Kinetics of Hormone-induced Tumor Cell Resistance to Killing by Antibody and Complement

Seymour I. Schlager, Sarkis H. Ohanian, and Tibor Borsos

Laboratory of Immunobiology, National Cancer Institute, NIH, Bethesda, Maryland 20014

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 with insulin, L-epinephrine, hydrocortisone, or prednisolone, the cells show a marked reduction in their susceptibility to antibody-complement-mediated killing within 15 to 60 min; this effect reverses within 4 hr in the continued presence of hormone. Maximal binding of the hormones to the line 1 cells was observed within 60 min. However, the hormones remained bound to the cells after 4 hr of incubation, suggesting that line 1 cells incubated in the continued presence of hormone revert to the susceptible state despite the persistence of cell-bound hormone. Hormone-treated tumor cells, washed free of hormone and reincubated in hormone-free medium, lost nearly all their bound hormone within 15 to 30 min of washing. These cells, however, remained resistant to antibody-complement-mediated killing for up to 2 hr after washing. Line 1 cells, reverted in the continued presence of hormone, remained susceptible to killing by antibody and guinea pig complement after reexposure to the same, but not to a different, hormone. Hormone-treated cells reverted after prolonged incubation in hormone-free media; however, they were rendered resistant to killing after reexposure to the same hormone. The temporary refractoriness of reverted cells to further hormone stimulation was not due to an inability of the cells to bind hormone.

INTRODUCTION

Line 1, a diethylnitrosamine-induced guinea pig hepatoma, is susceptible to killing by anti-Forssman antibody and GPC2 (23). In a previous report we have shown that line 1 cells treated with selected hormones were rendered resistant to killing by antibody and complement (30). The development of the resistant state was dependent upon hormone concentration, temperature, and time for which the cells were incubated with hormone; the effect reversed within 4 hr in the continued presence of hormone or within 2 hr if the cells were washed free of hormone (30). Less active or inactive hormone analogs used at up to 10,000-fold higher concentrations were ineffective (30).

Since the time of incubation of the line 1 cells with hormone was the most critical factor in the development of their resistance to antibody-complement-mediated killing, we have examined in greater detail the relationship of binding and persistence of hormone to the expression of the resistant state. In this report, we present data indicating that binding of hormone on line 1 tumor cells is necessary for the expression of the resistant state and that reversion to the susceptible state occurs despite the continued presence of cell-bound hormone. We also present evidence that hormone-treated cells that have reverted to the susceptible state are temporarily refractory to further exposure to the same, but not to a different, hormone.

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). The diethylnitrosamine-induced hepatic tumor, line 1, passed in strain 2 guinea pigs, was collected as described (24, 35, 36). Cells were used between the 92nd and 97th passage in animals. The cells were suspended in RPMI 1640-20% FCS.

All cytotoxicity assays were performed in VBS-gel (26).

Quantitation of Hormone Bound to Line 1 Tumor Cells.

Two hundred fifty thousand line 1 cells suspended in 0.5 ml of RPMI 1640-20% FCS containing [6,7-3H]prednisolone (New England Nuclear, Boston, Mass.; 40 Ci/m mole), [1,2,6,7-3H]hydrocortisone (New England Nuclear; 91 Ci/m mole), L-[7-14C]bitartrate-DL-epinephrine (New England Nuclear; 59.4 mCi/m mole), or 125I-labeled porcine insulin (New England Nuclear; 99.9 μCi/μg) were incubated for various lengths of time at 37° under 5% CO2-air. After preliminary dose-response experiments were performed, the quantities of labeled hormone to be added to the cells (see text for concentrations) were chosen to yield readily measurable amounts of activity taken up by the cells. After incubation, the cells were washed 2 times with 4 ml ice-cold Hanks’ balanced salt solution and collected onto 0.45-μm Millipore filter discs utilizing a Millipore vacuum sampling
manifold. The discs were washed 4 times with 4 ml ice-cold Hank's balanced salt solution and placed in 10 ml of Aquasol; the activity was determined in a Beckman liquid scintillation counter (actual counting efficiency is 42% for $^3$H and 70% for $^{14}$C). In experiments in which cells were cultured in medium containing $^{125}$I-labeled insulin, the cells were washed 2 times as above, and the activity associated with the cell pellet was determined in a Packard auto-gamma scintillation spectrometer (counting efficiency is 75% for $^{125}$I).

Controls consisting of line 1 tumor cells cultured in RPMI 1640-20% FCS alone or the tissue culture medium containing the radioactive hormone alone were prepared concurrently. All experiments were carried out at least 3 times in duplicate, and reported values have been corrected for the controls of medium plus radioactive hormone alone. These values were <15% of the experimental values.

**Treatment of Tumor Cells with Unlabeled Hormones.**

The hormones used in these studies were: hydrocortisone sodium succinate (The 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, 24.3 IU/mg); and L-epinephrine methyl ether HCl (Sigma, Lot 84B-5140). Tumor cells (5 x $10^6$ cells/ml) were cultured in 10 ml RPMI 1640-20% FCS containing the appropriate concentration (see text) of hormone in 100-x 15-mm plastic bacteriological Petri dishes (Falcon Plastics Co., Oxnard, Calif.) at 37° under 5% CO$_2$-air. After incubation for the appropriate length of time, aliquots of the cell cultures were drawn off; the cells were washed 5 times in 5 ml VBS-gel and tested for their susceptibility to killing by antibody and complement.

**Sera and Antisera.** Rabbit antiserum to sheep Forssman antigen was prepared as described previously (26).

Normal GPC (JEM Research, Inc., Kensington, Md.) and normal human complement (absorbed at 0° with sheep erythrocytes, 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 (24). Briefly, 0.1 ml of diluted antisera was added to 0.1 ml of tumor cells (10$^6$/ml in VBS-gel). After incubation for 30 min at 30°, the cells were washed with VBS-gel and incubated with 0.1 ml GPC (diluted 1:8) for 60 min at 37°. One-tenth ml 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; these values remained below 10% throughout. All cytotoxicity values reported have been corrected for complement controls.

**RESULTS**

**Kinetics of Hormone Binding and the Appearance of the Resistant State.** Line 1 cells (5 x $10^5$/ml) were incubated at 37° in 10 ml RPMI 1640-20% FCS or in medium containing $[^3]H$prednisolone, $[^3]H$hydrocortisone, $[^14]C$epinephrine, or $^{125}$I-labeled insulin (see Chart 1 for concentrations). Fifteen, 30, 60, 120, and 240 min later, 0.5-ml aliquots of each suspension were removed, the cells were washed, and the amount of $^3$H, $^{14}$C, or $^{125}$I bound to the cells was determined. The results of a representative experiment shown in Chart 1 indicate that hormone binding to the line 1 cells was rapid and that maximal binding occurred within 30 to 60 min. The maximal amounts of added hydrocortisone, prednisolone, epinephrine, and insulin bound to the cells were 0.06, 0.1, 0.5, and 0.7%, respectively (Chart 1). Similar experiments were performed in which the cells were incubated with labeled hormones at 0°. The amount of the steroid hormones bound at 0° was approximately half that bound at 37°, whereas binding of epinephrine and insulin at 0° was not markedly affected (Table 1).

Additional experiments were performed in which line 1 cells cultured at 37° with labeled hormones were tested for their susceptibility to killing by anti-Forssman IgM antibody and GPC, as well as for the quantity of $^3$H or $^{125}$I bound to the cells. Chart 2 shows the results of a representative experiment. The maximal binding of labeled hormone occurred within 30 to 60 min; concomitantly, the cells were rendered resistant to killing by antibody and GPC within 15 to 30 min after exposure to the radioactive hormones (Chart 2). However, although the hormones persisted on the cells throughout the 4-hr incubation, the cells reverted to the susceptible state within 2 hr (hydrocortisone and prednisolone).
Binding of radiolabeled hormone to line 1 cells at 0°

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Activity (cpm/2.5 × 10^5 cells) bound</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 × 10^{-7} M [3H]prednisolone</td>
<td>4,839 ± 97</td>
<td>39</td>
</tr>
<tr>
<td>2 × 10^{-7} M [3H]hydrocortisone</td>
<td>4,301 ± 86</td>
<td>52</td>
</tr>
<tr>
<td>3.4 × 10^{-8} M [14C]epinephrine</td>
<td>7,735 ± 154</td>
<td>8</td>
</tr>
<tr>
<td>4 × 10^{-9} M [125I]-labeled insulin</td>
<td>141,711 ± 382</td>
<td>8</td>
</tr>
</tbody>
</table>

* Activity bound after 60 min of incubation. Average of 3 experiments ± S.D. Corrected for radioactive blank control.

**Kinetics of Hormone-induced Resistance to Killing**

Table 1

The results with insulin-treated cells are shown in Chart 3. Within 15 min after suspension in hormone-free media, approximately 70% of the bound hormone had dissociated from the line 1 cells; after 30 to 60 min, 80 to 85% of the hormone had been lost from the cells (Chart 3). In contrast, the cells remained resistant to killing by antibody and GPC for at least 2 hr. These results indicate that, even after >80% of cell-bound hormone had dissociated from the line 1 cells, they remained resistant to killing for at least an additional 60 min. Similar results were obtained with cells pretreated with [3H]prednisolone, [3H]hydrocortisone, [14C]epinephrine, or [125I]-labeled insulin. After this time, the cells were washed 5 times with 10 ml RPMI 1640-20% FCS, resuspended in RPMI 1640-20% FCS (5 × 10^6 cells/ml), and reincubated at 37°. Immediately and after 15, 30, 60, 120, and 240 min of incubation, the cells were washed and tested for their susceptibility to killing by anti-Forssman antibody and GPC and for the amount of 3H, 14C, or 125I bound to the cells.

The results with insulin-treated cells are shown in Chart 3. Within 15 min after suspension in hormone-free media, approximately 70% of the bound hormone had dissociated from the line 1 cells; after 30 to 60 min, 80 to 85% of the hormone had been lost from the cells (Chart 3). In contrast, the cells remained resistant to killing by antibody and GPC for at least 2 hr. These results indicate that, even after >80% of cell-bound hormone had dissociated from the line 1 cells, they remained resistant to killing for at least an additional 60 min. Similar results were obtained with cells pretreated with [3H]prednisolone, [3H]hydrocortisone, [14C]epinephrine, or [125I]-labeled insulin. After this time, the cells were washed 5 times with 10 ml RPMI 1640-20% FCS, resuspended in RPMI 1640-20% FCS (5 × 10^6 cells/ml), and reincubated at 37°. Immediately and after 15, 30, 60, 120, and 240 min of incubation, the cells were washed and tested for their susceptibility to killing by anti-Forssman antibody and GPC and for the amount of 3H, 14C, or 125I bound to the cells.

**Response of Hormone-treated Reverted Line 1 Tumor Cells after Reexposure to Hormone.** Five million line 1 tumor cells in 10 ml RPMI 1640-20% FCS alone or in medium containing the appropriate concentrations of hormones were incubated at 37°. Aliquots of each suspension were removed 30 min and 4 hr later, and the cells were washed and tested for their susceptibility to killing by antibody and GPC. In addition, at the 4-hr time interval, aliquots of the suspensions were removed; the cells were washed, resuspended at 5 × 10^6 cells/ml in RPMI 1640-20% FCS or in medium containing the appropriate concentration of fresh hormone, and incubated for 30 min at 37°. At this time, the cells were tested for their susceptibility to killing by antibody and GPC.

Chart 4 shows the pooled results of 3 separate experiments. As expected (30), line 1 cells in culture with hormone for 30 min were rendered resistant to killing by antibody and GPC; after 4 hr of incubation in the continued presence of hormone, they had reverted to the sensitive state (Chart 4). When the reverted cells were reexposed to culture medium containing the same hormone, they remained susceptible to killing. However, when the reverted cells were cultured with a different hormone, they were rendered resistant to killing by antibody and complement. The only exceptions were hydrocortisone-treated reverted cells cultured with prednisolone and vice versa; these cells remained in the susceptible state (Chart 4).

Experiments were next performed to determine whether...
Hormone Binding by Reverted Cells. Experiments were performed to determine if the refractory state of hormone-treated reverted cells to further hormone exposure was due to lack of hormone binding by these cells. Line 1 cells (5 x 10⁶/ml) were cultured at 37° in RPMI 1640-20% FCS alone or in medium containing the appropriate concentration of hormone. After 4 hr, the cells were washed and resuspended in RPMI 1640-20% FCS containing [³H]prednisolone, [³H]hydrocortisone, or ¹²⁵I-labeled insulin. After 60 min of incubation at 37°, the amount of [³H] or ¹²⁵I bound to the cells was determined.

The data from a representative experiment shown in Table 2 indicate that hormone-treated reverted line 1 cells are capable of binding the hormone which no longer renders them resistant to killing by antibody and complement. Only 23, 24, and 33% inhibition of binding was observed for [³H]prednisolone, [³H]hydrocortisone, and ¹²⁵I-labeled insulin, respectively, by the reverted cells. Based on the molar concentrations of hormones that were added to the cell suspensions, the hormone-treated reverted line 1 cells are capable of binding concentrations of hormones that have the tumor cells require a "recovery time" to become resistant after reexposure to the same hormone. Five million line 1 tumor cells were incubated at 37° in 10 ml RPMI 1640-20% FCS alone or in medium containing the appropriate concentration of hormone. After 30 min, the cells were washed 10 times with 10 ml RPMI 1640-20% FCS, resuspended at 5 x 10⁶ cells/ml in RPMI 1640-20% FCS without added hormone, and reincubated at 37°. Immediately and 60, 120, and 240 min later, aliquots of the suspensions were removed, and the cells were tested for their susceptibility to killing by anti-Forssman antibody and GPC. At the 120- and 240-min time intervals, additional aliquots of the cell suspensions were removed; the cells were washed, resuspended at 5 x 10⁶ cells/ml in RPMI 1640-20% FCS or in medium containing the appropriate concentration of hormone, and incubated for an additional 30 min at 37°. After this time, the cells were tested for their susceptibility to killing by antibody and GPC.

Representative results are illustrated in Chart 5. Line 1 tumor cells that had reverted to the sensitive state after 2 and 4 hr in medium without added hormone could be rendered resistant to killing by antibody and GPC after exposure to the same hormone. This indicates that the refractory state of hormone-treated reverted cells to further hormone exposure is time dependent.
activity (dpm/2.5 × 10⁶ line 1 cells) bound by cells preincubated in medium alone for 4 hr at 37° × 100

activity (dpm/2.5 × 10⁶ line 1 cells) bound by cells preincubated in medium alone for 4 hr at 37° × 100

Table 2 Binding of hormone by line 1 cells reverted after 4 hr in the continuous presence of hormone

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>¹³¹I-Labeled insulin (2.5 μCi/ml)</th>
<th>¹³¹I-Labeled hydrocortisone (40 μCi/ml)</th>
<th>¹³¹I-Labeled prednisolone (20 μCi/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁷ M L-epinephrine</td>
<td>96</td>
<td>96</td>
<td>105</td>
</tr>
<tr>
<td>10⁻⁷ M hydrocortisone</td>
<td>85</td>
<td>76</td>
<td>123</td>
</tr>
<tr>
<td>10⁻⁸ M insulin</td>
<td>105</td>
<td>92</td>
<td>67</td>
</tr>
<tr>
<td>10⁻⁸ M prednisolone</td>
<td>77</td>
<td>92</td>
<td>121</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Activity bound in 60 min* (% of control)²

* Activity (dpm/2.5 × 10⁶ line 1 cells) bound by cells preincubated with hormone for 4 hr at 37°

² Activity bound in controls: [¹³¹I]prednisolone, 12,782 dpm; [¹³¹I]hydrocortisone, 11,112 dpm; [¹³¹I]insulin, 10,718 dpm.

been shown to be effective in rendering them resistant to killing by antibody and GPC (30).

**DISCUSSION**

We have previously shown that incubation of line 1 guinea pig hepatoma cells with the polypeptide hormone, insulin; the catecholamine, L-epinephrine-HCI; or the glucocorticoid steroids, hydrocortisone or prednisolone; renders these cells resistant to killing by antibody and complement (30). This effect was shown to be time dependent and reversed even in the continued presence of hormone (30). The results reported here confirm and extend our previous observations that line 1 cells were rendered resistant to killing by anti-Forssman antibody and GPC within 30 to 60 min of incubation with the hormones. Comconitantly, in the continued presence of hormone, maximal binding of the radio-labeled hormones occurred within 60 min, and the hormones persisted on the cells up to the time the experiment was terminated. Although hormone remained bound to the cells for 4 hr, the cells reverted to the sensitive state within that period of time. Thus, hormone binding to the line 1 cells is, in itself, not sufficient to account for their resistance to antibody-complement-mediated killing.

In addition, hormone-treated tumor cells, washed free of hormone and reincubated in medium alone, remained resistant to antibody-complement-mediated killing for up to 2 hr. The majority of hormone bound to the cell, however, had dissociated within 15 to 30 min of washing. This implies that persistence of bound hormone is not necessary for persistence of the resistant state. Both these observations suggest that the observed resistance of line 1 cells to humoral immune attack following hormone treatment is not due to a direct physical action by the hormone on the cell membrane.

The use of radio-labeled hormones to measure hormone binding enabled us to calculate the minimal number of hormone-binding sites on the line 1 cells. Utilizing the maximal number of counts bound as shown in Chart 1 and assuming that each hormone molecule has 1 binding site on the cell, our data indicate that each line 1 tumor cell has approximately 3 × 10⁴ binding sites for prednisolone, 1 × 10⁵ binding sites for hydrocortisone, 2 × 10⁶ binding sites for epinephrine, and 4 × 10⁶ binding sites for insulin. These results are similar to observations in other cell systems (7, 9, 13, 16, 19). In addition, the binding of radiolabeled hormones to the line 1 cells could be specifically inhibited up to 90% by the presence of the corresponding unlabeled hormone (S. I. Schlagel, unpublished observations).

Our observations also indicate that hormone-treated line 1 cells that have been rendered resistant to killing by antibody and complement and have reverted to the sensitive state in the continued presence of the hormone remain sensitive to killing after reexposure to the same hormone. These cells become responsive to hormone treatment only when exposed to the same hormone after prolonged incubation in hormone-free media. This refractory state was not due to an inability of the cells to bind sufficient amounts of the hormone. There are several possible explanations for this phenomenon stemming from observations in other cell systems. First, hormone-induced metabolic products or membrane alterations may inhibit further hormone action (6, 8, 10, 29). Second, the metabolic action of hormones on a cell is unaffected by addition or removal of the hormone (17, 20, 27). This suggests that a hormone effect on a cell, once initiated, proceeds independently of further hormone stimulus (20). Finally, cells cultured in the prolonged presence of hormone retain hormone binding sites but have lost the capacity to respond to the hormone stimulus (9, 13, 16, 28).

Our data have also shown a metabolic dissociation between hormone binding and hormone action. Although it was observed that hormone binding occurs at 0°, none of the hormones tested was able to render the cells resistant to killing by antibody and complement at this temperature (30). Furthermore, line 1 cells inhibited in their metabolism
after treatment with selected chemotherapeutic drugs will bind hormone but are not rendered resistant to antibody-complement-mediated killing by the hormone. These apparent temporal and metabolic distinctions between hormone binding and action support the hypothesis of at least a 2-step interaction of line 1 cells with hormone: the reaction of the hormone with its membrane or cytoplasmic combining site (7, 8, 19, 29) and the physiological effect of the hormone on the cell. This latter step most probably involves several consecutive distinct events (8, 22, 25) and can apparently occur within 15 to 30 min after the cells are exposed to hormone.

It has been postulated that glucocorticoid steroids (6, 14, 25) and polypeptide hormones (10, 32) may exert their biological effect through a direct modification of the cell membrane (2, 3, 4, 9, 13, 15, 16, 18, 33) and/or through modification of a metabolic pathway(s) in the cell (1, 5, 10—12, 17, 20—22, 27, 32, 34). Our data strongly suggest that the direct physical presence of the hormones used in this study on the line 1 cell membrane is not sufficient to explain the observed resistance of the cells to killing by antibody and complement. A metabolic and temporally separate event(s) appears to be required for the appearance of the resistant state. Further studies in this system are in progress in an attempt to elucidate these latter metabolic events.

REFERENCES

34. Young, D. A. Interrelationships between Carbohydrate, Protein, and Adenine Nucleotide Metabolism and Cortisol Effects on These Functions In Vitro. J. Biol. Chem., 244: 2210-2215, 1969.

Kinetics of Hormone-induced Tumor Cell Resistance to Killing by Antibody and Complement

Seymour I. Schlager, Sarkis H. Ohanian and Tibor Borsos


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/37/3/765

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.