Megakaryocytopoiesis: Incorporation of Tritiated Thymidine by Small Acetylcholinesterase-Positive Cells in Murine Bone Marrow During Antibody-Induced Thrombocytopenia

By Karen M. Young and Leon Weiss

The ability of small acetylcholinesterase-positive (SACHE) cells to incorporate tritiated thymidine ($^3$H-TdR) was studied in the bone marrow of mice made acutely thrombocytopenic by injection of guinea pig antimitouse platelet serum (APS) and in control mice injected with normal guinea pig serum (NS). $^3$H-TdR was administered in vivo, and bone marrow was collected at various time points thereafter. The initial labeling index (LI) for both groups was about 30%. After four hours, the LI increased and reached peak values at 48 hours, but was lower in thrombocytopenic animals. The lower peak LI in APS-treated animals may be the result of both a faster rate of influx of unlabeled cells into the acetylcholinesterase (AChE) positive cell compartment and efflux of more heavily labeled, and probably polyploid, SACHE cells into the compartment of recognizable megakaryocytes. In both groups the increasing LI occurred concomitantly with a decreasing mean grain count. This may indicate cellular division by some fraction of SACHE cells. Heterogeneity in nuclear morphology was also demonstrated, and the frequency of individual morphologies was altered in APS-treated animals. In addition, cells that incorporated $^3$H-TdR were larger than those that did not. This group of small cells appears to represent a pivotal point in megakaryocytopoiesis in which the switch from mitosis to endomitosis is occurring.

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Address reprint requests to Karen M. Young, VMD, PhD, University of Wisconsin-Madison, School of Veterinary Medicine, Department of Pathobiological Sciences, 2015 Linden Dr West, Madison, WI 53706.

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FROM THE UNIVERSITY OF PENNSYLVANIA SCHOOL OF VETERINARY MEDICINE.

IN THE PATHWAY of megakaryocyte differentiation, a group of cells intermediate between the colony forming unit-megakaryocyte (CFU-M) and morphologically recognizable megakaryocytes has recently been detected. In certain species, including the mouse, these cells contain acetylcholinesterase, a cytochemical marker highly selective for megakaryocytes. 1, 2 A proportionate increase in the number of small acetylcholinesterase-positive (SACHE) cells among all acetylcholinesterase-positive cells in bone marrow occurs in response to antibody-induced thrombocytopenia 3 and after administration of a thrombopoiesis-stimulating factor, 4 and suppression of SACHE cells follows transfusion-induced thrombocytosis. 5 The early response of these cells indicates that the first detectable response to changes in platelet mass may occur at this level.

SACHE cells appear to play a pivotal role in megakaryocytopoiesis with regard to cellular proliferation/endomitosis and feedback control by platelets, making critical an elucidation of their role in the dynamics of megakaryocytopoiesis. Few recognizable megakaryocytes initially incorporate tritiated thymidine ($^3$H-TdR), but by 72 hours, greater than 90% of these cells are labeled, 6, 7 even when reutilization of $^3$H-TdR is minimized. 8 Since recognizable megakaryocytes do not divide, 9, 10 we hypothesized that SACHE cells, precursors to recognizable megakaryocytes, would have an initially high labeling index (LI) and that their maturation would result in the subsequent rise in the LI of recognizable megakaryocytes. In addition, we hypothesized that the kinetics would be different in thrombocytopenic animals, since thrombocytopenia has been shown to result in a more rapid rise in the labeling index of recognizable megakaryocytes 1, 2, 11 and in an increase in the fraction of CFU-M in cycle. 12, 13

Our studies on the incorporation of $^3$H-TdR by SACHE cells indicate a heterogeneity with respect to cell cycle status and suggest that thrombocytopenia results in a more rapid rate of influx of cells from a precursor compartment and/or efflux of SACHE cells into the compartment of recognizable megakaryocytes. In addition we provide information on the morphology of SACHE cells that incorporate $^3$H-TdR.

MATERIALS AND METHODS

Mice. BDF1 mice (Jackson Laboratories, Bar Harbor, ME), 2 to 4 months old, were used in these studies. The animals were maintained under conventional conditions.

Antiplatelet serum. Antiplatelet serum (APS) was the generous gift of Dr Shirley Ebbe (Lawrence Berkeley Laboratory, University of California, Berkeley) and was prepared in the following manner: mouse platelets mixed with 0.15 mol/L sodium chloride (isotonic saline) and equal volumes of complete Freund’s adjuvant were injected into guinea pigs. The first injection was given in the foot pad and subcutaneously (SC), and three subsequent injections were given SC only. The injections were administered over a period of one month. One week following the last injection, serum was collected from the guinea pigs and heat-inactivated at 56 °C for 30 minutes. The serum was adsorbed three times with equal volumes of saline-washed mouse red cells and stored at −20 °C in aliquots of 1 mL until use. Thawed aliquots were diluted to 3 mL with isotonic saline, and each of 18 experimental mice received 0.1 mL intraperitoneally (IP). Eighteen control animals each received 0.1 mL of normal guinea pig serum (NS) IP. NS was heated to 56 °C for 30 minutes and diluted to 3 mL with isotonic saline prior to administration.

Tritiated thymidine. Tritiated thymidine ($^3$H-TdR) was obtained from New England Nuclear (Boston) as a sterile aqueous solution and had a specific activity of 2.0 Ci/mmole. The solution was diluted with sterile isotonic saline and injected IP at a dose of 1.1 μCi/g body weight four hours after injection with either APS or NS. This time point was chosen because thrombopoiesis-stimulating factor levels have been reported to be high four hours after APS administration. 14

Blood counts. Blood for counts was collected from the retro-orbital sinus directly into potassium-EDTA tubes (Sarstedt, Princeton, NJ). Platelet counts were performed on a calibrated hemocytometer at a dilution of 1:100 with 1% ammonium oxalate. Red blood cell (RBC) and white blood cell (WBC) counts were performed with a Coulter electronic particle counter, Model ZBI.
Pretreatment blood counts were performed on 26 mice. Counts were repeated four hours after injection with either APS or NS. Platelet counts in APS-treated animals had to be less than 5% of control and pretreatment values in order for the animal to be included in the experiment. This degree of thrombocytopenia appears necessary to effect quantitative changes in SACHE cells. Exchange transfusions with platelet-poor plasma have been ineffective in producing this effect quantitatively.

Bone marrow samples. Animals were killed by cervical dislocation at 30 minutes and at 4, 12, 24, 48, and 72 hours after administration of 3H-TdR. Bone marrow smears were made from split femora on acid-alcohol cleaned slides with a sable brush. Smears were heat-fixed at 50°C for five minutes and stained for acetylcholinesterase.

Acetylcholinesterase staining. Acetylcholinesterase (AChE) activity was detected using the method of Karnovsky and Roots, as modified by Jackson and Long and Williams. Smears were incubated at 22°C in the following medium, made freshly each day, for 6.0 to 6.5 hours: 0.5 mg/mL acetylthiocholine iodide (Sigma, St Louis), 75 mmol/L dibasic sodium phosphate (pH 6.0), 5 mmol/L sodium citrate, 3 mmol/L copper sulfate, and 0.5 mmol/L potassium ferricyanide. Slides were then gently rinsed with distilled H2O and fixed for ten minutes in 95% ethanol. They were counterstained in Harris’ hematoxylin for 30 seconds, rinsed gently in running tap H2O, blued in saturated lithium carbonate, and rinsed a final time in H2O. Cytochemical control smears were incubated in media lacking substrate and were negative for reaction product in all cells.

A recent study indicated that eosinophils may contain AChE when examined by phase microscopy. To examine the selectivity of the AChE marker, 100 μm sections of bone marrow fixed in 1.5% gluteraldehyde/1.25% paraformaldehyde in 0.1 mol/L cacodylate buffer were stained for AChE activity by the method of Lewis and Knight using acetylthiocholine iodide as the substrate. Silver/silver-gold sections were examined with a EM10CA transmission electron microscope (Zeiss, West Germany).

Mapping and 3H-TdR-labeling of small AChE-positive Cells. Counterstained smears were coverslipped and the positions of 200 to 250 SACHE cells/mouse were located on the slides. This procedure was necessary since processing the slides for autoradiography decolorizes the AChE reaction product (see below). SACHE cells were located with phase microscopy (Zeiss) under a 100× oil-immersion objective and had to fit the following criteria: (a) a cell diameter of less than 18 μm (measured with a calibrated stage micrometer), since morphologically recognizable megakaryocytes were always greater than 18 μm; (b) the presence of cytoplasmic AChE reaction product, which appears black and granular under phase microscopy and diffusely golden brown under bright field microscopy; and (c) a high nuclear–cytoplasmic ratio. The cell diameter and nuclear morphology of each SACHE cell was recorded. To map each cell, stage micrometer readings were noted, and the field was photographed.

After the cells for all 36 mice were mapped, the coverslips were removed from the slides, which were then cleaned in fresh xylene. The dried smears were dipped in NTB-2 film emulsion (Kodak) diluted 1:1 with distilled H2O. After thorough drying the slides were exposed in light-tight boxes containing desiccant at 4°C for 30 days. The slides were then developed in fresh D-19 diluted 1:1 with distilled H2O for five minutes (with agitation every 30 seconds), rinsed in distilled H2O for one minute, and fixed in undiluted Kodak fixer for five minutes. The entire developing procedure was carried out at 18°C, and all slides were processed on the same day. It is this development procedure that causes decolorization of the AChE reaction product. The slides were then rinsed in running H2O for 15 minutes and stained with a Romanovsky stain.

Mapped SACHE cells were located on each slide using the stage micrometer readings to find the correct field and then the photog-raph to locate the reaction product-positive cell (now decolorized) within the field. For each SACHE cell, the number of grains over the nucleus was counted under a 100× oil-immersion objective (Fig 1), and the nuclear morphology was recorded again.

Background counts were estimated in two ways: (a) grain counts were done for 500 cells/slide on ten slides treated identically to the experimental autoradiograms, except that the smears were made with bone marrow from mice that were not injected with 3H-TdR; and (b) grain counts were done on the experimental autoradiograms over areas away from the cells. Background counts determined by both methods were low (mean grain count was less than one grain/cell). For a cell to be considered labeled, it had to have a grain count of at least three times the background count.

The grain count data were analyzed at each time point after administration of 3H-TdR to calculate the LI (percentage of SACHE cells that were labeled) and the mean grain count of the labeled SACHE cells. The data were analyzed statistically using a three-way analysis of variance.
RESULTS

Platelet counts. Platelet counts are presented in Fig 2. For all experimental animals the platelet count was less than 5% of both the pretreatment and control values. Thereafter, the platelet count increased and reached pretreatment values by 76 hours after APS administration. NS had no effect on the platelet count.

Labeling index of SACHE cells. The labeling index (LI) for SACHE cells was defined as the percentage of SACHE cells that were labeled and was plotted against time after $^3$H-TdR administration for experimental and control animals (Fig 3). The initial LI (at 30 minutes) for both groups was about 30% (29% for experimental and 31% for controls). At four hours there was no change (29% for experimental and 32% for controls), but by 12 hours the LI reached about 50% in both groups (52% in experimental and 51% in controls). For the next three time points experimental and control values differed. At 24 hours the LI was 42% for experimental animals and 69% for controls. Both groups attained peak values at 48 hours (77% in experimental and 54% in controls, respectively). The difference between experimental and control groups was significant ($P < 0.02$).

Mean grain counts. The mean grain count (MGC) for each animal was calculated by averaging the grain counts of all the labeled cells for that animal. For comparison, the mean platelet count (MGC) at 30 minutes was assigned a value of 1.0, and each subsequent MGC was expressed as a proportion of the initial value. The actual values corrected for the number of cells counted were: at 3 minutes, 1.0; at 4 hours, 1.3; at 24 hours, 0.9; at 72 hours, 0.6. The two groups demonstrated a rise in MGC at four hours, and the MGC decreased at each subsequent time point. The difference in MGC between groups was not significant ($P > 0.1$).

To summarize, in both experimental and control groups, the LI increased after four hours, peaked at 48 hours, and then decreased. After 12 hours the LI for the experimental group was lower than for the control group. In both groups the MGC decreased sharply at first and thereafter at a slower rate.

Morphology of SACHE cells. The nuclear morphology of SACHE cells was sometimes difficult to assess with certainty, as round and tightly lobulated nuclei could not always be distinguished. If the nuclear morphology could be determined unequivocally, the cell was included in the analysis. The three morphologic types described by Long and Williams were noted in the present study, although the percentages were slightly different. Examples of SACHE cells before autoradiographic processing are presented in Fig 1.

In APS-treated mice, there was an altered frequency distribution of nuclear morphologies that varied with time (Table 1). As indicated by these data, experimental mice had decreased percentages of cells with a round and indented nucleus and an increased percentage of cells with a lobulated nucleus at early time points. Later on the percentages of cells with a round or indented nucleus was increased along with a decrease in the proportion of cells with a lobulated nucleus ($P < 0.05$).

The nuclear morphology of cells initially labeled was compared to that of unlabeled cells (Table 2). The frequency distribution of nuclear morphologies of both labeled and unlabeled cells was similar to that of all SACHE cells at 30 minutes after $^3$H-TdR or 4.5 hours after APS administration, indicating that uptake of $^3$H-TdR was not favored by a
INCORPORATION OF 3H-TdR BY SACHE CELLS

Table 1. Nuclear Morphology of SACHE Cells

<table>
<thead>
<tr>
<th>Time after APS (h)</th>
<th>n</th>
<th>Round</th>
<th>Indented</th>
<th>Lobulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>976</td>
<td>1.35 ± 0.02</td>
<td>6.50 ± 0.07</td>
<td>92.14 ± 0.08</td>
</tr>
<tr>
<td>8</td>
<td>663</td>
<td>4.79 ± 0.08</td>
<td>11.65 ± 0.14</td>
<td>83.56 ± 0.22</td>
</tr>
<tr>
<td>16</td>
<td>683</td>
<td>3.91 ± 0.08</td>
<td>10.92 ± 0.13</td>
<td>85.17 ± 0.20</td>
</tr>
<tr>
<td>28</td>
<td>560</td>
<td>1.96 ± 0.05</td>
<td>12.39 ± 1.80</td>
<td>85.60 ± 0.08</td>
</tr>
<tr>
<td>52</td>
<td>664</td>
<td>9.27 ± 0.06</td>
<td>21.68 ± 0.21</td>
<td>69.05 ± 0.21</td>
</tr>
<tr>
<td>76</td>
<td>623</td>
<td>9.35 ± 0.06</td>
<td>22.58 ± 0.15</td>
<td>68.07 ± 0.14</td>
</tr>
<tr>
<td>Control</td>
<td>3,849</td>
<td>5.06 ± 0.03</td>
<td>16.81 ± 0.08</td>
<td>78.13 ± 0.11</td>
</tr>
</tbody>
</table>

Values are mean percent ± SEM; n, number of SACHE cells evaluated.

cell with a particular nuclear morphology. In APS-treated mice, the percentage of labeled cells with a round nucleus showed a greater increase with time than that of unlabeled cells. By 72 hours after 3H-TdR administration, 12.15% ± 0.09% had a round nucleus (n = 337; mean ± SEM) as compared to 5.54% ± 0.39% (n = 286) in unlabeled cells and 9.35% ± 0.10% (n = 623) in all SACHE cells from APS-treated mice at 72 hours (P < 0.02).

SACHE cells were subject to both shrinkage and swelling due to the effects of fixation, histochemical reactions, and autoradiographic processing. Cell diameters could not, therefore, be considered absolute measures of cell size and were more useful for relative comparisons among groups of cells. The mean diameter of SACHE cells from APS-treated animals was 12.59 ± 0.23 μm (n = 4,609; mean ± SD) and for NS-treated animals was 12.63 ± 0.18 μm (n = 4,387; mean ± SD). There were no significant differences between time points or treatment groups (P > 0.1). In APS-treated mice the cell diameter of initially labeled cells was significantly greater than that of unlabeled cells (14.24 ± 1.69 μm and 11.96 ± 1.59 μm, respectively; P < 0.001). Results from control animals were similar.

Electron microscopy. The AChE reaction product was present in mature megakaryocytes and in a population of small cells but was absent in eosinophils at all stages of maturation (Fig 5).

DISCUSSION

The following conclusions can be made from the present study: (a) SACHE cells are heterogeneous with respect to cell cycle, with only 30% initially incorporating 3H-TdR. The percentage increases with time, peaking at 48 hours, but is lower in APS-treated animals after 12 hours. The MGC decreases concomitantly with the increasing LI. (b) SACHE cells are heterogeneous with respect to nuclear morphology, and the lobulated nucleus is the most prevalent form, followed by the indented and round forms. Following injection of APS, the percentages of round and indented forms decrease below control values but are higher than control values after two days. Cells with all three nuclear morphologies are capable of incorporating 3H-TdR, and the percentage of cells with a round nucleus is greater in labeled than in unlabeled cells by 72 hours. (c) While the size of cells in the compartment of SACHE cells remains stable, cells that initially incorporate 3H-TdR are larger than unlabeled cells.

We had hypothesized that a high proportion of SACHE cells would incorporate 3H-TdR based on the increased LI of mature megakaryocytes by 24 hours and the apparent susceptibility of precursors of mature megakaryocytes to cytotoxic drugs. Since only 30% of SACHE cells were labeled in this study, the possibility that mature megakaryocytes have a more immediate precursor than the SACHE cell was considered. This is unlikely in view of the studies on density profiles and the relationship of SACHE cells to megakaryocytes. A more likely explanation for the initially low labeling index is that SACHE cells are heterogeneous with respect to cell cycle status.

The lower peak LI in thrombocytopenic animals is probably the result of both an influx of cells from an initially unlabeled compartment and an efflux of labeled cells into the compartment of recognizable megakaryocytes, with both of these events occurring at a more rapid rate in thrombocytopenic animals. Under conditions of acute thrombocytopenia similar to those in the present experiments, the increase in

Table 2. Nuclear Morphology of Labeled and Unlabeled SACHE Cells 30 Minutes Post-3H-TdR Administration

<table>
<thead>
<tr>
<th>Time after APS (h)</th>
<th>Labeled</th>
<th></th>
<th></th>
<th></th>
<th>Unlabeled</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Round</td>
<td>Indented</td>
<td>Lobulated</td>
<td>n</td>
<td>Round</td>
<td>Indented</td>
<td>Lobulated</td>
<td></td>
</tr>
<tr>
<td>APS-treated</td>
<td>275</td>
<td>1.58 ± 0.08</td>
<td>5.26 ± 0.23</td>
<td>93.17 ± 0.22</td>
<td>701</td>
<td>1.31 ± 0.02</td>
<td>6.98 ± 0.10</td>
<td>91.72 ± 0.09</td>
</tr>
<tr>
<td>NS-treated</td>
<td>252</td>
<td>4.09 ± 0.17</td>
<td>14.39 ± 0.80</td>
<td>81.53 ± 0.76</td>
<td>683</td>
<td>3.45 ± 0.13</td>
<td>11.49 ± 0.14</td>
<td>85.06 ± 0.24</td>
</tr>
</tbody>
</table>

Values are mean percent ± SEM; n, number of SACHE cells evaluated.
CFU-M in S phase occurred by 24 hours and reached a maximum value of 49% at 40 hours. While a slight increase occurred at 12 hours, no change was observed at six hours. Thus, the majority of CFU-M would be unlabeled by H-TdR administered four hours after APS. As these unlabeled cells begin to synthesize DNA, they would eventually acquire the AChE marker and would enter the SACHE cell compartment. These unlabeled cells would decrease the LI. In control animals, influx of unlabeled cells may also occur, but at a slower rate. Efflux of labeled SACHE cells into the compartment of recognizable megakaryocytes is also likely to occur at a more rapid rate in thrombocytopenic animals, as labeled cells appear in the compartment of Stage I megakaryocytes more rapidly in thrombocytopenic animals.11,12

Antibody-induced damage of SACHE cells is an unlikely cause for the lower peak LI in experimental animals. Previous studies have suggested that damage to mature megakaryocytes is minimal or lacking when small single doses of antiserum are used.14,16,26-29 In addition, administration of thrombopoietin or APS had similar effects on SACHE cells with respect to changes in the percentage of these cells in the bone marrow. Also, no reduction in CFU-M occurred when bone marrow cells were preincubated with APS prior to plating or when APS was included in the plating mixture.14

The decrease in LI at 72 hours in both groups of mice can be explained by the maturation of labeled SACHE cells into recognizable megakaryocytes and the entry of unlabeled cells into the SACHE cell compartment by 72 hours. This is consistent with reports that recognizable megakaryocytes attain peak labeling at three days due to an influx of labeled precursors and maturation of cells through the compartment.6,7

In both experimental and control groups in this study, the MGC decreased while the LI increased. Cellular division, resulting in the distribution of labeled DNA among an increased number of cells, is one explanation for this phenomenon. However, while some SACHE cells may be capable of cellular division, most are not proliferating but are undergoing endomitosis. Other factors that may have contributed to the concurrent increasing LI and decreasing MGC are efflux of more heavily labeled SACHE cells into the compartment of recognizable megakaryocytes and influx of lightly labeled cells from the CFU-M compartment. Further studies are needed to explore these various possibilities.

Consistent with a previous report,19 we found that the most frequent nuclear morphology is the lobulated form. If this is also the most mature SACHE cell, the higher percentage present four to 24 hours after APS may represent a "shift to the right" in this population of cells. This is also suggested by the larger size of initially labeled cells.

In an earlier study, hydroxyurea, an inhibitor of DNA synthesis that lethally damages cells in S phase, was found to reduce only SACHE cells with a round nucleus, which represented less than 2% of the total SACHE cell compartment.19 Our studies indicate that uptake of H-TdR, which labels cells in DNA synthesis but is not cytotoxic at the doses used, is not limited to cells with a round nucleus. In fact, the highest percentage of cells that initially label have a lobulated nucleus. The marked difference in the mechanisms of action of these agents makes comparison difficult; however, the discrepancy in the percentage and morphology of cells affected may be the result of a higher resistance of polyploid SACHE cells, which incorporate H-TdR during endomitosis, to the lethal effects of hydroxyurea.

Recently a question was raised about the positive staining of eosinophils at the light microscopic level.4 In the present study it is unlikely that the AChE-positive cells represent eosinophils for the following reasons: (a) eosinophils that have a lobulated or segmented nucleus are postmitotic and therefore would not incorporate H-TdR; (b) cytoplasmic reaction product was visualized as black and granular with phase microscopy and diffusely golden brown (Hatchet's brown) under bright field microscopy; after decolorization of the AChE reaction product, no eosinophilic granules were seen in the Romanovsky-stained smears; and (c) in the examination of marrow sections by electron microscopy, we found that with the more sensitive technique of ultrastructural localization of AChE reaction product, positive eosinophils were never found at any stage of maturation.

These data are consistent with the following model of megakaryocytopoiesis: the CFU-M, normally in a relatively quiescent state, replenishes its own pool while feeding the SACHE cell compartment. SACHE cells are a heterogeneous population of cells with respect to cell cycle status and morphology, and are immediately responsive to marked changes in platelet mass. At least some proportion of SACHE cells are capable of cellular proliferation, while the most mature have begun the process of endomitosis. This compartment of cells is replenished by the CFU-M, and this occurs at a more rapid rate after the stress of thrombocytopenia. In turn, SACHE cells feed the compartment of recognizable megakaryocytes as the most mature SACHE cells, which are larger and have a lobulated nucleus, become Stage I megakaryocytes. Differentiation of cells committed to megakaryocytopoiesis may be directed by local and systemic mechanisms. SACHE cells constitute a heterogeneous group of cells in which the key element of differentiation is the change from mitosis to endomitosis, regulated at least in part by the end product of mature megakaryocytes.

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