Influence of Insulin Administration on Growth of the 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinoma in Intact, Oophorectomized, and Hypophysectomized Rats

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SUMMARY

Administration of insulin for 6 weeks at a daily dose of 2.5 i.u./100 g body weight, together with a 10% glucose solution as drinking fluid, increased tumor growth 8.3-fold as compared with a matched, untreated control group. Administration of insulin alone or of a 10% glucose solution alone produced a smaller yet statistically significant increase (4.8- and 2.2-fold, respectively). In oophorectomized rats, administration of the same dose of insulin, together with the 10% glucose solution for 4 weeks, failed to prevent tumor regression resulting from oophorectomy. On the other hand, in hypophysectomized rats, administration of insulin for 3 weeks at a daily dose of 0.4 to 0.8 i.u./100 g body weight significantly reactivated tumor growth, as compared with a matched control group, when started 21 days after hypophysectomy. Both insulin-treated and control groups received, in addition, a 10% glucose solution and daily s.c. injections of 1.5 mg ovine prolactin; the latter proved by itself incapable of significantly reactivating tumor growth.

It is concluded that insulin administered in vivo appears to display intrinsic growth-stimulating properties on the mammary tumor tissue, similar to those previously demonstrated in organ culture (7–9, 11), could also be demonstrated in vivo. Thus, tumors which were insulin dependent in vitro regressed in the rat after induction of alloxan diabetes and, conversely, only the few tumors that were insulin independent in vitro were able to grow in diabetic rats.

In order to investigate further this effect of insulin, the present paper examines whether administration of insulin to tumor-bearing rats stimulates tumor growth and formation and whether such stimulating effect is a direct one at the tumor tissue level, similar to that occurring in organ culture, or one which is mediated through other endocrine glands.

MATERIALS AND METHODS

Mammary carcinomas were induced in random-bred, female Sprague-Dawley rats, at age 50 days, by a single feeding of 20 mg of DMBA dissolved in sesame oil (14).

Oophorectomy was carried out by the dorsolateral route. Hypophysectomy was achieved by the transphenoidal route. On the day of operation and on the following day, rats received 1 mg of hydrocortisone acetate s.c. and a solution of 5% glucose and 0.9% NaCl as drinking fluid; they were then allowed to drink a 0.9% NaCl solution for an additional period of 4 days.

The insulin used was Novo-Lente (amorphous insulin, 12 i.u.; crystallized insulin, 28 i.u.; and zinc chloride, 0.08 mg, respectively, per ml of water; Novo A/S, Copenhagen, Denmark). It was given to intact and oophorectomized rats in amounts of 2.5 i.u. per 100 g body weight s.c. daily, 6 days/week. Some groups of rats received in addition a 10% glucose solution as drinking fluid in order to protect them from lethal hypoglycemia; the rats consumed large amounts of this glucose solution. Ovine prolactin (NIH-P-S9; 30.3 i.u./mg) was administered at the dose of 1.5 mg s.c. daily, 6 days/week.

Tumor size was measured by means of a caliper and organ culture (7–9, 11), could also be demonstrated in vivo. Thus, tumors which were insulin dependent in vitro regressed in the rat after induction of alloxan diabetes and, conversely, only the few tumors that were insulin independent in vitro were able to grow in diabetic rats.

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Tumor size was measured by means of a caliper and
expressed as "surface" by multiplying 2 perpendicular diameters. Total tumor surface per rat designates the sum of the surfaces of individual tumors.

Statistical analysis was carried out by Student's *t* test and by nonparametric methods specified in the text (20, 21).

**RESULTS**

**Effect of Insulin in Intact Tumor-bearing Rats.** Fifteen weeks after DMBA administration, 68 tumor-bearing rats were arrayed by order of increasing total tumor surface, divided in successive lots of 4 rats in this array, and randomized within these lots into the following treatment groups of 17 rats each, Groups A, B, C, and D.

Group A served as control and received no particular treatment. Group B received a 10% glucose solution as drinking fluid. Group C was treated with insulin. Group D received both insulin and a 10% glucose solution. Duration of the experimental period was 6 weeks. At the end, the rats were killed and autopsied. All tumors were subjected to histological examination, and only carcinomas were taken into consideration. Six fibroadenomas were found in the entire experiment and were discarded from the results because of their very different properties of hormone dependence (4). The results are presented in Chart 1 and in Tables 1 and 2. Chart 1 presents the mean tumor growth (increment of total tumor surface per rat) in each treatment group. It shows that tumor growth was significantly greater in Groups B, C, and D, than in control group A, and that the stimulating effect significantly increased from Group B to Group C and to Group D (*p* < 0.05). The maximum effect was observed in Group D (insulin plus 10% glucose), in which an 8.3-fold increase in tumor growth occurred, as compared with the control Group A. The increases in Groups B and C were 2.2- and 4.8-fold, respectively, as compared with control Group A.

Table 1 shows the individual course of the tumors in the 4 treatment groups. The tumors are classified as regressive, static (less than 10% change), progressive, and newly formed. It can be seen that the number of regressive tumors was smaller, while that of progressive and newly formed tumors was greater in Groups B and C compared with Group A, and in Group D compared with Groups A, B, and C. Statistical analysis shows that these differences are significant (Table 1, Footnote a). Table 1 illustrates the known fact that a certain proportion of the DMBA-induced rat mammary carcinomas undergo spontaneous regression (22). Treatment D completely overcame this process, since no tumor regression was observed in this group.

Table 2 lists the effects of treatments, at the end of the experimental period, on body weight, fluid consumption, serum glucose, and weight of organs. Analysis of these data leads to the following conclusions.

1. Insulin, with or without glucose, increased the body weight by 16 to 17%, while glucose alone was without effect.

2. The simple addition of 10% glucose to the drinking water increased the fluid consumption 3-fold; concomitant administration of insulin increased it another 2-fold. Insulin administration, without glucose solution, increased the fluid consumption more than 2-fold. The amount of glucose consumed in the drinking water daily was 4.7 and 9 g in Groups B and D, respectively.

3. The blood serum glucose measured 24 hr after the last insulin injection was not significantly elevated in Group B, while it was markedly elevated in Groups C and D, as compared with control Group A. When measured 4 hr after the last insulin injection, it was markedly lowered in both Groups C and D, although to a significantly lesser extent in Group D, which received the 10% glucose solution in addition to insulin.

4. The weight of the different endocrine glands was not significantly different in the 4 groups of animals (*p* > 0.10).

5. The weight of the liver was found to be increased by about 20% in the insulin-treated groups, with (Group D) or without (Group C) glucose. The weight of the kidneys was decreased in Group B, receiving the glucose solution, but was increased in Groups C and D with respect to the corresponding control Groups A and B.

All tumors in the 4 groups were histologically identical. The tumors were classified by use of the histological criteria described by Archer and Orlando (1). Most tumors belonged to the well-differentiated "type B." There was no distinct increase in cell secretory activity or in the proportion of tumors of the secretory "type D."

**Effect of Insulin in Oophorectomized Rats.** This experiment was devised to determine whether insulin could prevent mammary tumor regression following oophorectomy, in other words, whether it could exert its stimulating effect in the absence of the ovarian secretions.

Seventeen weeks after DMBA administration, 3 groups of 10 tumor-bearing rats each were formed, with the use of the
Table 1
Effect of administration of insulin, of 10% glucose solution as drinking fluid, and of concomitant administration of both treatments on the course of individual tumors

These results pertain to the same experiment shown in Chart 1.

<table>
<thead>
<tr>
<th>Group (no. of rats at end of experiment)</th>
<th>Treatment</th>
<th>Regressive</th>
<th>Static (Δ ≤ 10%)</th>
<th>Progressive</th>
<th>Newly formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (14)</td>
<td>Control</td>
<td>13</td>
<td>2</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>B (15)</td>
<td>10% glucose solution</td>
<td>10</td>
<td>2</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>C (13)</td>
<td>Insulin</td>
<td>5</td>
<td>0</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>D (15)</td>
<td>10% glucose solution + insulin</td>
<td>0</td>
<td>2</td>
<td>27</td>
<td>37</td>
</tr>
</tbody>
</table>

* The treatment effects were analyzed by ascribing a score to each rat, based on the course of individual tumors. The rat scores were obtained by determining the algebraic sum of tumor scores, based on their behavior and expressed in arbitrary units, as follows: regressive tumors, -1; static tumors, 0; progressive or newly formed tumors, +1. Tumor scores are based on the assumption that regressing tumors have the inverse biological meaning of progressive or newly formed ones. Statistical analysis, using the Fisher randomization test (21), 2-tailed, yields the following levels of significance: B - A, p = 0.05; C - A, p = 0.06; D - A, p < 0.01; D - B, p = 0.02; D - C, p = 0.02.

Table 2
Effect of administration of insulin, of 10% glucose solution as drinking fluid, and of concomitant administration of both treatments on various parameters, as measured at the end of experiment or at autopsy

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Rat weight (g)</th>
<th>Fluid consumption (ml/24 hr)</th>
<th>Before insulin</th>
<th>4 hr after insulin</th>
<th>Pituitary (mg)</th>
<th>Adrenals (mg)</th>
<th>Ovaries (mg)</th>
<th>Uterus (mg)</th>
<th>Liver (g)</th>
<th>Kidneys (mg)</th>
<th>Spleen (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>249</td>
<td>± 6</td>
<td>88</td>
<td>39</td>
<td>11.2</td>
<td>45</td>
<td>561</td>
<td>10.2</td>
<td>928</td>
<td>573</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10% glucose solution</td>
<td>249</td>
<td>± 1.9</td>
<td>± 0.5</td>
<td>± 2.1</td>
<td>± 3.2</td>
<td>± 41</td>
<td>± 0.4</td>
<td>± 28</td>
<td>± 29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Insulin</td>
<td>289a</td>
<td>± 4</td>
<td>± 4.1</td>
<td>± 3.3</td>
<td>11.2</td>
<td>44</td>
<td>52</td>
<td>249</td>
<td>12.9</td>
<td>1.033</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>10% glucose solution + insulin</td>
<td>292a</td>
<td>± 4</td>
<td>± 4.8</td>
<td>± 3.3</td>
<td>11.6</td>
<td>41</td>
<td>50</td>
<td>622</td>
<td>13.1</td>
<td>921</td>
<td></td>
</tr>
</tbody>
</table>

All values were obtained with Student's t test.

| CD - C, D - A, and D - B, p < 0.001. |
| BD - A, p < 0.10; D - B, p < 0.10; C - A, p < 0.001; D - A, p < 0.01. |
| DC - C, p < 0.01. |
| CD - C, D - A, and D - B, p < 0.01. |
| A - B, D - B, and C - A, p < 0.05. |

randomization design described under "Effect of Insulin in Intact Tumor-bearing Rats." The 1st group served as control; the 2nd and 3rd groups were oophorectomized. The 3rd group received, in addition, insulin injections and 10% glucose in the drinking water. The experiment lasted for 4 weeks.

The mean change in total tumor surface per rat in each group is shown in Chart 2. The 1st group had a normal rate of tumor growth. In the 2nd and 3rd groups, there was an almost complete regression of nearly all tumors that was totally uninfluenced by insulin and glucose administration.

**Effect of Insulin in Hypophysectomized Rats.** This experiment was carried out to determine whether the stimulating effect of insulin on mammary tumor growth in intact rats could have been mediated through an increased secretion of pituitary hormones, such as for instance growth hormone or prolactin.

The experiment was conducted as follows. Thirteen weeks after DMBA administration, 37 tumor-bearing rats were hypophysectomized. Three weeks after hypophysectomy, 23 rats were selected because they appeared effectively hypophysectomized (criteria: loss of at least 30 g body weight and marked tumor regression; the completeness of hypophysectomy was verified at the end of experiment by histological examination of the sella turcica, as detailed
Chart 2. Effect of administration of insulin and 10% glucose solution to oophorectomized rats on size of mammary tumors. The period of observation was 4 weeks. Three matched groups of 10 rats were constituted according to the randomized-blocks design described in the text. A, untreated control group; B, oophorectomized rats; C, oophorectomized rats receiving 10% glucose solution and daily s.c. injections of insulin, 2.5 I.U./100 g body weight. Numbers in parentheses, number of rats at the end of experiment; ■, mean change in total tumor surface per rat during the observation period. Normal tumor growth occurred in control Group A; tumor regression occurred in Groups B and C, uninfluenced by insulin.

below). The rats were then paired on the basis of the reduction of total tumor surface after hypophysectomy and were distributed, by randomization within pairs, into 2 groups of 10. Both groups of rats received prolactin and 10% glucose in the drinking water. The experimental group received insulin in addition. Although the doses of insulin were very small (see "Materials and Methods"), 3 rats died after the 1st injection. Therefore, the corresponding controls and 3 rats kept in reserve were paired according to the initial criterion and were randomly distributed into the 2 groups. Two additional rats of the insulin-receiving group died on the 2nd and 5th day; 1 of the corresponding controls was then selected at random and allocated to the insulin group. Still, an additional insulin-treated rat died from hypoglycemia, leaving 8 pairs that could be evaluated at the end of the 3-week period of the prolactin-glucose and prolactin-glucose-insulin treatments. It is worth remembering that all rats received prolactin and glucose for an identical period, while only one-half of those in the insulin-treated group received insulin during the entire 3-week period.

The results, given in Chart 3, demonstrate that, in the insulin-treated group, although insulin was not given either in full amounts, or during the entire experimental period to all rats, a significant increase in tumor growth was observed as compared with the control group. Resumption of growth in the insulin-treated group, however, was only partial; about one-third of the tumor surface lost after hypophysectomy was restored. In the control group, restoration by prolactin and glucose was less than 10% and was not statistically significant.

Chart 3. Reactivation of tumor growth by administration of insulin 21 days after hypophysectomy. The period of observation was 3 weeks. Two matched groups of rats were constituted by randomization within pairs, as described in the text. A, hypophysectomized rats receiving 10% glucose solution and daily s.c. injections of 1.5 mg ovine prolactin; B, hypophysectomized rats receiving the same treatment plus daily s.c. injections of insulin, 0.4 to 0.8 I.U./100 g body weight. Eight matched pairs of rats survived at the end of experiment. ■, mean values of tumor growth during treatment. Statistical analysis, with the Wilcoxon test (20) on the paired differences, 1-tailed, yields p < 0.025.

At the end of the experiment, the rats were killed and autopsied, to check the completeness of hypophysectomy. The ovaries, uterus, and adrenals were weighed and examined histologically; all were in a state of profound atrophy. The sella turcica was fixed, decalcified, and examined on serial sections; no hypophyseal tissue whatsoever could be found in these sections.

DISCUSSION

The experiments reported here show that administration of insulin intensely stimulates growth of the rat mammary carcinoma. This effect is the opposite of that resulting from insulin deprivation in our previous experiments on alloxan-diabetic rats (12).

This action is probably one of stimulation of tumor cell proliferation by insulin in vivo. This interpretation is based on earlier experiments on tumor tissue explants in organ culture, in which it was shown that insulin strongly increased DNA synthesis and cell proliferation, presumably through enzyme induction (7–9, 11).

Although the conditions prevailing in organ culture are entirely different from those existing in vivo, it is useful to compare the concentration of insulin reached under both conditions. The minimum concentration which effects the maximum stimulation of DNA synthesis in the tumor explants in vitro is about 1 µg/ml, i.e., 25 milliunits/ml (J-C. Heuson, N. Legros, and R. Heimann, unpublished data). This concentration is greater than the plasma level in the
Sprague-Dawley rat: this level varies from 50 microunits in the resting state to 250 microunits/ml under the effect of various stimuli (6). In our experiments, the plasma level reached after administration of insulin has not been measured. However, comparison with reported data on endogenous insulin secretion is relevant to this question. Endogenous secretion has been evaluated by various methods. One method, based on the determination of such parameters as insulin space, turnover rate, and mean plasma concentration, yielded the value of 0.1 i.u. /100 g body weight/day (5). However, methods based on insulin administration either to intact rats (3) or to alloxan-diabetic rats (15; J-C. Heuson, N. Legros, and R. Heimann, unpublished data) yielded higher values: 1.0 and 1.2 to 1.5 i.u./100 g body weight/day, respectively. It is thus apparent that the doses of insulin administered to the intact and oophorectomized rats in the present experiments (2.5 i.u./100 g body weight/day) were largely supraphysiological. An additional aspect to be considered is the discontinuity of administration of insulin, which was injected as single daily doses. This discontinuity was partly alleviated by the use of a long-acting insulin preparation. Nonetheless, single daily injections of this preparation produced a deep hypoglycemia within 4 hr, whereas hyperglycemia occurred after 24 hr. This hyperglycemia indicates that the preceding dose was exhausted, while endogenous secretion was depressed (16). A consequence of the intermittent administration of supraphysiological doses of insulin is that, at times, very high levels of insulinemia are reached. The latter may conceivably be of the same order of magnitude as that used in organ culture. Although the same concentration of insulin is not expected to be required to produce equal effects both in vitro and in vivo, concentrations close to those which are active in organ culture may well have been achieved in the present in vivo experiments. It is concluded that there exists no argument, based on concentration, to refute the interpretation that insulin administered in vivo may directly stimulate cell proliferation in the mammary tumor tissue, by mechanisms similar to those involved in organ culture (9, 11), thus accounting for the intense stimulation of tumor growth observed here.

Nevertheless, these considerations do not rule out the possibility that the stimulating action of insulin on tumor growth was indirect, through stimulation of endocrine secretions. Thus it was found that the presence of the ovaries was required for insulin to exhibit its stimulating properties. It is believed that this is simply the reflection of the complexity of the endocrine control of mammary tumor growth which probably involves several hormones. However, it appears unlikely that the action of insulin in nonoophorectomized rats was merely a result of ovarian stimulation. The reasons are that insulin is not known to increase ovarian secretion markedly, that insulin did not significantly increase ovarian weight in the present experiments and, of still greater import, that administration of estrogens and progesterone, while they support tumor growth in oophorectomized rats (23), does not stimulate tumor growth to any marked extent in intact rats (22).

The possibility of an indirect effect through stimulation of the pituitary secretions should now be discussed. It is known that insulin-induced hypoglycemia increases growth hormone secretion. However, it seems unlikely that growth hormone played an important part here for several reasons. First, we found in a small number of experiments on insulin-dependent tumors in organ culture that growth hormone failed to stimulate cell proliferation to any significant extent (J-C. Heuson, N. Legros, R. Heimann, unpublished data). In vivo, it was shown that bovine growth hormone completely failed to reactivate tumor growth from their regressed state in "triply operated rats" (ablation of the ovaries, adrenals, and pituitary) (18), in adrenoovariectomized rats (17), or after spontaneous regression (22). Finally, in the present experiments, administration of a 10% glucose solution as drinking fluid to rats increased tumor growth by itself, while producing a significant hyperglycemia. This in turn is expected to decrease growth hormone secretion and, on the contrary, to raise that of insulin (2). In fact, the latter might well be responsible for the stimulating effect of the 10% glucose solution administered alone. Moreover, the glucose solution enhanced the stimulating effect of insulin, although it decreased the insulin-induced hypoglycemia, which is the alleged stimulus to growth hormone secretion. An important role of growth hormone, in the present experiments, is therefore unlikely.

In contrast, a possible part played by prolactin is of greater interest in this discussion. It has been reported that prolactin used alone reactivates mammary tumor growth in triply operated rats (18) or in adrenoovariectomized rats (17). Moreover, perphenazine, which stimulates prolactin secretion, allows DMBA-induced mammary carcinogenesis to take place in oophorectomized and adrenalectomized rats (19). These data show that prolactin markedly influences tumor growth under various conditions. The observation on the effect of perphenazine is of particular interest in the present discussion because, if insulin was acting on tumor growth by increasing prolactin secretion, it should then be expected, by analogy with perphenazine, to stimulate tumor growth in oophorectomized rats. This was not the case, and it is therefore suggested that, if insulin failed to reactivate tumor growth under such conditions, this may have resulted from cessation of prolactin secretion in oophorectomized rats, which could not be reactivated by insulin, in contrast to results with perphenazine. These data are consistent with the view expressed by Pearson et al. (18, 19) that prolactin is necessary for the maintenance of growth of the rat mammary carcinoma. This tumor, with rare exceptions, also has an absolute requirement for insulin to grow in vivo, as shown in our previous experiments in alloxan-diabetic rats (12).

In the present experiments, it was shown that insulin retains its stimulating effect on tumor growth in hypophysectomized rats receiving replacement doses of prolactin. Prolactin was administered to both the control and insulin-treated rats, because of the claimed absolute requirement for prolactin referred to above (18, 19). Somewhat unexpectedly, prolactin alone did not measurably reactivate tumor growth. This can probably be explained by insufficient amounts of administered prolactin. Nagasawa and Yanai (17) recently reported experiments wherein doses of prolactin higher than those used by Pearson (18) and by ourselves were necessary for reactivation of tumor growth in adrenoovariectomized rats. Notwithstanding these discrepancies, the important point is the present observation of an unequivocal, intrinsic
growth-stimulating effect of insulin on mammary tumors in rats that had been totally hypophysectomized. This effect is particularly significant in view of the minuteness of the doses of insulin used. The latter may explain, perhaps in part, that insulin restored only one-third of the tumor surface lost after hypophysectomy.

Insulin therefore appears to display intrinsic growth-stimulating properties on the DMBA-induced rat mammary carcinoma in vivo that seems not to be indirectly mediated through other endocrine secretions but rather to be identical with the corresponding property previously demonstrated in organ culture. The present data, together with earlier data on the same subject (7, 12), provide concordant evidence suggesting that the rat mammary tumor, in addition to being estrogen (13) and prolactin (19) dependent, is also insulin dependent.

REFERENCES
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