Advances in Brief

N-Methyl-N-nitrosourea Alters Thymocyte Subset Distribution and Targets Immature CD4\(^{-8}\) Cells for Lymphoma Development\(^1\)

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Abstract

The majority of N-methyl-N-nitrosourea (MNU)-induced lymphomas in AKR/J mice express a CD4\(^{-8}\) phenotype. The CD4\(^{-8}\) subset in normal thymus contains functionally mature medullary cells and immature cycling cells. This study demonstrates that MNU-induced lymphomas correspond to the immature CD4\(^{-8}\) subset. In addition, specific changes in the distribution of thymocyte subsets defined by CD4 and CD8 expression were observed after MNU treatment. Cortical thinning and selective depletion of immature CD4\(^{-8}\) and CD4\(^{-8}\) subsets occur immediately after treatment. In contrast, immature CD4\(^{-8}\) progenitors and mature medullary CD4\(^{-8}\) and CD4\(^{-8}\) subsets are relatively resistant to cytotoxicity. Normal thymic architecture and subset distribution are restored within 2 weeks after which selective expansion of the immature CD4\(^{-8}\) subset occurs. The data suggest that MNU induces neoplastic conversion in progenitor cells corresponding to the CD4\(^{-8}\) or immature CD4\(^{-8}\) stages of thymocyte maturation.

Introduction

MNU\(^3\) is a potent carcinogen that induces various types of neoplasms depending on the dosage administered and the experimental animal species tested. In mice, injection of MNU results in a high incidence of thymic lymphomas (1). The AKR strain is particularly susceptible to MNU-induced lymphomagenesis. When 6-week-old AKR mice are given a single injection of MNU (75 mg/kg), approximately 80% develop thymic lymphoma prior to 180 days of age (1–3). Untreated AKR mice have a high incidence of spontaneous thymic lymphoma at 8–12 months of age resulting from somatic integration of endogenous recombinant murine leukemia viruses referred to as mink cell focus-forming virusus due to their expanded host range (4,5). In contrast to spontaneous lymphomas, MNU-induced lymphomas of AKR mice appear prior to 6 months of age and do not contain mink cell focus-forming proviral integrations (6,7) suggesting that distinct mechanisms underlie the development of spontaneous versus MNU-induced lymphoma. This notion is consistent with the finding that in contrast to spontaneous lymphomas which are phenotypically heterogeneous, the majority of MNU-induced lymphomas express a CD4\(^{-8}\) phenotype (8). The present study was undertaken, in part, to determine if MNU-induced lymphomas represent neoplastic counterparts of mature CD4\(^{-8}\) cells or a recently described compartment of immature cycling CD4\(^{-8}\) cells that express high levels of heat-stable antigen and contain progenitors of the CD4\(^{-8}\) subset (9–11). Immunofluorescence studies were performed to determine the fate of thymocyte subsets after MNU-induced depletion, during regeneration, and throughout the latent period prior to development of lymphoblastic foci. The data provide new insight regarding changes in thymocyte subset distribution and the specific population targeted for neoplastic transformation after MNU treatment.

Materials and Methods

Mice and MNU Treatment. AKR/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lymphomas were induced by a single i.p. injection of MNU. This dose has been shown to induce thymic lymphomas in >80% of AKR/J mice by 6 months of age (2, 6–8).

Cell Suspensions, Immunofluorescence Staining, and Flow Cytometry. Single cell thymocyte suspensions were prepared and stained for direct and indirect immunofluorescence assays by standard techniques (6,8). To deplete CD4\(^{-8}\)-bearing cells, thymocytes were incubated with an optimal dilution of anti-CD4 mAb in the presence of rabbit complement (Low-Tox M; Cedarlane, Hornby, Ontario, Canada) as described (12). Fluorescein isothiocyanate-conjugated anti-CD8 (clone 53-6.7), phycoerythrin-conjugated anti-CD4 (clone GK 1.5) and phycoerythrin-conjugated streptavidin were purchased from Becton Dickinson (Mountain View, CA). The J11d hybridoma, producing mAb that recognizes an epitope of HSA, was obtained from Dr. M. Bevan (11). Immunofluorescence was analyzed on a fluorescence-activated cell sorter (FACS IV; Becton-Dickinson) using a 488-nm argon ion laser. Dead cells were excluded from analysis by forward light scatter. Data were collected using a three decade log amplifier, stored in the list mode of a Consort 40 PDP/11-based computer system (Becton-Dickinson FACS) and, for single color analysis, displayed as histograms with the log fluorescence intensity on the x-axis and relative cell number of the y-axis. For two color analysis, contour plots were generated with quadrants based on the profile of unstained cells and cells stained with a single mAb.

Results and Discussion

MNU-induced Alterations in Thymic Cellularity and Histology. Profound changes in thymic architecture and cellularity were observed shortly after MNU treatment. The number of viable cells recovered from MNU-treated mice was reduced by approximately 90% throughout the first week. Thereafter, thymic regeneration took place, resulting in a normal thymocyte yield by 14 days. In agreement with a previous report (13), histological analysis revealed substantial cortical thinning and hypocellularity with sparing of the medulla at 2 days (Fig. 1B). By 7 days, restoration of a discrete cortical region was evident (Fig. 1C) and at 2 weeks, regeneration of normal thymic architecture was complete (Fig. 1D). Thereafter, the regenerated thymus maintained a normal histological appearance until an increase in the ratio of blasts to small lymphocytes became apparent in the thymic cortex of some mice by 6 to 8 weeks after MNU injection. By 10 weeks, large areas of homogeneous lymphoblastic cells characterized by a high mitotic index were routinely observed (data not shown).
Alterations in Thymocyte Subset Distribution within 1 Week after MNU Treatment. MNU induced dramatic shifts in the relative proportions of thymocyte subsets defined by CD4 and CD8 expression. Table 1 summarizes the distribution of thymocyte subsets in normal and MNU-treated mice at various intervals. Most striking is the marked depletion of the immature CD4^+8^+ population which, at 3 days, represented only 20% of total thymocytes. This finding is consistent with the cortical thinning observed on histological survey. However, all immature subsets are not equally sensitive to the cytotoxic effects of MNU since a slight increase was observed in the proportion of cells in the CD4^−8^- precursor compartment at 2 and 3 days after treatment.

Sparing of the medullary compartment after MNU treatment was reflected by the relative increase in CD4^+8^- and CD4^−8^+ subsets, although an unexpected shift in their ratio (from 1:1
to 3:1) was observed on day 3. These data suggest that the CD4-8* subset is more sensitive to the cytotoxic effects of MNU than is the CD4-8' subset. Recent studies have shown that the CD4-8' population contains a component of immature cells that express high levels of cell surface HSA (15, 16). To determine if immature and mature CD4-8' cells differ in sensitivity to MNU-mediated cytotoxicity, CD4-bearing cells were eliminated from thymocyte suspensions by incubation with anti-CD4 mAb and complement. The depleted cells (<7% CD4+ cells) contained 60 to 70% CD4~8+ cells with the remainder belonging to the CD4-8' subset. Fig. 2A shows that the cell surface expression of HSA on CD4-8+ thymocytes from normal mice forms a continuum from negligible to high levels of expression. The data in Fig. 2B show that 3 days after MNU injection, there was a selective depletion of CD4~8* cells expressing high levels of surface membrane HSA. Therefore, the relative shift in the CD4-8'/CD4-8' ratio is due to the loss of the immature HSAhi component from the CD4-8' subset. Comparison of quadrants I and III in Fig. 2 show that selective depletion of HSAhi cells is also evident within the CD4-8' population.

Subset Distribution of Thymocytes Recovered during the Latent Period. The data in Table 1 show that even though the thymus remains hypocellular, a normal pattern of thymocyte subset distribution is restored by 7 days following MNU treatment coinciding with regeneration of the cortical region and normal thymic architecture. No gross abnormalities in subset distribution were noted until 6 to 8 weeks after MNU treatment, at which time a slight increase in the CD4-8' subset was observed in some mice. By 10 weeks a marked expansion in the percentage of CD4-8' cells was consistently observed. Thymocytes obtained at 10 weeks also contained an increased proportion of cycling cells detected by propidium iodide staining (data not shown) and a subset of large cells revealed by analysis of forward light scatter (Fig. 3A). Gating on the large cells revealed that the majority expressed a CD4-8' HSAhi phenotype (Fig. 3, B and C). Therefore, in contrast to the MNU-resistant CD4-8' subset present 3 days after treatment, the expanded CD4-8' population at 10 weeks expresses high levels of HSA suggesting selective expansion of the immature CD4-8' subset. Thymocytes obtained from age-matched untreated mice displayed a normal distribution of subsets defined by CD4, CD8, and HSA expression (data not shown). Finally, analysis of HSA expression on ten MNU-induced lymphomas consistently showed high levels of HSA expression (data not shown) confirming that the immature CD4-8' subset which is depleted shortly after MNU treatment is nevertheless the population that preferentially undergoes neoplastic expansion.

Since MNU is highly labile, it must exert its mutagenic effect on target cells shortly after administration. ras gene mutations are associated with MNU-induced murine thymic lymphomas and rat mammary carcinomas (3, 15-19). The presence of ras mutations in preneoplastic tissues suggests that this is a critical event in the initiation of MNU-induced tumors (1, 6, 19). MNU is also a potent cytotoxic agent that rapidly depletes the thymic cortex while sparing the medulla (13). Considering the 2 to 3-month latent period preceding lymphoma development, it seems likely that MNU-induced mutations are sustained in initiated cells until additional genetic defects occur leading to neoplastic transformation. Since the medulla is relatively resistant to MNU-mediated cytotoxicity, mature thymocytes of the medullary compartment might be expected to contain the target population that undergoes progression. However, this assumption is not consistent with the fact that the majority of MNU-induced lymphomas display a CD4-8' HSAhi phenotype corresponding to an immature cortical subset with progenitor function (8, 9-11). Therefore, it seems more likely that the initiated population exists within the sparse subset of CD4-8' HSAhi spared after MNU-mediated depletion. Alternatively, the target cells may reside in the CD4-8' population which contains the immediate precursors of the CD4-8' subset. Both subsets are plausible candidates for initiation since each contains cycling progenitors. In general, a cycling population would have a greater opportunity to accumulate additional genetic errors leading to progression even if ras or other initiating mutations are not subset specific.

If the initiated cells reside in the CD4-8' population, differentiation to the CD4-8' HSAhi stage must be a consistent and early event since we have not observed expansion of the CD4-8' subset during the latent period, and MNU-induced lymphomas with a CD4-8' phenotype have not been found. The CD4-8' HSAhi lymphomas appear to have limited differentiation potential since some monoclonal tumors contain CD4-8' as well as CD4-8' cells suggesting that the more primitive CD4-8' population gives rise to CD4-8' progeny (8). In support of this notion, we have established a CD4-8' lymphoma cell line that acquires stable CD4 expression after intrathymic transfer. This finding is consistent with studies demonstrating conversion of immature CD4-8' thymocytes to a CD4-8' phenotype (9, 10). Nevertheless, the differentiation capacity of MNU-induced lymphomas is restricted in that maturation be-

### Table 1 Thymocyte subset distribution at intervals after MNU treatment

<table>
<thead>
<tr>
<th>Interval post-MNU</th>
<th>No. of mice</th>
<th>CD4-8'</th>
<th>CD4-8</th>
<th>CD4-8</th>
<th>CD4-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td>4</td>
<td>9.5 ± 2.9</td>
<td>52.7 ± 1.6</td>
<td>24.2 ± 1.3</td>
<td>13.7 ± 3.7</td>
</tr>
<tr>
<td>3 days</td>
<td>4</td>
<td>7.5 ± 0.9</td>
<td>22.9 ± 2.2</td>
<td>52.8 ± 3.5</td>
<td>16.9 ± 0.6</td>
</tr>
<tr>
<td>5 days</td>
<td>4</td>
<td>5.7 ± 0.8</td>
<td>72.8 ± 4.6</td>
<td>13.4 ± 2.5</td>
<td>8.1 ± 1.6</td>
</tr>
<tr>
<td>7 days</td>
<td>3</td>
<td>4.0 ± 0.6</td>
<td>75.5 ± 2.2</td>
<td>9.3 ± 1.4</td>
<td>11.2 ± 1.0</td>
</tr>
<tr>
<td>2 wk</td>
<td>4</td>
<td>1.4 ± 0.1</td>
<td>85.8 ± 0.3</td>
<td>5.8 ± 0.5</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>6 wk</td>
<td>3</td>
<td>2.3 ± 0.4</td>
<td>80.3 ± 2.3</td>
<td>10.8 ± 2.5</td>
<td>6.7 ± 2.1</td>
</tr>
<tr>
<td>10 wk</td>
<td>3</td>
<td>2.1 ± 0.2</td>
<td>31.3 ± 12.9</td>
<td>1.5 ± 0.7</td>
<td>65.1 ± 12.9</td>
</tr>
<tr>
<td>Normal thymus</td>
<td>9</td>
<td>3.2 ± 0.8</td>
<td>79.9 ± 3.3</td>
<td>8.8 ± 2.5</td>
<td>8.1 ± 2.1</td>
</tr>
</tbody>
</table>

Fig. 2. HSA expression on CD4-8' and CD4-8" thymocytes from untreated (A) and MNU-treated (B) mice. After eliminating CD4-bearing thymocytes by anti-CD4 plus complement treatment, the remaining cells were assessed for simultaneous expression HSA (detected by J11d mAb) and CD8 molecules. Contour plots were generated from two color immunofluorescence analysis of reactivity with fluorescein isothiocyanate-conjugated anti-CD8 (abscissa) and biotinylated anti-J1Id plus phycoerythrin-conjugated avidin (ordinate).
yond the CD4\(^{-}\)\(8^{-}\) differentiation stage has not been observed.

We cannot rule out the possibility that the target population for MNU-induced lymphomagenesis resides in a bone marrow pre-T-cell compartment. However, this seems unlikely in view of a report showing that thymectomy prevented the appearance of MNU-induced T-cell neoplasms (20). Implants of neonatal thymic grafts into thymectomized recipients failed to restore thymic lymphoma development unless the grafted thymus was exposed to MNU. Furthermore, after exposure of the thymic implants to MNU, the resulting lymphomas in several animals were derived from the donor thymus (20). These findings implicate intrathymic cells as the target population in MNU-induced lymphoma. Taken together with the results in the present report, the data indicate that conversion to a malignant phenotype takes place in thymocytes at the CD4\(^{-}\)\(8^{-}\) or immature CD4\(^{-}\)\(8^{-}\)HSA\(^{hi}\) stages of differentiation. This information provides a rational approach for isolating specific thymocyte subsets at intervals after MNU injection in order to determine if genetic alterations, such as activation of specific oncogenes, are restricted to a particular stage of thymocyte development during the pathogenesis of MNU-induced lymphoma.

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References

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