A Study of Human Epidermoid Carcinoma (H.Ep. #3) Growing in Conditioned Swiss Mice*

II. Statistical Methods and Steroid Therapy

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SUMMARY

By the concept of quality control, two simple graphic methods have been used to analyze tumor-weight data obtained from a human epidermoid carcinoma (H.Ep. #3) growing in conditioned Swiss mice. These methods were not only used for the purpose of checking untreated tumor weights for erratic averages, excessive variations, or erratic individual tumor weights, but they also served as guides for judging test compound results.

The multiple performance of these graphic devices, which do not require excessive computation, has been found useful for handling large amounts of data gathered from screening programs.

With steroids used as model substances and assay groups of three mice, it was found that the corticoids produced consistent antitumor effects. These effects were judged to be nonspecific, however, because of associated toxicity to the host animal.

The general operating characteristics of a chemotherapy system, in which human carcinoma (H.Ep. #3) is grown in conditioned Swiss mice, have been presented in an earlier publication (2). Two analytical procedures found useful in the analysis of chemotherapy data are presented in this paper. Steroids have been used as model substances because of their interest to clinical study groups and the usefulness of certain corticoids in the treatment of clinical cancer (1).

MATERIALS AND METHODS

A detailed description of the transplantation, conditioning, and chemotherapy procedures has been published (2). However, a brief description of the method is presented for the purpose of orientation.

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Female Swiss Webster mice (Taconic Farms), weighing 15–22 gm., were used as hosts. Since three tumors, each weighing a minimum of 2.0 gm., were required in the transplant procedure and because of the difficulty of maintaining a continuous transplant line of H.Ep. #3 in Swiss mice, 7- to 14-day-old H.Ep. #3 growing in x-radiated and cortisone-treated rats was used. The tumor was first separated from the muscle and bone of the donor rat; it was minced with scalpels and then diluted in the ratio 1 part tumor:1 part saline fortified with potassium penicillin G (1,000 units/ml) and streptomycin sulfate (2 mg/ml). Minced suspensions were injected through a 16-gauge needle fitted to a 2-ml Luer Lok syringe. All transplantations were made intramuscularly into the right thigh. Immediately thereafter each mouse routinely received a single dose of cortisone acetate (Cortone-Merck) (150 mg/kg) subcutaneously in the nape of the neck. No further conditioning was required. In the previous publication (2), x-ray and cortisone were used in a combined conditioning procedure.
Chemotherapy trials were conducted against three tumor-bearing mice. Control groups also contained three mice. In general, one set of control mice served for three test groups. Therapy was usually started 34 hours after transplantation and continued for 7 days. Animals were sacrificed 9 days after transplantation, and net tumor weights were determined by subtracting the weight of the opposite uninjected thigh from the weight of the tumor-bearing thigh.

RESULTS

Quality control procedures with average tumor weight.—Chart 1 presents control data from 117 assays in chronological order of performance. Each point plotted on a logarithmic scale represents the average for three tumor-bearing mice receiving no chemotherapeutic treatment. The 117 assays were conducted over a 14-month period beginning in January, 1958. Preliminary experimental data, subsequently extended to larger series of tumor-inoculated mice, indicated that x-radiation could be discontinued in our experimental system and cortisone dosage changed to a single injection of the steroid given on the day of transplantation. Therefore, in the middle of December, 1958, x-radiation was discontinued as one of the conditioning agents. Thereafter, mice were simply given a single dose of cortisone acetate on the day of transplant. By inspection of Chart 1 it can be seen that, except for three instances (114 of the 117), all average tumor weights were between 1.5 and 3.8 gm., the median being about 2.3 gm. The later averages tended to be somewhat larger.

With the use of this basic control chart with a median value of 2.3 gm. and a lower limit of 1.5 gm., “activity-rating” bands have been arbitrarily defined in a series of graduations, each of approximately the same logarithmic width. Chart 2 shows, for each occasion of testing of “cortisone-like” (C-21) steroids, the average treated tumor weight. In any single assay, the average treated tumor size might alternatively be compared with the historical average over many experiments. Where the current local control average tumor weight for a test was less than the historical average of 2.3 gm., the bands were shown relative to the historical average and were drawn horizontal to the mean value of 2.3 gm. When the current control average was greater than 2.3 gm. the bands were shown relative to the current or local average and were drawn parallel to the line corresponding to equality for treated and control average treated tumor weights. The band within which results for assays fall identifies the following indicated levels of anti-tumor effect:

1. 0–36 per cent:
   - , little or no antitumor effect; relative tumor weight exceeds 80 per cent of controls; inhibition less than 20 per cent.
   ± , weak antitumor effect; relative tumor weight 64–80 per cent of controls; inhibition 30–36 per cent.

2. 36–60 per cent:
   ± , moderate antitumor effect; relative tumor weight 50–64 per cent of controls; inhibition 36–50 per cent.
   ±_§ , good antitumor effect; relative tumor weight 40–50 per cent of controls; inhibition 50–60 per cent.

3. 60 per cent and over:
   + , strong antitumor effect; relative tumor weight below 40 per cent of controls; inhibition in excess of 60 per cent.

The rating for a compound employing these bands was the higher of its ratings relative to the historical or the current control average. Such a rating should be interpreted as an indicator of possible, rather than definite, proof of antitumor activity. Any weakness of this procedure is mitigated by the practice in this laboratory of testing agents on several occasions and requiring consistent indications of antitumor effect.

It is recognized that, relative to the antitumor

![Chart 1: Average weights of nondrug-treated H.Ep. #3 tumors obtained from 117 consecutive bio-assays. Tumors from conditioned Swiss mice were harvested 9 days after transplantation.](chart1.png)
effects achieved with many test compounds in other experimental tumor systems, a 60 per cent inhibition, which is here rated as strong, is ordinarily only moderate. In actual procedure, compounds producing ± or + effects are studied further. Agents which have given lesser but consistent effects over 2 or more trials are also considered of interest. This can be seen more clearly in Table 1, which shows, for each of the “cortisone-like” steroids, the activity classification of each test occasion and the dosage level employed. Eight of the nine materials received a rating of ± or + or better on at least one occasion, and it is noteworthy that such a rating was in every case confirmed by the results of at least one other test. The lower activity rating for hydrocortisone reflects the difference between the unesterified and the esterified (acetate) form. More extensive titrations of these compounds have indicated a progressive antitumor effect with concomitant host toxicity with increasing dose level.

Table 2 presents data on one of the more potent corticoids to illustrate that the doses which produced the antitumor effects were associated with body weight loss and/or mortality to the host.

Table 1 shows, in addition, results of testing other C-21 steroids, each on three occasions. Test levels were generally high, 500 or 1000 mg/kg, but none of these steroids gave consistent indication of activity. Ratings were frequently −, and, with one exception, were ± or poorer. The one compound yielding a + rating on one occasion received a − rating on the two remaining occasions, suggesting that the high rating may have been due to some laboratory error. Not only were these steroids generally inactive, but they were also nontoxic to the tumor-bearing hosts.

**High-low quality control procedures.**—Chart 3 illustrates the application of an alternative quality control procedure, the averages alone for which have already been given in Chart 1. Each control set of three tumors is represented on Chart 3 by a single point, the abscissa and ordinate of the point corresponding, respectively, to the smallest and largest of the three tumors. Logarithmic scales are employed for individual extreme tumor weights. Lines have been drawn by inspection on Chart 3 in an attempt to define a region within which control data should ordinarily fall.

Line A is the line for equality between the largest and smallest tumors in a control set, and, of necessity, no points can fall below this line. Points only slightly above this line would indicate instances in which all three tumor weights of a control set fell within a limited logarithmic range. Such narrow ranges occur frequently enough so that no lower limit on the range can be prescribed. In any case, it is questionable whether with small samples extreme uniformity should be a basis for making data suspect.

Excessive variation in tumor weight is indicated by too great a range in the data as indicated...
TABLE 1

EFFECT OF C21 STEROIDS ON H.EP. #3 GROWING IN CONDITIONED SWISS MICE*

<table>
<thead>
<tr>
<th>Compound</th>
<th>1000 mg/kg</th>
<th>500 mg/kg</th>
<th>250 mg/kg</th>
<th>125 mg/kg</th>
<th>62 mg/kg</th>
<th>31 mg/kg</th>
<th>Ident. in figs.</th>
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</thead>
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<tr>
<td>Cortisone acetate</td>
<td>±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>±</td>
<td>±</td>
<td>± +</td>
<td>1</td>
</tr>
<tr>
<td>Hydrocortisone acetate</td>
<td></td>
<td>±</td>
<td>±</td>
<td>± ± ±</td>
<td>± +</td>
<td>± ± +</td>
<td>2</td>
</tr>
<tr>
<td>Hydrocortisone, free</td>
<td></td>
<td></td>
<td></td>
<td>± ± ±</td>
<td>± +</td>
<td>± ± +</td>
<td>3</td>
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<tr>
<td>Prednisone</td>
<td></td>
<td></td>
<td></td>
<td>±</td>
<td>±</td>
<td>± ± +</td>
<td>4</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>± +</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>±</td>
<td>± ± +</td>
<td>± ± +</td>
<td>5</td>
</tr>
<tr>
<td>9α-Fluorohydrocortisone acetate</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± + ±</td>
<td>± + ±</td>
<td>± + ±</td>
<td>± ± +</td>
<td>6</td>
</tr>
<tr>
<td>9α-Fluprednisolone acetate</td>
<td></td>
<td></td>
<td></td>
<td>± + ±</td>
<td>± + ±</td>
<td>± + ±</td>
<td>7</td>
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<tr>
<td>10α-Fluprednisolone</td>
<td>±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>±</td>
<td>± ± +</td>
<td>± ± +</td>
<td>8</td>
</tr>
<tr>
<td>Decadron</td>
<td>±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>±</td>
<td>± ± +</td>
<td>± ± +</td>
<td>9</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Deoxycorticosterone acetate</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
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<td>± ± ±</td>
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<td></td>
</tr>
<tr>
<td>Deoxyprednisolone, 21-monooacetate</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Deoxycorticosterone, 16a, 17a-epoxy</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Deoxycorticosterone-17α-hydroxy</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
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<td>Progesterone</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Progesterone, 16a, 17a-epoxy</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Progesterone, 17α hydroxy 17 acetate</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Progesterone, 16-a dehydro</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Progesterone, 4-chloro</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Progesterone, 2α-methyl</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Progesterone, 11-keto-2α-methyl</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
<tr>
<td>Progesterone, 21-fluoro-11β, 18α-dehydroxy</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td></td>
</tr>
</tbody>
</table>

* Inhibition of tumor growth:
- <20%
± 20-30%
± ± 36-50%
± + 50-60%
+ >60%

TABLE 2

EFFECT OF DECADRON (16α-METHYL-9α-FLUOROPREDNISOLONE) ON H.EP. #3 GROWING IN THE CONDITIONED* SWISS MOUSE

<table>
<thead>
<tr>
<th>Dose (mg/kg×7 SC)</th>
<th>No. trials</th>
<th>Mortality</th>
<th>Host body wt. change (gm.)</th>
<th>Av. tumor wt. (gm.)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>1</td>
<td>3/3</td>
<td>-1.4</td>
<td>1.0</td>
<td>±</td>
</tr>
<tr>
<td>62</td>
<td>4</td>
<td>3/12</td>
<td>-1.4</td>
<td>(0.9-1.1)</td>
<td>±</td>
</tr>
<tr>
<td>31</td>
<td>1†</td>
<td>2/3</td>
<td>+0.2</td>
<td>1.5</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>2†</td>
<td>0/6</td>
<td>+0.2</td>
<td>(1.1-1.8)</td>
<td>±</td>
</tr>
<tr>
<td>16</td>
<td>3†</td>
<td>2/9</td>
<td>-1.2</td>
<td>1.1</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1/12</td>
<td>-1.7</td>
<td>(0.7-1.4)</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0/21</td>
<td>+0.2</td>
<td>2.0</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>8†</td>
<td>1/24</td>
<td>+2.1</td>
<td>(1.6-2.6)</td>
<td>±</td>
</tr>
</tbody>
</table>

* X-radiation: 100 r total-body; cortisone acetate, four doses (1.5, 1.0, 1.0, 1.0 mg/mouse).
† No x-radiation; cortisone acetate, 1 dose, 3.0 mg/mouse (150 mg/kg).
D provide guides for the detection of such instances. Line C permits determining whether the largest value in a future group of animals is greater than it has generally been in the preceding groups, while line D permits a similar comparison of the smallest value in a group with the historical record of such smallest values.

The fact that the sample sizes of each group are relatively small permits employment of such a figure with only minor loss in statistical efficiency. For small samples, the relative efficiency of the mid-range, average of highest and lowest, is high compared with that of the over-all average. A given value of the mid-range (in this case, the logarithmic mid-range) corresponds to a line perpendicular to Line A. In the present instance, line E corresponds to a lower limit, line F to an upper limit on the logarithmic mid-range.

In total, the area defined by the lines A–F permits simultaneous statistical control, using past experience as a guide, for detecting shifts in central tendency (Lines E and F) or variability (Line B) and for identifying the presence of unusual individual values (Lines C and D).

The control chart described, although intended primarily for checking the untreated control results for erratic average, excessive variation, or erratic individual tumors, can also be used as a guide for judging test compound results. For this purpose, not all the control limits defined above remain useful, but rather only lines D and E—Line D permits judging whether the average tumor weight has been reduced, whereas Line E permits judging whether the tumor in an individual animal has been adversely affected without any necessary regard to the effect on the average.

Chart 4 presents the results of tests with the "cortisone-like" (C-21) steroids previously discussed. It can be seen that repeatable tumor inhibitions are demonstrated with this type of quality control figure. Once again, hydrocortisone is found to be classified inactive, while the acetate is considered active. No definite or consistent antitumor effect was found for 23 additional steroids.

Chart 4.—Effects of steroid therapy on H.Ep. #3 tumor weights:

Legend:

- #1—Cortisone acetate;
- #2—Cortisol acetate;
- #3—Cortisol;
- #4—Prednisone;
- #5—Prednisolone;
- #6—9α-Fluorocortisol;
- #7—9α-Fluoroprednisolone acetate;
- #8—9α-Fluoroprednisolone;
- #9—Decadron.

Dose Symbols:

- ▽ = 51 mg/kg.
- □ = 62.5 mg/kg.
- △ = 125 mg/kg.
- ○ = 250 mg/kg.
- ● = 500 mg/kg.
- □ = 1000 mg/kg.

Additional data on host body weight change and mortalities will be published elsewhere.

According to the above graphical methods, diameters of tumors in mice treated with these steroids were also found to be suitable for detecting antitumor effects. This suggests the usefulness...
of tumor size rather than weight as an end-point of effect where it is desirable not to sacrifice the animals.

DISCUSSION

Experimental chemotherapy programs require that antitumor agents display consistent and repeatable activity. Routine testing in this laboratory has been accomplished with as few as three conditioned mice bearing human tumors. Untreated control groups of mice have also been restricted to this size. With groups so small, the reliability with which the antitumor effect of an agent is ascertained in any one assay could be poor. Nevertheless, it has been found that the results obtained in the H.Ep. #3-Swiss mouse system have been relatively consistent. This corroborates the findings of other investigators (3) that groups of three mice per test can be adequate for judging test results.

Control limits on averages, based on over 100 sets of control data, were determined so as to exclude only a small number of the most extreme averages occurring. Unlike the usual control chart, the range of variation limits for the averages were arbitrarily drawn in by inspection rather than on the basis of variability between tumor weights in the same experiments: (three extreme values were eliminated from a total of 117 assay averages). The central line obtained in this way represents the median average and is, therefore, less sensitive than the arithmetic mean to any extreme average. By screening out those unusual results which occurred infrequently, realistic upper and lower limits were obtained which reflected actual day-to-day variation rather than variation on a day. If a strict procedure for setting limits based on day-to-day variation were adopted, excessively wide central limits could result if early data already contained aberrant results. A second control procedure based on the high and low tumor weights for erratic averages, excessive variation (and also for erratic individual results) in the single “high-low” control chart. For normally distributed variables, the efficiency of the mid-range (the average of the highest and lowest values of the sample) is theoretically better than 90 per cent relative to that for the average of all three sample values. The multiple performance of this graphic device would seem to be useful enough so that even with moderately larger samples it could be well employed despite a larger cost in statistical efficiency. Assay results, when plotted against the combined “high-low” chart, have yielded information identical with that obtained from the “activity-rating” band graph system.

The consistent antitumor activity of “cortisone-like” steroids parallels the results in certain transplantable animal tumor systems (4). Therefore, in this respect, the human tumor transplant is not unique. Since H.Ep. #3 tumor-bearing mice have already been conditioned with levels of cortisone so as to permit maximal growth of the heterologous tumor, further treatment with cortisone or cortisone-type agents could readily result in toxic effects to the host and, in turn, to the tumor. In the present study, antitumor effects were also associated with host toxicity. In the case of several of the more potent corticoids, doses close to the LD₅₀ were required to produce antitumor activity. In view of these considerations and because of the fact that “significant” tu-
mor inhibitions can also be obtained in the H.Ep. #3-tumor system when host body weight loss is greater than 15 per cent, it is likely that these active corticoids are producing their effects indirectly rather than via any specific action on the tumor cell per se.

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