Effects of Chronic Daily Exposure to Tobacco Smoke on the High Leukemic AKR Strain of Mice

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ABSTRACT

Daily exposure of the high leukemic AKR strain of mice to low levels of fresh tobacco smoke (TS) produces significantly different mortality profiles associated with both the sex of the animals and the age at which TS exposure commences. Females tend to be susceptible and die sooner than males, where a significant proportion of animals survives longer than age-matched controls. This prolongation of life appears to be due to a failure of the leukemic state to be mobilized in the TS-exposed males. Exposure of both the females and the males to the TS does not induce significant detectable immunological reactivity against the leukemic cells for several parameters tested, possibly due to a significant enhancement of suppressor activity in the serum of the chronically exposed animals over and above that which also occurs in age-matched control animals.

INTRODUCTION

Recently there has been increasing criticism of the use of transplantable cell lines in the study of the possible existence and role of an immune surveillance mechanism for the control of tumors (1–3). It has been argued that much of the failure to transfer experimental results to the primary tumor system, especially in humans, has been due to the use of models essentially incorporating atypical rapidly growing malignant cell lines (2). Therefore models based on animals growing their own primary and/or secondary tumors should form the more exacting experimental conditions required to examine the etiology of tumor growth and any concomitant immunity (2–4). However, limitations may still exist in this model as inherent characteristics appear often to determine the inducibility of tumors (4).

Our early studies, designed mainly to investigate the effects of chronic daily exposure to TS2 on adaptive immunity, leave little doubt that TS exposure significantly modifies this to common T- and B-cell requiring antigens. TS exposure also modifies antitumor-immune reactions, both humoral and cell mediated, to transplanted cell lines either to allow them to grow and kill the host or to be rejected (5–9). More recently our studies have used an animal model where primary alveologenic carcinomas have been induced by urethane in the presence or absence of TS exposure (4). In this model there is little evidence of adaptive immunity or natural killer cell recognition of the tumor cells in vitro; however, considerable natural cytotoxic cell activity is expressed in vitro against the tumor cells which in vivo grow to kill the host.4,5

The AKR mouse strain represents animals congenitally destined to develop a 100% fatal incidence of virus-induced leukemia despite the fact that potentially the cells are immunogenic (10, 11). While the development of immune tumors has been associated with the appearance of specific (12–16) or nonspecific suppressor cells (17, 18), the nature of the immune recognition and/or suppression of immunity observed in AKR leukemia is less clearly defined. Leukemic cells derived from leukemic AKR animals have been shown to suppress PHA responses and in vitro antibody production of normal spleen and lymph node cells (19–22). While these interactions appear to require cell to cell contact, the production of a soluble suppressor factor(s) also appears to accompany the growth of malignant cells in AKR mice and can be detected in the sera of leukemic animals (23, 24).

With this wide array of potential immune reactions available, we have investigated the effects of TS exposure on the development of leukemia in AKR mice, and the results are presented herein.

MATERIALS AND METHODS

Animals. AKR (4- to 6-wk-old) female and male inbred mice were obtained from Bantin & Kingman, United Kingdom, or from the Animal Resource Centre, Murdoch University, Western Australia. These animals were derived from the United Kingdom stock under specific-pathogen-free conditions and maintained under minimal disease conditions and fed sterile food ad libitum and acidified water.

Tobacco Smoke Exposure. All animals were randomly allocated to either daily exposure, 5 days/wk, to fresh TS from 30 cigarettes (±15 mg tar) in a 7:1 airsmoke ratio for 7 to 9 min in a Hamburg II small animal smoking machine (Heinrich Borgwaldt, West Germany) or a group of age-matched control animals. The cigarettes were king size filters supplied by the Australian Tobacco Research Foundation.

Animal Mortality Profiles. The results are expressed as the relative cumulative percentages of animals dying following TS exposure at any given time. This is determined as follows.

The relative cumulative number of any given day

\[
\text{(no. dead in the TS-exposed group on the day)} = \frac{\text{(total no. in the control group on the day)}}{\text{(total no. in the test group on the day)}},
\]

These individual relative cumulative numbers are expressed as the relative cumulative percentage for the TS-exposed group and compared statistically (\(x^2\) test) using Yates correction for continuity, to the cumulative percentage dying in the age-matched control group.

The results are also presented as the time for designated percentiles to be reached for both TS-exposed and age-matched control animals of both sexes and commencing TS exposure at various ages. The results are analyzed statistically by the raw \(x^2\) for differences in days to attained given percentages of deaths.

Preparation of Single Cell Suspensions. Spleens, inguinal lymph nodes, and thymus were removed aseptically from animals, and cells were expressed from the tissues using a sterile syringe and passed through stainless steel sieves to prepare single cell suspension.

Erythrocytes present in the single cell suspension were lysed by the addition of ACK lysis solution (8.2 g NH4Cl; 1 g KHCO3; 0.037 g EDTA per liter), and the remaining cells were washed twice in warm RPMI 1640 plus 10% FCS.

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Viable counts of the single cell suspension were performed using a Neubauer counting chamber and resuspended to the appropriate cell density.

Peritoneal Cells. Two female AKR mice of 6 to 8 wk of age were used as the peritoneal cell source at each sample time. The mice were sacrificed by cervical dislocation. Peritoneal cells were collected by washing the peritoneal cavity with 6 ml of warm RPMI 1640 plus 10% FCS using a syringe with a wide bore needle. The cells were washed twice in warm medium, and the viable cells were counted and resuspended to the appropriate cell density.

Preparation of Radioactive (51Cr) Target Cells. Pooled thymoma cells obtained from syngeneic AKR mice were incubated at the concentration of $4 \times 10^8$ cells per 100 $\mu$l of 51Cr (Na251CrO7, Radiochemical Centre, Amersham, United Kingdom) in 1 ml of RPMI 1640 plus 10% FCS at 37°C for 1 h. The resulting radioactive cells were washed once in a 10-ml tube (Falcon Plastic; No. 2001) with a FCS underlay and then twice without the underlay in a Beckman TJ-6 bench centrifuge at 1500 rpm for 5 min.

The viable labeled cells were then counted and resuspended to the required concentrations.

Serum. Blood, collected by heart puncture under deep anesthesia, was left to clot in glass tubes for 1 h in a 37°C water bath. The tubes were spun down in a Beckman bench centrifuge at 3000 rpm for 20 min, having been stabilized at 4°C for 2 h.

Clear sera were removed, filtered, and stored at -20°C until further use.

Complement. Fresh adult rabbit serum, prepared from peripheral blood, was used as the source of complement.

Humoral Cytotoxicity. Various dilutions of sera in 100 $\mu$l of RPMI 1640 from both TS-exposed or age-matched control AKR mice with or without rabbit complement (20 $\mu$l) were added to 100 $\mu$l of 2 $\times$ 108 of 51Cr-labeled thymoma cells in 96-well V-shaped tissue culture plates (Linbro IS-MVC-96). Trays were incubated at 37°C for 1 h and centrifuged at 200 x g for 10 min.

One hundred $\mu$l of the supernatant were harvested from each well and transferred to 3DT tubes, and the radioactivity present was determined by $\gamma$ spectrometry in a Packard β spectrometer (Model 5236).

The results are expressed as:

$$\% \text{ of specific cytotoxicity} = 100 \left(\frac{cpm \text{ test} - cpm \text{ spontaneous released}}{cpm \text{ total released}}\right)$$

The statistical significance of the results was analyzed by Student's $t$ test for the differences between group means.

Cellular Cytotoxicity. One hundred $\mu$l of single cell suspension of effector cells (from spleen, inguinal lymph nodes, thymus) in RPMI 1640 plus 10% FCS from both TS-exposed or age-matched control AKR mice were added to $2 \times 10^8$ of 51Cr-labeled thymoma cells at the ratio of 50 to 1, 100 to 1 (effector cell:target cell ratio), in 96-well V-shaped plates. The trays were incubated at 37°C in 5% CO2 for 4 h and centrifuged at 200 x g for 10 min.

One hundred $\mu$l of supernatant were then harvested from each well and transferred to 3DT tubes, and the radioactivity present was determined by $\gamma$ spectrometry.

The results are expressed, statistically analyzed as above, and presented as the percentage of specific cytotoxicity.

Antibody-dependent Cytotoxicity. Peritoneal cells, prepared as described previously, were seeded at 10$^5$ cells per well in a 96-well flat-bottomed tray (Falcon No. 3072) in 100 $\mu$l of RPMI 1640 plus 10% FCS for 1 h at 37°C in 5% CO2.

Each well of the tray was then washed vigorously 3 times with warm RPMI without FCS to remove nonadherent cells. The remaining adherent cells (70% of total cells) were opsonized with 100 $\mu$l of serum in RPMI 1640 from TS-exposed and age-matched control AKR mice for 24 h at 37°C in 5% CO2 prior to the addition of target cells; 100 $\mu$l of 51Cr-labeled pooled thymoma cells were added to each well at the ratio of 50 to 1, 100 to 1 (effector cell:target cell ratio). The trays were incubated for a further 4 h at 37°C in 5% CO2 and centrifuged at 200 x g for 10 min.

One hundred $\mu$l of the supernatant were harvested from each well and transferred to 3DT tubes, and the radioactivity present was determined by $\gamma$ spectrometry.

The results were expressed as before as the percentage of specific cytotoxicity.

Interferon Assay. An established technique (25) was used to assay for interferon-like activity in the sera and peritoneal cells of female AKR mice exposed to TS daily or age-matched controls. Encephalomyocarditis virus was used as the indicator virus because vesicular stomatitis virus is not permitted in Australia (25). The virus dilution used was sufficient to provide for a CPE following 16 to 18 h of incubation. The end point of interferon-like activity was recorded from the turbidity well showing 50% CPE. Three animals were tested in duplicate at each sample time, and the results were calculated on the basis that it required 1 unit of interferon to neutralize the CPE of the virus. The results are expressed as units of interferon-like activity per ml of test material.

A laboratory source of fibroblast interferon was prepared (26) and used as a positive control at each test.

The results were analyzed by Student's $t$ test for the significance between group means.

SeraSuppressor Activity Assay. This assay for measuring inhibitory factors existing in the sera of AKR mice of various ages following daily (5 days per wk) TS exposure or age-matched control animals is well documented (24).

The cells were finally harvested onto filter pads using a Skatron cell harvester. The filter pads were dried and transferred to vials containing scintillation fluid at the 27:1 ratio of Toluene and Pernfluelor. The radioactivity present was determined by $\beta$ spectrometry in a Packard $\beta$ spectrometer (Tricarb-2660).

The results are expressed as dpm $\times 10^{-3}$ of $6\text{H}$_{thymidine (Code TRA-61; Radiochemical Centre) trapped in cellular DNA, and Student's $t$ test was used to compare the group means between the sera of animals under TS exposure and age-matched controls.

Humoral Responses of AKR Mice to Sheep Erythrocytes. Washed sheep erythrocytes were resuspended to 1% (v/v) in phosphate-buffered saline via a micro-hematocrit reader (Hawksley, United Kingdom). TS-exposed or age-matched control AKR mice were immunized with 200 $\mu$l of 10$^8$ sheep erythrocytes per ml into the peritoneal cavity at Day 0 and Day 11.

At each sample time, 5 animals were sampled for blood by cardiac puncture (Days 0, 5, 7, 11, 15, and 18). Sera were separated from the blood by centrifugation and stored at -20°C until tested. Sera collected from the experiment were tested for the presence of antibody on the same day.

Antibody Titration. Twenty $\mu$l of 1:4 dilution of test serum were serially diluted in normal saline in the 96-well U-bottomed plates (Cooke, Alexandria, VA). To each well, 20 $\mu$l of 1% sheep RBC were added. The plates were washed with a damp paper towel and placed on wet Benchkote absorbent paper (Whatman, England). End point titers were read after 90 min of incubation at room temperature. In a parallel series, 20 $\mu$l of 1:4 dilution of test serum were added to 20 $\mu$l of 0.1 M 2-mercaptoethanol (BDH, United Kingdom) and allowed to incubate at room temperature for 30 min before serially diluting as above.

The titer for total serum antibody was taken as the dilution of the 2-mercaptoethanol-treated serum in the last well showing hemagglutination.

The serum IgG titer was read as the dilution of the 2-mercaptoethanol-treated serum in the last well showing hemagglutination.

The serum IgM titer was calculated as the difference between total and IgG titers.

All results are expressed in log$_2$ dilutions, and the results were analyzed by Student's $t$ test for the differences between means.

RESULTS

Effect of Daily TS Exposure (5 Days/Wk) on the Development of Fatal Leukemia in Inbred Female and Male AKR Mice

Mortality Profiles of Inbred AKR (Female) Mice Exposed Daily (5 Days/Wk) to TS from 48 to 56 Days of Age Compared to Age-matched Controls. Initial studies indicated that there...
were no significant differences in the mortality profiles of male and female AKR mice in the animals used for this study. Table 1 indicates that, if female AKR inbred mice commenced daily TS exposure at 48 days of age, they died in significantly higher numbers than age-matched control animals up to 300 days, when there was no significant difference in the mortality profiles between TS-exposed and TS-unexposed animals. However, if animals were first exposed to TS at 56 days of age, the onset of significant numbers of deaths over age-matched controls was delayed in the TS-exposed animals from 100 days to 250 days. Table 1 indicates that the mortality pattern was significantly different between 100 and 150 days of age if the TS exposure commenced at 48 days rather than 56 days of age.

It can be seen from Table 2 that the higher mortality was occurring at a significantly earlier time ($P < 0.001$) when animals commenced TS exposure at 48 days of age. This pattern was also seen if animals began their TS exposure at 56 days of age, when TS-exposed animals reached the 70th percentile significantly sooner than control animals (Table 2).

Observations at autopsy indicated that a high proportion of animals dying in the 100- to 200-day range following the commencement of TS exposure at 48 days of age had grossly enlarged malignant thymomas and enlarged spleens; their peripheral leukocyte numbers tended to be within the normal range for the strain. However, age-matched unexposed controls, while also exhibiting a similar degree of thymoma development, often presented with peripheral leukocyte counts up to $18 \times 10^9$/liter, especially by 200 days of age. A proportion of animals dying in this age bracket died of undefined causes.

Mortality Profiles of Inbred AKR (Male) Mice Exposed Daily (5 Days/Wk) to TS from 48, 56, or 84 Days of Age Compared to Age-matched Controls. Male AKR mice exhibited the same early significant increase in mortality up to 200 days over age-matched controls if daily TS exposure began at 48 days of age (Table 3). This significant incidence of mortality also occurred at an earlier time than in age-matched controls (Table 4). However, at the 300- to 500-day time scale, although more TS-exposed animals died, this was not statistically significant from age-matched controls (Table 3). There was no evidence that the animals that died were dying significantly sooner than controls (Table 4).

If the animals began their TS exposure at 56 or 84 days of age, they died later than age-matched controls. In the 56-day-old group, this became statistically significant after 300 to 400 days, and the time to death was significantly retarded from 250

### Table 1 Relative cumulative percentage of mortality of inbred female AKR mice commencing TS exposure at 48 or 56 days of age compared to age-matched controls

<table>
<thead>
<tr>
<th>Days of age</th>
<th>100</th>
<th>150</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>350</th>
<th>400</th>
<th>450</th>
<th>500</th>
<th>550</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm (48D)*</td>
<td>11</td>
<td>17</td>
<td>26</td>
<td>58</td>
<td>71</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sm (56D)</td>
<td>2</td>
<td>5</td>
<td>14</td>
<td>58</td>
<td>72</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSm control</td>
<td>3</td>
<td>5</td>
<td>16</td>
<td>32</td>
<td>49</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistics:

$x^2_{48/C}$, $x^2_{56/C}$,

$P < 0.05$, $0.01$, $0.001$ NS

NSm control, age-matched TS-unexposed animals; $x^2_{48/C}$, $x^2_{56/C}$, and $x^2_{56/48}$, statistical comparison of the animals commencing TS exposure on Day 48 to unexposed age-matched controls (C), etc.; NS, not significant.

* $P < 0.05$, statistical probability of the results.

<table>
<thead>
<tr>
<th>Relative cumulative percentile</th>
<th>10</th>
<th>15</th>
<th>25</th>
<th>50</th>
<th>70</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm (48D)*</td>
<td>94</td>
<td>135</td>
<td>200</td>
<td>245</td>
<td>265</td>
<td>550</td>
</tr>
<tr>
<td>Sm (56D)</td>
<td>185</td>
<td>205</td>
<td>210</td>
<td>240</td>
<td>265</td>
<td>560</td>
</tr>
<tr>
<td>NSm control</td>
<td>160</td>
<td>198</td>
<td>230</td>
<td>275</td>
<td>320</td>
<td>540</td>
</tr>
</tbody>
</table>

Statistics:

$x^2_{48/C}$, $x^2_{56/C}$,

$P < 0.001$, $0.01$, $0.001$ NS

NSm control, age-matched TS-unexposed animals; $x^2_{48/C}$, $x^2_{56/C}$, and $x^2_{56/48}$, statistical comparison of the animals commencing TS exposure on Day 48 to unexposed age-matched controls (C), etc.; NS, not significant.

* $P < 0.05$, statistical probability of the results.

### Table 3 Relative cumulative percentage of mortality of inbred male AKR mice commencing tobacco smoke exposure at 48, 56, or 84 days of age compared to age-matched controls

<table>
<thead>
<tr>
<th>Days of age</th>
<th>100</th>
<th>150</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>350</th>
<th>400</th>
<th>450</th>
<th>500</th>
<th>550</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm (48D)*</td>
<td>20</td>
<td>33</td>
<td>40</td>
<td>46</td>
<td>57</td>
<td>69</td>
<td>96</td>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sm (56D)</td>
<td>5</td>
<td>10</td>
<td>16</td>
<td>27</td>
<td>35</td>
<td>42</td>
<td>52</td>
<td>87</td>
<td>100</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Sm (84D)</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>11</td>
<td>24</td>
<td>31</td>
<td>36</td>
<td>55</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSm control</td>
<td>5</td>
<td>13</td>
<td>26</td>
<td>52</td>
<td>65</td>
<td>82</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistics:

$x^2_{48/C}$, $x^2_{C/56}$, $x^2_{C/84}$,

$P < 0.001$, $0.01$, $0.05$ NS

NSm control, age-matched TS-unexposed animals; $x^2_{48/C}$, $x^2_{C/56}$, and $x^2_{C/84}$, statistical comparison of the animals commencing TS exposure on Day 48 to unexposed age-matched controls (C), etc.; NS, not significant.

* $P < 0.001$, statistical probability of the results.
days until the final deaths occurred at between 500 and 600 days (Tables 3 and 4). This result represented a significant prolongation of life for a significant proportion of males of from 100 to 150 days at least (Tables 3 and 4). In animals that commenced TS exposure from 84 days of age, the mortality profile is significantly suppressed over the 150- to 500-day period when compared to age-matched controls. In addition the life expectancy of this group of animals was increased by TS exposure.

The gross pathological picture of male AKR mice dying throughout these trials was similar to that seen in the females. The extent of gross thymoma and enlarged spleens increased throughout the life span of the animals, with a greater tendency for a peripheral leukocyte pattern representing acute leukemia to occur with age. A large proportion of animals died from ill-defined causes.

**Immunological Assessment of Female AKR Mice Exposed to TS**

**Antibody-directed Cellular Cytotoxicity.** Peritoneal cells from 6- to 8-wk-old syngeneic AKR mice were incubated with the sera from TS-exposed or age-matched control animals, and their ability to lyse radioactive syngeneic thymoma cells was tested in vitro. The results are presented in Table 5. There was no evidence of an opsonizing antibody capable of inducing syngeneic cells to become specifically cytotoxic to thymoma cells.

**Direct Cellular Cytotoxicity.** Suspensions of thymus, spleen, and lymph node cells from animals exposed to TS and age-matched controls were used in a 4-h 51Cr-cytotoxic assay with syngeneic thymoma cells as the targets. The assay was established at effectortarget ratios of 50:1, 100:1, and 200:1, and the results of the 100:1 ratio are presented in Table 6. Initially a low but significant cytotoxic activity in the lymph nodes and spleen cells from TS-exposed animals was present; this became ineffective by 200 days of age.

**Humoral Activity.** Sera collected from TS-exposed and age-matched control animals were tested for their ability to kill 51Cr-labeled syngeneic thymoma cells in both the presence or the absence of rabbit complement. The results are presented in Table 7. There is some evidence for the appearance of a complement-dependent cytotoxicity from 290 days of age which was directed against syngeneic thymoma cells. However, there

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**Table 4** Time (days) to arrive at designated mortality percentiles of male AKR mice commencing TS exposure at 48, 56, and 84 days of age, compared to age-matched controls

<table>
<thead>
<tr>
<th>Relative cumulative percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td>Sm</td>
</tr>
<tr>
<td>Sm</td>
</tr>
<tr>
<td>Sm</td>
</tr>
<tr>
<td>NSm</td>
</tr>
</tbody>
</table>

**Table 5** Percentage of antibody-directed cellular cytotoxicity against 51Cr-labeled thymoma cells over a 4-h period, the source of sera being TS-exposed or age-matched control female SKR mice

All results are the means of three animals in duplicate at each sampling time. The results have been compared by Student's t test for the differences between group means, and there is no evidence of statistically significant differences.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum dilution</th>
<th>50</th>
<th>80</th>
<th>110</th>
<th>140</th>
<th>170</th>
<th>200</th>
<th>230</th>
<th>260</th>
<th>290</th>
<th>320</th>
<th>350</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm</td>
<td>1:20</td>
<td>-18.3</td>
<td>-4.5</td>
<td>-0.8</td>
<td>-2.1</td>
<td>-3.2</td>
<td>0.0</td>
<td>-2.9</td>
<td>-7.9</td>
<td>0.0</td>
<td>0.0</td>
<td>-0.3</td>
</tr>
<tr>
<td>Sm</td>
<td>1:40</td>
<td>-18.3</td>
<td>-2.1</td>
<td>-1.4</td>
<td>0.0</td>
<td>-1.4</td>
<td>-1.7</td>
<td>0.0</td>
<td>-4.6</td>
<td>-1.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NSm</td>
<td>1:20</td>
<td>-17.3</td>
<td>-1.0</td>
<td>-1.9</td>
<td>0.0</td>
<td>-0.4</td>
<td>0.0</td>
<td>-0.9</td>
<td>-7.2</td>
<td>0.0</td>
<td>0.0</td>
<td>NA</td>
</tr>
<tr>
<td>NSm</td>
<td>1:40</td>
<td>-17.3</td>
<td>0.0</td>
<td>-0.4</td>
<td>-0.8</td>
<td>-5.2</td>
<td>-0.3</td>
<td>0.0</td>
<td>-9.0</td>
<td>-1.3</td>
<td>0.0</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Sm, TS exposed; NSm, age-matched controls.

**Table 6** Percentage of cytotoxicity as 51Cr release in a 4-h assay using suspensions of thymus, spleen, and lymphocyte cells from female AKR mice exposed to TS from 48 days of age and age-matched controls, the target cells being syngeneic thymoma cells obtained from TS and age-matched controls and labeled with Na2CrO4

All assays were carried out in quadruplicate at 50:1, 100:1, and 200:1 effectortarget ratios, and the 100:1 ratio is presented here.

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>80</th>
<th>110</th>
<th>140</th>
<th>170</th>
<th>200</th>
<th>230</th>
<th>260</th>
<th>290</th>
<th>320</th>
<th>380</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>7.4</td>
<td>-2.5</td>
<td>2.9</td>
<td>-3.3</td>
<td>-13.6</td>
<td>-0.76</td>
<td>1.7</td>
<td>11.3</td>
<td>-2.3</td>
<td></td>
</tr>
<tr>
<td>NSm</td>
<td>-2.5</td>
<td>-0.3</td>
<td>-1.9</td>
<td>-3.9</td>
<td>-3.3</td>
<td>-4.4</td>
<td>-12.1</td>
<td>-15.9</td>
<td>7.1</td>
<td>-6.3</td>
</tr>
<tr>
<td>IGLN</td>
<td>13.8</td>
<td>2.8</td>
<td>5.8</td>
<td>11.1</td>
<td>2.6</td>
<td>2.6</td>
<td>-2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSm</td>
<td>2.4</td>
<td>6.3</td>
<td>1.3</td>
<td>0.65</td>
<td>11.1</td>
<td>-0.1</td>
<td>-3.5</td>
<td>-0.8</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>3.1</td>
<td>0.2</td>
<td>-2.3</td>
<td>-2.20</td>
<td>-7.2</td>
<td>-8.8</td>
<td>-11.2</td>
<td>-6.7</td>
<td>-11.1</td>
<td>-4.1</td>
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* Sm, TS exposed; NSm, age-matched controls.

* Student's t test; P < 0.05.
Interferon-like Activity of the Sera and Peritoneal Cell Extracts from Female AKR Mice TS Exposed Daily from 48 Days of Age and Age-matched Control Animals

Table 8 The units of interferon activity per ml detected in the sera and peritoneal cell extracts from female AKR mice TS exposed daily from 48 days of age and age-matched control animals

<table>
<thead>
<tr>
<th>Days of age</th>
<th>Serum</th>
<th>Sm*</th>
<th>NA</th>
<th>66</th>
<th>106</th>
<th>160</th>
<th>13.3</th>
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<th>Smlog4</th>
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Interferon-like Activity of the Sera and Peritoneal Cell Extracts from TS-exposed and Age-matched Control Animals. Sera and peritoneal cells were routinely obtained from TS-exposed and age-matched control animals and assessed for interferon-like activity in the encephalomyocarditis virus assay system. Table 8 indicates that, between 110 and 200 days, there was more interferon-like activity present in the sera of TS-exposed AKR mice than in age-matched control animals, and interferon-like activity was also detected in cell extracts from peritoneal washings from TS-exposed animals over 140 to 200 days. However, these results are not statistically significant.

Antibody Production to Sheep Erythrocytes and T-Cell-dependent Antigen in TS-exposed and Age-matched Control Animals. Fig. 1 illustrates the ability of TS-exposed and age-matched control animals to develop IgM and IgG antibodies to sheep erythrocytes. Initially at 80 days of age the TS-exposed animals developed significantly more IgG (P < 0.05) antibodies in the early secondary immune response to sheep erythrocytes than age-matched controls. However, as the animals aged (147 days) and hence the TS exposure time became longer, these animals produced significantly (P < 0.05) less IgM antibody than age-matched control animals in the primary immune response at Day 5 but significantly more (P < 0.05) by Day 7. By 178 days of age the TS-exposed animals were making less IgM in the primary response, but this was not statistically significant.

Inhibitory Effects of AKR Serum from Both TS-exposed and Age-matched Control Animals for the DNA Synthesis of Syngeneic Lymphocytes to PHA. Table 9 illustrates the inhibitory effects to DNA synthesis of the sera from TS-exposed or age-matched controls, the longer the animals undergo TS exposure.
TS EXPOSURE OF AKR MICE

80 DAYS OLD AKR MICE

112 DAYS OLD AKR MICE

147 DAYS OLD AKR MICE

178 DAYS OLD AKR MICE

DISCUSSION

Daily exposure of the high leukemic AKR strain of mouse to TS at levels known to initially generate between 5 and 8% carboxyhemoglobin 3 min after exposure (data unpublished) produces significantly different mortality profiles which appear to be associated with both the sex of the animals and the age at which TS exposure commences (Tables 1 to 4). Animals commencing TS exposure at 48 days of age died sooner and in greater numbers than in age-matched controls whether they were males or females (Tables 1 to 4). However, a proportion of TS-exposed animals survived up to 300 days when the mortality patterns were not significantly different from control animals. If female AKR mice commenced TS exposure at 56 days of age, the early mortality pattern was not seen until 250 days (Table 1). However, in male AKR mice the mortality pattern did not become significant until 350 days if TS exposure commenced at 56 days, when at this time the control animals were dying sooner than TS-exposed animals. If TS exposure commenced at 84 days of age this was further substantiated with animals surviving an additional 100 to 150 days (Tables 3 and 4). It appears that the TS exposure has effectively detected two populations of leukemic animals within the AKR strain; one population expresses its leukemia at between 100 and 200 days of age, and the second population exhibits a lymphatic leukemia which can be prolonged to 500 to 600 days in TS-exposed males.

An immunological assessment of female AKR mice exposed to TS from 48 days of age was compared to age-matched controls throughout their respective life spans. In these animals, which represent a population developing their primary leukemias with an additional stress of daily chronic TS exposure, there was no evidence of significant tumor-specific immunity in the long term. There was no evidence of antibody-directed cellular immunity (Table 5); while complement-dependent cytotoxicity could be detected early in the leukemia development, there were no significant differences between TS-exposed or age-matched control animals (Table 7). Low but statistically significant in vitro cellular cytotoxicity existed in TS-exposed animals in the early stages of leukemia development (Table 6); however, this was not detected after 200 days of age. To some degree this is indicative of the early immunological responses seen in the transplantable cell model used previously (5-7). There was no evidence of significant interferon induction in TS-exposed animals over that found in age-matched controls (Table 8). However, over the time spans involved in these trials, both the TS-exposed and age-matched control animals were shown to be capable of initiating and maintaining significant IgM and IgG humoral responses to sheep erythrocytes, where the TS-exposed animals tended to respond significantly better in the initial stages (Fig. 1). This again mimics humoral responses seen in strains of mice not bearing primary malignant tumors (5, 27).

AKR mice have been shown to develop suppressor activity in their sera for the PHA induced lymphoproliferative response (23, 24) with time, and this is enhanced in TS-exposed animals (Table 9).

Therefore, while early exposure to TS promotes a significant number of deaths compared to age-matched control animals up to 250 to 300 days of age, this does not persist in older animals. This promoter activity is delayed if animals commence TS exposure at a later time, and in males, a significant proportion survives for up to 100 to 150 days longer than age-matched controls. This appears to be due to the TS-exposed animals failing to mobilize their leukemias but persisting with a chronic lymphatic leukemia. Importantly, there is little evidence of immunity to the leukemias either in the TS-exposed or age-matched control animals supporting the concepts, which suggest that in vivo in the primary tumor situation significant tumor immunity may not exist (1-3). The animals have been
shown to be capable of significant humoral responses to a T- and B-cell requiring antigen which is enhanced by TS exposure initially (Fig. 1). In vitro suppressor activity, which is enhanced by TS exposure, has also been shown to exist in the sera of the leukemic animals, thus supporting earlier work (23, 24). The significance of this suppressor activity or the presence of cellular suppressor activity of the leukemic cells themselves (22) in vivo has yet to be studied. Studies are planned to purify the suppressor factor and attempt to locate its origins with the view to possible development of the factor, through genetic engineering techniques, as an immunological modulating agent.

ACKNOWLEDGMENTS

We thank Julie McBride for careful technical assistance.

REFERENCES

Effects of Chronic Daily Exposure to Tobacco Smoke on the High Leukemic AKR Strain of Mice

Dinh Tam Nguyen and David Keast