Effect of Combined Selenium and Retinyl Acetate Treatment on Mammary Carcinogenesis

Henry J. Thompson, L. David Meeker, and Peter J. Becci

MATERIALS AND METHODS

Virgin female Sprague-Dawley rats obtained from Taconic Farms, Germantown, N. Y., at 35 days of age were used. The animals were housed in stainless steel wire mesh-bottomed cages (4 rats/cage) in a controlled environment with temperature maintained at 22 ± 1° (S.D.) and a 12-hr light-dark cycle. Animals were provided with Purina 5001 laboratory chow diet and tap water ad libitum. The chow diet contained 0.2 μg selenium and 15 IU of retinyl acetate per g. Levels of selenium in the drinking water were below the limits of fluorometric analysis.

At 50 days of age, the rats received an i.v. injection of MNU dissolved in 0.9% NaCl solution. The carcinogen was administered at the level of 50 mg MNU per kg body weight as described previously (15). Seven days after injection of MNU, the animals were randomized into 4 groups of 25 rats. The resulting groups were each fed one of 4 diets: a placebo chow diet containing gelatinized beadlets to which no retinyl acetate was added; chow diet supplemented with 4 mg of selenium per kg of diet as sodium selenite and placebo gelatin beadlets; chow diet supplemented with 300 mg of retinyl acetate per kg of diet as a stable gelatinized beadlet preparation; and a chow diet supplemented with 300 mg of retinyl acetate and 4 mg of selenium per kg diet.

All animals were palpated for the detection of mammary tumors twice each week and were weighed weekly. The length of the estrous cycle was determined in 10 rats per group throughout the study. The study was terminated 130 days following carcinogen treatment. At necropsy, all tumors, as well as areas of uninvolved mammary tissue, were removed and processed for histopathological evaluation. This permitted histological classification of the tumors palpated during the study. In addition, sections of ovary, oviduct, uterus, and liver
were taken and evaluated for pathological changes. Of the tumors palpated during the study or detected at necropsy, only those which were confirmed as mammary gland adenocarcinomas were used in the statistical evaluation of the data.

**Statistical Analysis.** Differences in cancer incidence among treatment groups were analyzed using the \( \chi^2 \) test with Yates correction for continuity (13). Approximate 95% confidence intervals for the median time-to-first tumor for the four treatment groups were calculated from the sign test as described by Gibbons (2). The empirical distribution functions of the time-to-first-tumor distributions were compared using the one-sided Kolmogorov-Smirnov 2-sample test, as described by Gibbons (2). Goodness of fit of the observed number of tumors per rat to a Poisson distribution was determined by the usual \( \chi^2 \) test (13).

The mean number of tumors in the treatment groups were compared using the likelihood ratio test based on their Poisson distributions (7). Analysis of variance (11) of the number of tumors per rat (tumor count) was performed on data after square root transformation (13).

**RESULTS**

The tumors induced by i.v. administration of MNU were predominantly mammary gland adenocarcinomas. Of the 270 tumors found at necropsy, only 2 were fibroadenomas. Data concerning histopathologically confirmed adenocarcinomas were tabulated and analyzed in an investigation of the effects of diet treatment upon cancer incidence, latency to tumor appearance, and average number of tumors per rat.

The incidence of cancer 130 days after administration of the carcinogen is shown in Table 1. Differences in the number of tumor-bearing rats among the treatment groups PL, SE, and RA at the termination of the study were insignificant. A comparison between the PL and the RA + SE groups showed a significant difference \((p < 0.05)\).

Previous studies (8, 9, 14, 15) have indicated that tumor latency is increased by the addition (in sufficient quantities) of either retinyl acetate or selenium to an animal's diet. The data arising from this experiment support this hypothesis (Table 1, Chart 1). Table 1 shows approximate 95% confidence intervals for the median time-to-first tumor for each of the four treatment groups. While differences between PL and SE or between RA and RA + SE were not significant, there was strong evidence that the tumors latency associated with treatments RA and RA + SE were longer than that of PL. This hypothesis was further strengthened by comparison of the empirical time-to-first-tumor distributions which indicated a significant difference \((p < 0.005)\) between groups PL and RA and PL and RA + SE, and between Groups RA and RA + SE \((p < 0.07)\). Groups PL and SE were not significantly different \((p > 0.9)\).

The estimated mean number of tumors per rat in each treatment group is shown in Table 1. Poisson distributions provide a good fit to the observed number of tumors per rat. The usual \( \chi^2 \) test shows a good fit \((p > 0.2)\) for Groups PL, SE, and RA and a marginal fit \((p > 0.1)\) for Group RA + SE. In the latter instance, the fit was made difficult because of the presence of 2 very susceptible rats (outliers) who, between them, account for 20 of the 43 observed tumors in the (RA + SE) group and thus greatly inflate the sample variance.

The mean number of tumors in Groups SE, RA, and RA + SE were each compared with PL. Of these comparisons, only the first (PL and SE) was insignificant \((p > 0.1)\). Similar comparisons of the mean number of tumors in Groups RA and RA + SE yielded a significant result \((p < 0.07)\). It should be

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**Table 1**

<table>
<thead>
<tr>
<th>Diet and treatment</th>
<th>No. of rats</th>
<th>Tumor-bearing rats</th>
<th>No. of tumors</th>
<th>Median time to first tumor</th>
<th>Body wt (g)</th>
<th>Length of estrous cycle (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>25</td>
<td>25 (100)</td>
<td>52 (3.68)</td>
<td>52 ≤ Med(4) ≤ 76</td>
<td>260 ± 4</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>SE</td>
<td>25</td>
<td>25 (100)</td>
<td>71 (2.84)</td>
<td>55 ≤ Med(4) ≤ 78</td>
<td>243 ± 3</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>RA</td>
<td>23 (92)</td>
<td>61 (2.44)</td>
<td>90 ≤ Med(4) ≤ 117</td>
<td>241 ± 4</td>
<td>5.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>RA + SE</td>
<td>17 (68)</td>
<td>43 (1.72)</td>
<td>90 ≤ Med(4) + SE</td>
<td>236 ± 3</td>
<td>8.4 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

*Approximate 95% confidence interval for median time to first tumor (days postcarcinogen).

*Numbers in parentheses, percentages.

*Numbers in parentheses, estimated mean number of tumors per rat.

*Med, median.

*Mean ± S.E.

*Significantly different from placebo group \((p < 0.5)\).

*Significantly different from placebo group \((p < 0.01)\).

*Significantly different from retinyl acetate group \((p < 0.07)\).
remarked that this significance level is markedly increased (p < 0.005) if the 2 outliers, previously mentioned, are excluded from the RA + SE group.

Table 2 presents a summary of the analysis of variance of the transformed tumor count data. The results of this 2 × 2 factorial analysis indicate a significant contribution due to diet with a highly significant component identified with the retinyl acetate factor and a significant component identified with the selenium factor. The variance component associated with the interaction between the 2 diet additives was insignificant.

The analysis of variance was repeated in order to compare the animals’ response to the treatments during the early portion of the experiment. The data consisted of the number of tumors observed for each rat in the period 0 to 84 days postcarcinogen (84 days was the median time-to-first-tumor appearance in the placebo group). Comparisons of the estimated mean number of tumors per rat indicated no significant difference between the PL and SE groups and between the RA and RA + SE groups. Significant differences were indicated between SE and RA (p < 0.05). The analysis of variance of transformed tumor counts indicated a highly significant variance component associated with the retinyl acetate factor (p < 0.001) and insignificant contributions arising from the selenium factor and the interaction component.

At the termination of the study, the group mean body weights of animals fed diet supplemented with either SE, RA, or RA + SE were 96, 95, and 93% that of the controls, respectively (Table 1). Weight loss was not observed in this study and no external signs of selenium or retinyl acetate toxicity other than reduced food intake and rate of gain were evident. The length of the estrous cycle was prolonged in animals fed RA + SE (Table 1).

At necropsy, the livers of rats treated with either PL or SE alone were essentially normal when evaluated by conventional histological methods (5-μm sections stained with hematoxylin and eosin). The livers of animals receiving RA and RA + SE had mild generalized perilobular degenerative changes with necrosis of random hepatocytes in the degenerative regions. Over 50% of the animals in the RA + SE group had ovaries with marked fibrosis and chronic and acute inflammation. In those animals with pathological ovarian changes, oviducts had marked fibrous thickening of the walls with extensive chronic and acute inflammation including small focal abscesses. The uterine wall of these animals was thickened and contained chronic and acute inflammatory infiltrates.

DISCUSSION

The results of this study represent the first report of an additive inhibitory effect arising from combined treatment with retinyl acetate and selenium. This observation is particularly significant since the level of selenium fed was not, in itself, high enough to have a significant inhibitory effect on the carcinogenic process. As noted, the level of selenium chosen was 20% less than that previously shown to inhibit MNU-induced tumorigenesis. Loss of antineoplastic activity also results when the level of retinyl acetate is reduced by 20%.

As reported by Harr (4), the feeding of selenium in a laboratory chow diet ameliorates the occurrence of selenium toxicity. No evidence of selenium toxicity other than reduced food intake was observed in this study. Retinyl acetate at the level fed is also known to pose the problem of subchronic toxicity (14). There was evidence of pathological changes in the livers of animals given retinyl acetate, as well as an effect on rate of body weight gain somewhat greater than that caused by selenium. Some degree of liver pathology was also observed in animals treated with RA + SE; in addition, there were fibrotic changes in ovary, oviduct, and uterus.

Animals receiving either PL, SE, or RA had essentially normal estrous cycling throughout the study. Those animals placed on the RA + SE diet (7 days after carcinogen treatment) had highly variable cycle lengths with an average of 8.4 days between the occurrence of estrus.

Although the inhibition of tumorigenesis by treatment with RA + SE was marked, the explanation of this effect is far from clear. The role which was played by changes in estrous cycling caused by RA + SE cannot be determined from the data. Nonetheless, hormone suppression has been shown to enhance the antineoplastic activity of retinyl acetate (17). The fact that inhibition of tumorigenesis in the RA and RA + SE groups was similar over the first 84 days postcarcinogen would, however, suggest that factors other than the hormonal status of the animals were involved in the observed tumor inhibition. Furthermore, factorial analysis of the tumor count data (average number of tumors per rat) indicated that no significant interaction could be attributed to treatment with RA + SE. Rather, the analysis suggested that the inhibitory activity produced by retinyl acetate and selenium was additive.

Reduced rates of body weight gain, which have been associated with an inhibitory influence on the development of spontaneously occurring tumors (16), could have played a role in the study. The extent of voluntary food restriction was, however, smaller than that reported to inhibit tumorigenesis. Furthermore, the animals treated with selenium alone had a rate of body weight gain similar to those given the combined treatment (RA + SE), yet a significant inhibition of tumorigenesis was not observed in the SE group.

Thus far in the discussion, the role which may have been played by the toxic effects of treatment with RA + SE has been stressed. It is also possible that the observed inhibition of tumorigenesis resulted from a pharmacological response to RA + SE. It has been reported that either selenium (1, 12) or vitamin A (10), when given in pharmacological amounts, can have a potentiating effect on an animal’s immune system. Other mechanisms have been suggested for tumor inhibition by derivatives of vitamin A (6). It is interesting to note that (as

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>d.f.</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diets</td>
<td>9.54</td>
<td>3</td>
<td>3.18</td>
<td>6.47</td>
</tr>
<tr>
<td>Retinyl acetate</td>
<td>6.74</td>
<td>1</td>
<td>6.74</td>
<td>13.71</td>
</tr>
<tr>
<td>Selenium</td>
<td>2.40</td>
<td>1</td>
<td>2.40</td>
<td>4.86</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.40</td>
<td>1</td>
<td>0.40</td>
<td>0.81</td>
</tr>
<tr>
<td>Total</td>
<td>56.72</td>
<td>99</td>
<td>0.49</td>
<td></td>
</tr>
</tbody>
</table>

* Significant (p < 0.005).
  b Significant (p < 0.05.).
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Chart 2. The effect of diet treatments on the average number of mammary cancers per animal confirmed histopathologically. Animals were fed either a placebo diet (•) or diet supplemented per kg with either 300 mg retinyl acetate (A), 4 mg selenium as sodium selenite (O), or 300 mg retinyl acetate plus 4 mg selenium (△).

suggested by Chart 2) the effectiveness of treatment with RA was declining during the latter part of the study (after Day 84) whereas in the RA + SE group the effect was maintained. The significance of this observation cannot be determined. The response, however, is compatible with the work of Milner who reported retardation of growth of L1210 cells in selenium-treated mice (3).

In conclusion, significance of combined treatment with retinyl acetate and selenium as an inhibitor of mammary carcinogenesis cannot be stated until additional studies of the role of toxicological versus pharmacological effects resulting from such treatment are differentiated. The results of this investigation do, however, warrant further study.

REFERENCES

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