4</td>
<td></td>
</tr>
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<td>3</td>
<td>3.8 6.2</td>
<td>3.3 13.8 0.40</td>
<td></td>
</tr>
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<tr>
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<td>None</td>
<td>5.2 1.7</td>
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<td>5.2 1.9 0.87</td>
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<td>5.5 1.7 0.67</td>
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<td>5.5 1.7</td>
<td>5.5 11.7 0.36</td>
<td></td>
</tr>
<tr>
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<td>None</td>
<td>4.6 20.1</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>4.8 7.3</td>
<td>5.5 1.7 0.67</td>
<td></td>
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<tr>
<td>18</td>
<td>5.7 12.0</td>
<td>5.7 12.0 0.77</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>5.4 10.3</td>
<td>5.4 10.3 0.82</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>5.0 15.6</td>
<td>5.0 15.6 0.27</td>
<td></td>
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<tr>
<td>21</td>
<td>5.0 12.5</td>
<td>5.0 12.5 0.68</td>
<td></td>
</tr>
</tbody>
</table>

IHG-2 was included in subsequent experiments as a positive control. In each experiment GSI results are adjusted to growth suppression obtained with IHG-2.

exhibited clumping (SK-L2 and SK-L3) were shaken to disperse the cells before adding the dye.

2-Mercaptoethanol Treatment. IHG pools were diluted 1:1 with PBS, and 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N.Y.) was added to a final concentration of 0.1 M. The solutions were incubated at room temperature for 2 hr followed by dialysis against PBS for 20 hr at 4°.

Determination of GSI. Duplicate cultures were prepared in 2-oz prescription bottles by suspending approximately 6 X 10^6 viable EB3 cells in either 12.0 ml EMEM-10 FCS (for controls) or 12.0 ml EMEM-10 FCS plus 0.5% (v/v) IHG (for immune globulin-exposed cells). Viable cell titers of the cultures were determined immediately and at 24-hr intervals thereafter. The average difference in viable cell content of the treated cultures compared to the control cultures after 96-hr incubation was selected as the index of growth suppression.

Calculation of GSI. The GSI was calculated as follows:

\[
GSI = \frac{(\text{average 0 time IHG-treated culture viable cell titer}) \text{ minus} \ (\text{average 0 time control culture viable cell titer})}{(\text{average 96-hr IHG-treated culture viable cell titer}) \text{ minus} \ (\text{average 96-hr control culture viable cell titer})}
\]

Thus, for example, from Table 2, IHG-2 Experiment 1, the GSI is:

\[
\frac{(4.6 \times 10^5) - (1.9 \times 10^5)}{(4.6 \times 10^5) - (16.5 \times 10^5)} = 0.88
\]

Table 3

Fluorescence potency and GSI of 20 lots of IHG

<table>
<thead>
<tr>
<th>IHG preparation no.</th>
<th>Fluorescence titer*</th>
<th>GSI†</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1:640</td>
<td>1.10</td>
</tr>
<tr>
<td>14</td>
<td>1:480</td>
<td>0.93</td>
</tr>
<tr>
<td>2</td>
<td>1:640</td>
<td>0.88</td>
</tr>
<tr>
<td>15</td>
<td>1:640</td>
<td>0.87</td>
</tr>
<tr>
<td>11</td>
<td>1:320</td>
<td>0.83</td>
</tr>
<tr>
<td>19</td>
<td>1:640</td>
<td>0.82</td>
</tr>
<tr>
<td>1</td>
<td>1:320</td>
<td>0.80</td>
</tr>
<tr>
<td>18</td>
<td>1:320</td>
<td>0.77</td>
</tr>
<tr>
<td>8</td>
<td>1:320</td>
<td>0.68</td>
</tr>
<tr>
<td>21</td>
<td>1:160</td>
<td>0.68</td>
</tr>
<tr>
<td>17</td>
<td>1:320</td>
<td>0.67</td>
</tr>
<tr>
<td>4</td>
<td>1:160</td>
<td>0.62</td>
</tr>
<tr>
<td>5</td>
<td>1:160</td>
<td>0.60</td>
</tr>
<tr>
<td>13</td>
<td>1:160</td>
<td>0.53</td>
</tr>
<tr>
<td>3</td>
<td>1:160</td>
<td>0.51</td>
</tr>
<tr>
<td>12</td>
<td>1:160</td>
<td>0.40</td>
</tr>
<tr>
<td>16</td>
<td>1:160</td>
<td>0.36</td>
</tr>
<tr>
<td>20</td>
<td>1:160</td>
<td>0.27</td>
</tr>
<tr>
<td>7</td>
<td>1:80</td>
<td>0.27</td>
</tr>
<tr>
<td>6</td>
<td>1:80</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*Highest dilution which produced positive immunofluorescence.
†Rearranged from Table 2.
Therefore, EB3 cells exposed to IHG-2 grew to only 12% of the titer of the untreated control cells by 96 hr. For GSI determinations on the other 19 lots of IHG, IHG-2 was included in each experiment and results were adjusted to those obtained with IHG-2.

![Chart 1. Results of daily viable cell titer determination for 5 IHG-treated cultures.](image)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IHG addition</th>
<th>Viable cell titer/ml (× 10^6) after incubation time (hr) of 0 96</th>
<th>GSI (adjusted)</th>
<th>Immunofluorescence: fluorescing cells in culture (%)</th>
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</thead>
<tbody>
<tr>
<td>EB3</td>
<td>None</td>
<td>7.9 27.6</td>
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<td>0.68</td>
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<td>7.1 2.0</td>
<td></td>
<td></td>
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<tr>
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<td>5.2 16.2</td>
<td>0.69</td>
<td>0.38</td>
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<tr>
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<td>4.3 2.3</td>
<td></td>
<td></td>
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<tr>
<td>SK-L2</td>
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<td>2.9 3.4</td>
<td>0.59</td>
<td>&lt;0.1</td>
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<td>3.0 1.5</td>
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<td>0.43</td>
<td>&lt;0.1</td>
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<tr>
<td></td>
<td>2</td>
<td>5.0 4.9</td>
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<td></td>
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<tr>
<td>AL3</td>
<td>None</td>
<td>3.8 5.2</td>
<td>0.40</td>
<td>N.D.*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.3 3.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4

**Suppression of growth of other continuous cell lines by IHG-2**

* N.D., not determined.

**RESULTS**

Chart 1 presents results of daily viable cell titer determination for 5 IHG-treated cultures. Each lot of IHG suppressed EB3 cell culture growth when incorporated into the tissue culture medium at a concentration of 0.5% (v/v). Fifteen additional lots were examined similarly; these results are presented in Table 2.

**Fluorescence Titer (Potency) of IHG Preparations.** The 20 IHG lots were also examined by the indirect immunofluorescence procedure for reactivity to EB3 cells. The EB3 strain of cells produced positive reactions with Burkitt tumor patient serum in 0.68% of the culture cells. Two-fold dilutions (PBS) of the IHG preparations, ranging from 1:10 through 1:1280, were examined. The results, presented in Table 3, indicate a positive correlation between immunofluorescence potency and GSI of the IHG preparations.

**Absorption of IHG.** HA and EB3 cells were used in an attempt to absorb the globulin(s) responsible for immunofluorescence and for growth suppression. The results of this experiment are reported in Table 5 and indicate that absorption with EB3 cells, but not with HA cells, partially reduces both the GSI and the fluorescence titer of IHG. The increased GSI obtained with HA-absorbed IHG may indicate the removal of nonspecific globulins which obscure the cytotoxic reaction.

**Effect of 2-Mercaptoethanol on Growth Suppression by IHG.** 2-Mercaptoethanol has been reported to cause dissociation of globulins IgM and IgA while leaving globulin IgG intact (12). Immediately following dialysis, the 2-mercaptoethanol-treated IHG was incorporated (0.50%,...
IHG-2 (nondialyzed)
IHG-2 (2-mercapto-
v/v) into the EMEM-10 FCS media, EB3 cells were
were found to suppress growth of EB3 cells and other
cells derived from Burkitt's lymphoma and human leu-
hemias. The active globulin was nondialyzable, was par-
tially sensitive to 2-mercaptoethanol, was not dependent
upon complement, and was capable of staining cells con-
taining EB antigen. The pools of globulins with a high
fluorescence titer also contained a higher titer for growth
suppression. No similar relationship has been demon-
strated for polio, diphtheria, pertussis, or typhoid anti-
bodies.

Heat Inactivation. Heat inactivation (56°, 30 min) of ei-
ther IHG or fetal calf serum or both did not appreciably
affect the growth suppression potency of IHG, indicating
that complement is not required for either reaction.

DISCUSSION

Twenty lots of commercially pooled human globulins
were found to suppress growth of EB3 cells and other
cells derived from Burkitt's lymphoma and human leu-
kemias. The active globulin was nondialyzable, was par-
tially sensitive to 2-mercaptoethanol, was not dependent
upon complement, and was capable of staining cells con-
taining EB antigen. The pools of globulins with a high
fluorescence titer also contained a higher titer for growth
suppression. No similar relationship has been demon-
strated for polio, diphtheria, pertussis, or typhoid anti-
bodies.

The variations in titer of active globulins in the differ-
ent lots were surprisingly great. We cannot account for
this variation except to note that lots from the same man-
ufacturer tended to be consistently high, or low, in activ-
ity suggesting the involvement of a manufacturing pro-
cedure.

A number of the cell lines used in this study were ini-
tially established in media which contained human serum.
This suggests that sensitivity to a growth-suppressing globulin has arisen since discontinuation of the use of hu-
man serum in the culture medium or, alternatively, a fortuitous choice of human serums with low growth-sup-
pressing globulin content. Because of the large numbers
of donors involved in the preparation of each lot of human
immune globulin, the individual donor input of active
globulins cannot be estimated.

The growth-suppressing and fluorescence-producing
activity of the globulin pools was partially absorbed by
EB3 cells but not by HA cells. Also, the extent of growth
suppression was generally related to the severity of EB
antigen infection of the cell strains tested. Both observ-
ations suggest the involvement of virus-related cellular anti-
gens.

The IHG pools represent globulins from a large num-
ber of donors with broad antigenic experiences. Henle et al. (8) have implicated EB antigen in the etiology
of infectious mononucleosis. They have also used hyper-
immune human globulins to stain cells derived from Bur-
kitt tumor patient serum has been reported by Osunkoya
cence tests.

The presence of a cell growth-inhibitory factor in Bur-
kitt tumor patient sera has been reported by Osunkoya
(14). However, no correlation could be shown between
growth inhibition potency and cell membrane fluores-
cence potency.

Observations reported here indicate that there are globu-
lins in human globulin pools specific for EB antigen-con-
taining cells which suppress the in vitro growth of these
cells and that the globulins responsible for specific flu-
orescence may be the same globulins responsible for in vitro growth suppression.

The observations of temporary regression of Burkitt
tumors following infusion of serum from Burkitt tumor
patients in chemotherapeutic remission (1, 13) suggest
that passive immunity can be successfully transferred.
Observations presented here suggest that similar results
might be obtained with "normal" immune human globu-
lin.

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