Inhibition of Antibody-Complement-mediated Killing of Tumor Cells by Hormones

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SUMMARY

Line 1, a chemically induced guinea pig hepatoma, is susceptible to killing by anti-Forssman immunoglobulin M antibody and guinea pig complement. When these tumor cells are pretreated at 37°C with 10^{-4} to 10^{-11} M concentrations of the polypeptide hormone insulin, with the catecholamine L-epinephrine-HCl, or with the glucocorticoid steroids hydrocortisone sodium succinate or prednisolone sodium succinate, the cells show a marked reduction in their susceptibility to killing by antibody and guinea pig complement; pretreatment at 0°C is ineffective. Similar results were obtained with another antigenically distinct guinea pig hepatoma (line 10) when tested with anti-Forssman immunoglobulin M or specific antitumor antibodies and human complement. The ability of the hormones to render the cells resistant is dependent on time, temperature, and hormone concentration. The effect of hormone treatment is maximal between 30 and 60 min and is reversible within 4 hr even in the continued presence of hormone. Treatment of line 1 cells with up to 10,000-fold greater concentrations of the less biologically active or inactive analogs, DL-epinephrine, β-estradiol, testosterone, or proinsulin has no effect on the susceptibility of the cells to killing by antibody and guinea pig complement. The effect of hormone treatment is not due to a direct inactivation of bound or fluid-phase complement components by the hormones or to a decrease in the ability of the cells to bind complement-fixing antibody.

INTRODUCTION

The diethylnitrosamine-induced guinea pig hepatoma, designated line 1, is susceptible to killing by anti-Forssman antibody and GPC but not by specific antitumor antibody and GPC (24). The antigenically distinct line 10 hepatoma, when sensitized with either antibody, is susceptible to killing by HUC but not by GPC (24). Pretreatment of the tumor cells with certain metabolic inhibitors (29, 31, 32) renders the cells susceptible or increases their susceptibility to killing by antibody and GPC. It was concluded from these studies that resistance to cytotoxicity may be due to intrinsic properties of the cells to prevent complement-mediated damage to their surface (5, 6, 29-32).

Antibody-complement-mediated attack results in morphological and chemical changes of the cell membrane (15). Since steroid and polypeptide hormones have been shown to increase the stability and/or to decrease the permeability of cell membranes (1, 2, 10, 14, 16, 19, 22, 36, 38-40), a number of these hormones were examined for their ability to modify the effects of in vitro humoral immune attack. The purpose of this paper is to document the various aspects of hormone treatment of tumor cells and to show that, following treatment with certain hormones, the cells are less susceptible to killing by antibody and complement.

MATERIALS AND METHODS

Animals. Inbred Sewall-Wright strain 2 male guinea pigs were obtained from the Frederick Cancer Research Center, Frederick, Md. The animals weighed approximately 250 g. Cells and Reagents. Sheep erythrocytes were collected and washed as previously described (26).

Two antigenically distinct diethylnitrosamine-induced hepatic tumors (ascites form), line 1 and line 10 passed in strain 2 guinea pigs, were collected as described (25, 42, 43) and suspended in RPMI 1640-20% FCS. All cytotoxicity assays were performed in VBS-gel.

The hormones used in these studies were: hydrocortisone sodium succinate (Upjohn Co., Kalamazoo, Mich.; Lot 343-BR); prednisolone sodium succinate (National Cancer Institute, Cancer Therapy Evaluation Branch, Bethesda, Md.; NSC 9151); bovine insulin (Sigma Chemical Co., St. Louis, Mo.; Lot 24C-3130; 243 IU/mg); L-epinephrine methyl ether HCl (Sigma; Lot 848-5140); DL-epinephrine (Sigma; Lot 65C-0011); Δ1,3,5(10)-estratrien-3,17β-diol (β-estradiol; Sigma; Lot 45C-0100); Δ4-androsten-17β-ol-3-one (testosterone; Sigma; Lot 113C-1460); pork insulin, chicken insulin, pork proinsulin, pork DAA-insulin and the A and B chains of pork insulin (courtesy of Dr. Pierre De Meyts, Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH).

Treatment of Tumor Cells with Hormones. Unless otherwise specified, tumor cells (5 x 10^5 cells/ml) were cultured in 10-ml volumes of RPMI 1640-20% FCS with the appropriate concentrations of hormone in 100- x 15-mm plastic
bacteriological Petri dishes (Falcon Plastics, Oxnard, Calif.) at 37° under 5% CO₂-air.

After incubation, aliquots of the cell cultures were drawn off, dispersed into 12- x 75-mm polypropylene capped tubes (No. 2063; Falcon), washed 5 times in VBS-gel, and tested for their susceptibility to killing by antibody and complement. All cells that were to be reincubated in culture medium without added hormone were first washed, transferred to a new set of 12- x 75-mm capped tubes, and resuspended in fresh RPMI 1640-20% FCS.

**Sera and Antisera.** Rabbit antiserum to sheep Forssman antigen was prepared as described previously (26). Ten ml were heated for 30 min at 56° and passed through a column of Sephadex G-200. The IgM-rich fraction was collected, pooled, and stored at −20° until used.

Tumor-specific rabbit anti-line 1 and anti-line 10 antisera were prepared as previously described (4). The antisera were heated for 30 min at 56°, absorbed with sheep erythrocytes and with the appropriate tumor cells, filtered, and stored at −20°.

Normal GPC (JEM Research Products, Inc., Kensington, Md.) and normal HUC, absorbed at 0° with sheep erythrocytes and line 1 and line 10 tumor cells, were stored at −30°.

**Antibody-Complement-mediated Cytotoxicity of Tumor Cells.** This method for determining antibody-complement-mediated killing has been described (25). Briefly, 0.1 ml of diluted antiserum was added to 0.1 ml of tumor cells (10⁶/ml). After incubation for 30 min at 30° the cells were washed with VBS-gel and incubated with 0.1 ml GPC (diluted 1:8) or HUC (diluted 1:10) for 60 min at 37°. One-tenth ml of 0.4% trypan blue was added, and those cells taking up the dye were counted visually. Controls included cells plus complement alone and cells plus buffer alone. The error of the test is less than ±10%.

**RESULTS**

**Antibody-Complement-mediated Cytotoxicity of Hormone-treated Tumor Cells.** Five million line 1 tumor cells in 10 ml RPMI 1640-20% FCS, containing various concentrations of L-epinephrine-HCl, insulin, prednisolone sodium succinate, or hydrocortisone sodium succinate, were incubated at 37° under air-5% CO₂. Control cultures were prepared concurrently with cells in tissue culture medium alone. After 30 min, the cells were washed 3 times with 10 ml RPMI 1640-20% FCS and 2 times with 10 ml VBS-gel, resuspended to 10⁶ cells/ml in VBS-gel, and assayed for their susceptibility to killing by anti-Forssman antibody and GPC. Chart 1 illustrates the combined results of 3 experiments. Hormone-treated line 1 cells were less susceptible than were the control cells to killing by anti-Forssman antibody and GPC.

Resistance to killing by GPC was observed with cells coated with limiting, near excess, or excess amounts of anti-Forssman antibody (Chart 1). Similarly, hormone-treated line 1 cells, which are more susceptible to killing by HUC when coated with antibody, demonstrated increased resistance to killing. However, this occurred only when the cells were coated with limiting dilutions of anti-Forssman or specific anti-line 1 antibody (data not shown). Hormone-treated line 10 cells also showed a marked increase in resistance to killing by either anti-Forssman or specific anti-line 10 antibodies and HUC, compared with untreated cells (data not shown). In all cases with both tumor cell lines, complement control and cell control values remained below 10%. Since the overall effect of the hormones on the line 1 and line 10 cells was similar regardless of the antibody or complement source used, we used line 1 cells treated with anti-Forssman IgM antibody and GPC as the system for all further experiments.

**Effect of Hormone Concentration in Decreasing the Sensitivity of Tumor Cells to Antibody-Complement-mediated Killing.**

Dose-response experiments were performed to determine the optimal concentration of each hormone that was most effective in rendering the line 1 tumor cells resistant to antibody- and GPC-mediated killing. Serial 100-fold dilutions of hormones between 10⁻⁴ and 10⁻¹⁵ M were tested in 5-ml volumes of RPMI 1640-20% FCS, containing 5 x 10⁶ line 1 cells/ml. The cells were cultured for 30 min at 37°, washed, and examined for their susceptibility to killing by antibody and GPC. The results of a representative experiment are presented in Chart 2. Line 1 cells, incubated with concentrations of L-epinephrine-HCl, hydrocortisone, or
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To the loss of hormone activity. Hormones incubated for 4 hr at 37° in RPMI 1640-20% FCS alone or supernatant fluids from 4-hr cultures of line 1 cells plus hormone were still capable of rendering fresh line 1 cells resistant to killing by antibody and complement.

The reversion of line 1 cells to the susceptible state in the continued presence of hormone was now contrasted with their reversion when washed free of hormone. Line 1 cells (5 x 10⁶/ml) were incubated in RPMI 1640-20% FCS with hormones for 30 min at 37°. The cells were then washed 10 times with 10 ml of RPMI 1640-20% FCS and resuspended at 5 x 10⁶ cells/ml in RPMI 1640-20% FCS without added hormone. The cells were tested for susceptibility to killing immediately and after incubation for an additional 30, 60, 120, and 240 min at 37°. The results in Chart 4 show that the cells remained resistant to killing for up to 60 min at 37° after transfer to hormone-free medium but reverted to the sensitive state within 2 hr. The reversion to the sensitive state could not be prevented by culturing the washed cells at 0°.

Effect of Temperature on Hormone Treatment of Tumor Cells. Line 1 tumor cells were cultured at 0° or 37° in RPMI 1640-20% FCS with and without hormones. After 30-, 60-, and 120-min incubations, the cells were washed and tested for their susceptibility to killing by antibody and GPC. Table

to prednisolone as low as 10⁻¹¹ M, were markedly depressed in their susceptibility to killing. Insulin was effective at concentrations as low as 10⁻¹³ M (Chart 2).

The steroid hormones at concentrations of 10⁻⁴ M or greater were toxic, whereas neither of the polypeptide hormones was toxic at 10⁻³ M, the highest concentration tested (Chart 2). On the basis of these results we concluded that the lowest concentrations of hormone resulting in the maximal effect on the cells were 10⁻⁷ M hydrocortisone, epinephrine, and insulin and 10⁻⁹ M prednisolone. All further experiments were performed with these concentrations.

Effect of Time of Hormone Treatment of Tumor Cells. Line 1 tumor cells were cultured in RPMI 1640-20% FCS with and without hormones. After 15, 30, 60, 120, and 240 min of incubation, aliquots of the cell suspensions were removed and the cells were washed and examined for their susceptibility to killing by antibody and GPC. Chart 3 illustrates that the hormone-treated cells begin to show resistance to killing within 15 min and that maximal resistance is reached within 30 to 60 min in culture. After 60 min of incubation, the cells begin to revert to their sensitive state and are indistinguishable from control cells within 4 hr (Chart 3). Cell control (line 1 cells alone) and complement control (cells plus GPC alone) values remained less than 10% throughout the experiments.

The reversion of the line 1 cells to a susceptible state in the continued presence of hormone could not be attributed

**Chart 2.** Effect of hormone concentration on the susceptibility to killing of hormone-treated line 1 cells by antibody and GPC. A, L-epinephrine-HCl; B, insulin; C, prednisolone sodium succinate; D, hydrocortisone sodium succinate; *, cells plus anti-Forssman IgM antibody diluted 1:10 plus GPC diluted 1:8; †, cells plus GPC diluted 1:8; □, cells plus buffer alone.

**Chart 3.** Effect of time of incubation of line 1 cells with hormone on the susceptibility of the cells to killing by anti-Forssman IgM antibody (1:10) and GPC (1:8). A, 10⁻⁷ M L-epinephrine-HCl; B, 10⁻⁷ M insulin; C, 10⁻⁷ M hydrocortisone sodium succinate; D, 10⁻⁹ M prednisolone sodium succinate; †, hormone-treated cells; ‡, complement control values (line 1 cells plus GPC alone) for hormone-treated cells; *, line 1 cells not treated with hormone (values corrected for complement control). Values represent mean ± S.D. for 3 separate experiments.
1 shows that cells cultured with hormone at 0° for as long as 2 hr remained as sensitive to killing as the untreated controls. Subsequent experiments have shown that line 1 cells cultured with hormones at 0° for as long as 4 hr remain susceptible to killing (data not shown). As expected, cells cultured at 37° with these hormones were rendered resistant to killing after 30 to 60 min and began to revert to their susceptible state within 2 hr (Table 1).

**Effect of Less Physiologically Active Hormone Analogs on Tumor Cell Killing.** For each hormone used in this study, there exists an analog that physiologically is either less active or inactive (10, 23, 27, 36). The analogs of L-epinephrine HCl (β-epinephrine) and of hydrocortisone succinate and prednisolone succinate (β-estradiol and testosterone) were tested for their effect on the susceptibility of line 1 cells to killing by antibody and GPC. Line 1 cells were incubated in RPMI 1640-20% FCS at 37° with 10⁻³ M L-epinephrine-HCl, with 10⁻² M hydrocortisone sodium succinate, or with 100- or 10,000-fold higher concentrations of their less active analogs. Controls included cells in RPMI 1640-20% FCS without added hormone. After 30-min, 2-hr, 4-hr, and 17-hr incubations, aliquots of the cell suspensions were collected and the cells were washed and tested for their sensitivity to killing by antibody and GPC. Table 2 shows that only those cells cultured in L-epinephrine-HCl or hydrocortisone were rendered resistant to killing. At the times studied, no increase in resistance to killing by antibody and GPC was observed with cells cultured in medium containing the hormone analogs (Table 2).

The results obtained with insulin from 3 species (bovine, chicken, and pig), the less physiologically active analogs of pork insulin (DAA-insulin and proinsulin), and the isolated A and B chains of pork insulin are presented in Table 3. Bovine, chicken, and pork insulins were all effective in rendering the line 1 cells resistant to killing by anti-Forssman antibody and GPC (Table 3). Pork DAA-insulin at 10⁻³ M was marginally effective in rendering the cells resistant; pork proinsulin at 10⁻⁵ M and the isolated insulin A and B chains at 10⁻⁶ M were ineffective (Table 3).

**DISCUSSION**

These studies are a continuation of efforts to analyze the interaction of tumor cells with antibody and complement (5, 8, 9, 24, 25, 29-32). The data presented in this paper show that line 1 tumor cells incubated in medium containing the polypeptide hormone, insulin, the catecholamine L-epi-

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>30 min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>60 min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>120 min&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0°</td>
<td>37°</td>
<td>0°</td>
</tr>
<tr>
<td>L-Epinephrine-HCl</td>
<td>10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>Hydrocortisone sodium</td>
<td>10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>succinate</td>
<td>45</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Insulin</td>
<td>10⁻³</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Prednisolone sodium</td>
<td>10⁻⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>succinate</td>
<td>39</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>49</td>
<td>5</td>
</tr>
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</table>

<sup>a</sup> Time of incubation of line 1 cells with hormone.

<sup>b</sup> TAC, antibody-coated line 1 cells plus GPC (anti-Forssman IgM 1:10 plus GPC 1:8); TC, complement control (line 1 cells plus GPC). Cell controls (line 1 cells alone) also remained below 10%.
Table 2

Effect of less active or inactive hormone analogs on antibody-complement-mediated killing of line 1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>30 min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>120 min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>240 min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>17 hr&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>TAC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TC</td>
<td>TAC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TC</td>
</tr>
<tr>
<td>10&lt;sup&gt;-2&lt;/sup&gt; M L-epinephrine-HCl</td>
<td>8</td>
<td>4</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>10&lt;sup&gt;-3&lt;/sup&gt; M DL-epinephrine</td>
<td>49</td>
<td>3</td>
<td>57</td>
<td>6</td>
</tr>
<tr>
<td>10&lt;sup&gt;-3&lt;/sup&gt; M hydrocortisone sodium succinate</td>
<td>41</td>
<td>4</td>
<td>44</td>
<td>4</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; M β-estradiol</td>
<td>22</td>
<td>2</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>10&lt;sup&gt;-6&lt;/sup&gt; M testosterone</td>
<td>44</td>
<td>3</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>None</td>
<td>37</td>
<td>4</td>
<td>50</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Time of incubation of line 1 cells with hormone.

<sup>b</sup> TAC, antibody-coated line 1 cells plus GPC (anti-Forssman IgM 1:10 plus GPC 1:8); TC, complement control (line 1 cells plus GPC).

Table 3

Effect of insulin obtained from several species, insulin analogs, and insulin components on antibody-complement-mediated killing of line 1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% cells stained with trypan blue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAC&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-2&lt;/sup&gt; M bovine insulin</td>
<td>15</td>
</tr>
<tr>
<td>10&lt;sup&gt;-3&lt;/sup&gt; M bovine insulin</td>
<td>17</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt; M chicken insulin</td>
<td>12</td>
</tr>
<tr>
<td>10&lt;sup&gt;-11&lt;/sup&gt; M chicken insulin</td>
<td>20</td>
</tr>
<tr>
<td>10&lt;sup&gt;-9&lt;/sup&gt; M pork insulin</td>
<td>27</td>
</tr>
<tr>
<td>10&lt;sup&gt;-9&lt;/sup&gt; M pork proinsulin</td>
<td>49</td>
</tr>
<tr>
<td>10&lt;sup&gt;-7&lt;/sup&gt; M pork proinsulin</td>
<td>45</td>
</tr>
<tr>
<td>10&lt;sup&gt;-9&lt;/sup&gt; M pork DAA-insulin</td>
<td>29</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt; M pork insulin A-chain</td>
<td>54</td>
</tr>
<tr>
<td>10&lt;sup&gt;-4&lt;/sup&gt; M pork insulin B-chain</td>
<td>49</td>
</tr>
<tr>
<td>None</td>
<td>40</td>
</tr>
</tbody>
</table>

<sup>a</sup> TAC, antibody-coated line 1 cells plus GPC (anti-Forssman IgM 1:10 plus GPC 1:8); TC, complement control (line 1 cells plus GPC).

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The action of the hormones on the line 1 cells is not due to a decrease in the amount of detectable Forssman or tumor-specific antigen on the cell surface. According to the C1 fixation and transfer test (26), hormone-treated line 1 cells that were resistant to killing by antibody and GPC could bind as much C1-fixing anti-Forssman or specific antitumor antibody as could untreated control cells (S. I. Schlager, unpublished observation).

It has been proposed that the observed resistance of erythrocytes to lysis by antibody and complement may be due to a direct anticomplementary effect by high concentrations of hydrocortisone on fluid-phase complement when the complement source is added to the cells with the hormone (13). In this report, however, an inhibitory effect by the hormones on fluid phase complement is unlikely. GPC diluted in any of the 4 hormones used in our studies killed antibody-coated line 1 cells to the same degree as did GPC diluted in hormone-free media. In addition, the 4 hormones used were effective at concentrations ranging from 10<sup>-3</sup> to 10<sup>-8</sup> M; this is within the detectable physiological range of concentrations for these hormones (10, 35).

Finally, the tumor cells were routinely washed to remove unbound hormone prior to testing for susceptibility to killing.
To test the effect of hormone treatment on membrane-bound complement components, the hormones were added to line 1 cells in the T* state. The T* state is an intermediate in which the tumor cell has at least one membrane-bound antigen-antibody complex and all the necessary complement components bound for killing to occur (7). Preliminary experiments indicated that the hormones reversibly inhibit the transformation of line 1 cells in the T* state to dead cells. These results suggest that the hormones do not directly affect membrane-bound complement components.

The mechanism by which hormone treatment decreases the sensitivity of the cells to attack by antibody and complement is not understood. It has been shown that hormones (especially the glucocorticoid steroids) increase the stability and integrity of cell membranes (1, 3, 14, 16, 17, 19, 22, 33, 35, 36, 38, 39, 41, 42). This may occur by direct interaction of the hormone with a membrane component (2, 22, 39) or indirectly through specific binding to intracellular hormone receptors (2, 3). Further studies with the line 1 system offer an opportunity to examine these possibilities critically.

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12. Fell, H. B., and Weiss, L. The Effect of Antiserum, Alone and with Complement Components Bound for Killing to Occur (7). Preliminary experiments indicated that the hormones reversibly inhibit the transformation of line 1 cells in the T* state to dead cells. These results suggest that the hormones do not directly affect membrane-bound complement components.

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