Ontogenic Development of Thymocyte in Normal and Bis-diamine Treated Rats

—Immu-no- and Enzymo-histochemical Studies—

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The ontogenic development of rat thymocytes was studied immunohistochemically for the expression of cell surface antigens and enzymohistochemically for the activity of lysosomal enzymes in both normal and bis-diamine-induced hypoplastic thymuses.

In normal thymus, W 3/13 antigen was expressed first on thymocytes on gestation day 16, and followed simultaneously by W 3/25, MRC OX8 and MRC OX7 antigens on gestation day 18. The thymocytes bearing these antigens increased rapidly in number during the late fetal life, and the thymocytes with the same antigenic profiles as mature peripheral T cells seemed to occur within the medulla from day 20 of gestation when the cortex and medulla could be distinguished. A possible maturation pathway of T cell surface antigens within the thymus is discussed. Positive thymocytes for AcP, B-G and ANAE activities were demonstrated in varying frequency in all fetal thymuses examined, and they were distributed in much higher frequency in the medulla than in the cortex after birth. The data suggests that the presence per se of AcP, B-G and ANAE activities is independent of ontogenic thymocyte differentiation.

Significantly hypoplastic thymuses were induced by treatment with bis-diamine in rat. These thymuses showed a retardation in ontogenic differentiation of thymocyte surface antigens, which was well correlated with the delay in morphologic development of thymuses. Furthermore, the immunopathological correlation between the bis-diamine-induced malformation complex in rat and the DiGeorge’s syndrome in man is discussed.
INTRODUCTION

The thymus is an essential organ for production and differentiation of T lymphocytes. Recently, many studies have been reported concerning the differentiation of thymic lymphocytes (thymocytes) employing techniques of immuno- and enzymo-cytochemistry, proliferation kinetics and functional assays. However, most of the studies have dealt with the thymocytes from the stage of child or young adult, there have been a few reports concerning the ontogenic thymocyte differentiation in man and mouse, but not in rat.

In the present study, the thymuses from rat fetuses and newborns were examined immunohistochemically for the expression of several surface antigens as well as enzymohistochemically for the activity of several lysosomal enzymes in order to analyze the ontogenic development of thymocytes within the thymus.

On the other hand, a bis(dichloroacetyl)diamine is a drug which was first shown to suppress spermatogenesis and to be an effective oral abortifacient in rat. The administration of this drug to pregnant rats could produce high incidence of congenital malformations including aplastic or hypoplastic thymus and cardiovascular anomalies. Recently, more detailed studies of the bis-diamine-induced anomalies in rat have demonstrated the resemblance with the anomalies in the DIGEORGE’s syndrome in man.

From this viewpoint, in the present study, the effect of bis-diamine on the ontogenic development of rat thymocytes was also examined, and the immunopathological correlation between the bis-diamine-induced malformation complex in rat and the DIGEORGE’s syndrome in man is discussed.

MATERIALS AND METHODS

1. Experimental animals

The female Wistar rats weighing between 200 and 250g (Kyudo breeding farm, Saga, Japan) were caged with the male overnight, and the following day when sperm was confirmed in vaginal smears was designated as gestation day 0. The animals were given commercial rat chow (Labo MR breeder, Nihon Nosan Kogyo, Co. Ltd., Kanagawa, Japan) and water ad libitum during the period examined.

2. Administration of drug

The drug N,N’-bis-(dichloroacetyl)-1,8-octamethylenediamine (Win, 18,446, abbreviation: bis-diamine, Sigma Chemical Co., St. Louis, U.S.A.) was blended with a 1% aqueous suspension of gum tragacanth to give a mixture of 100 mg/ml. A single daily dose of 2 ml of the mixture (200 mg of bis-diamine) was given to pregnant rats on days 9 and 10 of gestation by a gastric tube. Control rats received gum tragacanth alone or none.

3. Macroscopic observations

The fetuses were removed under ether anesthesia on days 16, 18 and 20 of gestation.
Implantation sites were counted macroscopically. Furthermore, the newborns were obtained immediately after birth. Number, viability and weight of the offspring were examined. Thereafter, the thymus and spleen were removed under the stereomicroscopy, and they were fixed in periodate–lysine–paraformaldehyde (PLP) fixative as soon as they were weighed. The thymuses from 7 to 84 days old healthy untreated rats were also examined.

4. Tissue preparation for immuno-and enzymo-histochemistry

The thymuses and spleens fixed in PLP fixative at 4°C for 12–24 hours were rinsed with several changes of cold 10% sucrose added 0.05M phosphate buffered saline, pH7.2 at 4°C for 48 hours and then they were dehydrated in graded series of ethanol and cleared in xylene. After permeation with 3 changes of paraffin (melting point 42–44°C, Merck, Darmstadt, Germany) at 45°C for 30 minutes each, the specimens were embedded in paraffin. The tissue blocks were stored at 4°C until use. 5 µm sections were mounted on glass slides coated with albumin, dewaxed as usual to examine immuno- and enzymo-histochemically as well as histologically.

5. Immunohistochemistry

Antibodies (Table 1): The mouse monoclonal antibodies against rat T cells W 3/13a, W 3/25b, MRC OX8c and MRC OX7d were purchased from Sera Lab, Sussex, England, and used for the marker of thymic lymphocyte differentiation as shown in Table 1. For negative control, mouse anti-human histocompatibility antigen monoclonal antibody W 6/32e (Sera Lab, Sussex, England), which does not react with rat tissue, was used. Other antibodies used were peroxidase conjugated F(ab')2 goat anti-mouse IgG (Cappel Lab., Inc., PA, U.S.A.), IgG fraction goat anti-mouse IgG (Cappel Lab., Inc., PA, U.S.A.) and mouse F(ab')2 peroxidase-anti-peroxidase (Jackson Immuno Research Lab., Inc., Pennsylvania, U.S.A.).

Procedures of immunohistochemistry: Immunoperoxidase techniques were adopted in order to clarify the distribution of cells labelled with the monoclonal antibodies. W 3/13, MRC OX8 and MRC OX7 antibodies stained thymocytes strongly by the indirect peroxidase method, whereas W 3/25 antibody could label thymocytes intensely only by the

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Lymphocytes labelled with the antibody</th>
<th>Role of antigen positive T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>W 3/13a</td>
<td>All thymocytes and periphera’T cells</td>
<td>All T cell function</td>
</tr>
<tr>
<td>W 3/25b</td>
<td>86% thymocytes, 70% peripheral T cells</td>
<td>Helper/inducer</td>
</tr>
<tr>
<td>MRC OX 8</td>
<td>88% thymocytes, 30% peripheral T cells</td>
<td>Cytotoxic/suppressor</td>
</tr>
<tr>
<td>MRC OX 7 d</td>
<td>96% thymocytes, few peripheral T cells</td>
<td>Not known</td>
</tr>
</tbody>
</table>

a) W3/13 antigen is also present on brain, neutrophils, plasma cells and megakaryocytes.
b) W5/25 antigen is also present on macrophages.
c) MRC OX 7 antigen (rat Thy 1.1) is also present on brain.
peroxidase-anti-peroxidase (PAP) method probably because of the minority of W 3/25 antigens on thymocytes\textsuperscript{13}. The optimal dilution for W 3/13, W 3/25, MRC OX8 and MRC OX7 antibodies was 1:200, 1:200, 1:100 and 1:200 respectively. Diaminobenzidine reaction was carried out as described by \textsc{Graham} and \textsc{Karnovsky}\textsuperscript{7}). The slides were lightly counterstained with 0.1\% methyl green in 0.1M acetate buffer, pH 4.2. For negative control stain, the sections were incubated with W 6/32 antibody diluted equally and which gave low levels of non-specific staining.

6. Enzymohistochemistry

Activities of acid phosphatase (AcP), $\beta$-glucuronidase (B-G) and $\alpha$-naphthyl acetate esterase (ANAE) were investigated. For demonstration of AcP activity, the dewaxed sections were incubated in a freshly prepared medium described by \textsc{Egashira}\textsuperscript{5}). Activity of B-G was ascertained by incubation in a modified medium described by \textsc{Hayashi}, \textit{et al.}\textsuperscript{11).} ANAE activity was revealed after incubation in a modified medium described by \textsc{Horwitz}, \textit{et al.}\textsuperscript{12).} The optimal time and temperature of incubation for AcP, B-G and ANAE were 2 hours at 37\textdegree C, 6 hours at 37\textdegree C and 1.5 hours at room temperature respectively. After incubation, the slides were washed in running distilled water at room temperature for 15 minutes. They were then lightly counterstained with 0.1\% methyl green.

7. Determination of percentage of positive thymocytes for the monoclonal antibodies and the lysosomal enzymes

In the thymus, more than 100 thymocytes were counted in relation to their localization, depending upon the number of positive cells encountered, and the percentage of positive cells was estimated in both control and treated rats.

\textbf{RESULTS}

1. Macroscopic observations

Table 2. shows the number of implantations, resorbed and dead offspring, and survived offspring of untreated control and bis-diamine treated rats. The rate of resorbed and dead offspring in the treated groups was significantly high in comparison with that in the control groups.

Control thymus was seen as a pyramid-shaped and fused bilobe of the equal size lying ventral to the lower portion of trachea on all days examined. In the treated groups, on the other hand, the thymus was formed by two separate lobes lying on the middle portion of trachea usually on day 16, and occasionally on day 18 of gestation, and the organ was frequently formed by a small irregular mass which both lobes were fused on the ventral to the middle or lower portion of trachea on days 18 and 20 of gestation and at birth. In this study, aplasia of thymus was not observed.

Table 3. shows the body and thymus weights in the survived offspring of control and treated rats. In comparison with the control, the thymus weight and the ratio of thymus to body weight in the treated were significantly low on all days examined.
Table 2. Status of pregnant rats and offspring untreated and treated with bis-diamine

<table>
<thead>
<tr>
<th>Day observed</th>
<th>Number of dam</th>
<th>Number of implantation (mean)</th>
<th>Number of resorbed and dead offspring (%)</th>
<th>Number of survived offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation day 16</td>
<td>4</td>
<td>55 (13.8)</td>
<td>4 (7.3)</td>
<td>51 (92.7)</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>52 (13.0)</td>
<td>0 (0.0)</td>
<td>52 (100.0)</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>51 (12.8)</td>
<td>3 (5.9)</td>
<td>48 (94.1)</td>
</tr>
<tr>
<td>At birth</td>
<td>5</td>
<td>65 (13.0)</td>
<td>0 (0.0)</td>
<td>65 (100.0)</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>223 (13.1)</td>
<td>7 (3.1)</td>
<td>216 (96.9)</td>
</tr>
<tr>
<td>Gestation day 16</td>
<td>6</td>
<td>85 (14.2)</td>
<td>12 (14.1)</td>
<td>73 (85.9)</td>
</tr>
<tr>
<td>18</td>
<td>5</td>
<td>62 (12.4)</td>
<td>7 (11.3)</td>
<td>55 (88.7)</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>66 (13.2)</td>
<td>9 (13.6)</td>
<td>57 (86.4)</td>
</tr>
<tr>
<td>At birth</td>
<td>5</td>
<td>64 (12.8)</td>
<td>11 (17.2)</td>
<td>53 (82.8)</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>277 (13.2)</td>
<td>39 (14.1)*</td>
<td>238 (85.9)*</td>
</tr>
</tbody>
</table>

* Significantly different from the control value, p<0.01 (χ² test)

Table 3. Body and thymus weights (mean ± SD) in offspring of untreated and bis-diamine treated rats

<table>
<thead>
<tr>
<th>Day observed</th>
<th>Body weight (g)</th>
<th>Thymus weight (mg)</th>
<th>Thymus weight / Body weight × 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation day 16</td>
<td>0.60 ± 0.08</td>
<td>0.79 ± 0.17</td>
<td>1.36 ± 0.38</td>
</tr>
<tr>
<td>18</td>
<td>1.65 ± 0.18</td>
<td>2.39 ± 0.53</td>
<td>1.44 ± 0.26</td>
</tr>
<tr>
<td>20</td>
<td>3.93 ± 0.70</td>
<td>6.90 ± 1.95</td>
<td>1.76 ± 0.41</td>
</tr>
<tr>
<td>At birth</td>
<td>5.51 ± 0.63</td>
<td>12.14 ± 2.92</td>
<td>2.20 ± 0.42</td>
</tr>
<tr>
<td>Gestation day 16</td>
<td>0.60 ± 0.09</td>
<td>0.32 ± 0.11*</td>
<td>0.50 ± 0.19*</td>
</tr>
<tr>
<td>18</td>
<td>1.65 ± 0.20</td>
<td>0.77 ± 0.27*</td>
<td>0.46 ± 0.14*</td>
</tr>
<tr>
<td>20</td>
<td>3.61 ± 0.36</td>
<td>2.64 ± 1.34*</td>
<td>0.72 ± 0.34*</td>
</tr>
<tr>
<td>At birth</td>
<td>4.87 ± 0.43*</td>
<td>3.96 ± 0.82*</td>
<td>0.82 ± 0.18*</td>
</tr>
</tbody>
</table>

* Significantly different from the control value, p<0.001 (Student’s t-test)

As shown in Fig. 1, the gain of thymus weight in the control rats was very rapid during the fetal life, whereas that in the treated animals was less.

2. Histology of the thymus

Control rats: On day 16 of gestation, the thymus was lobulated and surrounded by loose fibrous connective tissue, and consisted mainly of reticular epithelial cells and large lymphocytes, but the cortex and medulla could not be distinguished (Fig. 2a). Epithelial cells were easily distinguished morphologically from large lymphocytes. Mitoses of epithelial cells and large lymphocytes were frequently seen throughout the organ. A small number of large lymphocytes, neutrophils and macrophages were scattered in the surrounding connective tissue on all days examined.

On day 18 of gestation, the thymus showed further lobulation with the development of connective tissue septa. Although narrow light zones which consisted mainly of epi-
Thymelial cells began to appear in the innermost part, the differentiation of cortex and medulla was not yet distinct (Fig. 2 b). In intercellular spaces of an open meshwork formed by epithelial cells, many large lymphocytes and a few small lymphocytes were observed. Mitoses of lymphocytes were frequently seen.

On day 20 of gestation, further lobulation was noted and the differentiation of cortex and medulla was evident (Fig. 2 c). The thymus showed a structure which was fundamentally similar to that seen in young adults. Large lymphocytes were seen mainly in the subcapsular region and medulla, and small lymphocytes predominated in the inner cortex. Mitoses of lymphocytes were scattered mostly in the cortex. A number of small vessels developed within lobules, especially at the cortico-medullary junction.

At birth, histological structure of the thymus was similar to that on gestation day 20, but both areas of cortex and medulla were expanded with an increase of lymphocytes (Fig. 2 d).

The postnatal thymus from 7 to 84 days old healthy untreated rats showed fundamentally similar findings to those of newborns. The thymus was well lobulated and each lobule showed obvious differentiation of the cortex and medulla. In the cortex, small lymphocytes were densely packed, and in addition, a small number of large lymphocytes were collected in the subcapsular narrow region and were scattered throughout. The percentage of large lymphocytes in the cortex gradually decreased with increasing age. In the med-

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**Fig. 1.** Thymus weights in offspring of untreated and bis-diamine treated rats
ulla, on the other hand, many large lymphocytes were intermingled with epithelial cells.

Thyroid rats: On day 16 of gestation, the thymus was smaller and less lobulated compared with that of the control. It consisted mainly of epithelial cells and large lymphocytes, and mitoses of epithelial cells and large lymphocytes were frequently seen throughout.

On day 18 of gestation, the thymus was smaller and less lobulated than that of the control (Fig. 2e), and resembled structurally that of the control on gestation day 16.

On day 20 of gestation, the thymus did not yet show the differentiation of cortex and medulla, but small light areas which consisted mainly of epithelial cells began to appear in the innermost part of the organ (Fig. 2f). Accordingly, the thymus corresponded structurally to that of the control on gestation day 18.

At birth, the thymus obviously showed the differentiation of cortex and medulla. Furthermore, a number of small vessels developed within lobules, especially at the cortico-medullary junction. The thymus resembled histologically that of the control on gestation day 20.

In the treated fetuses and newborns, there could be found neither glandular nor cystic formation as seen in the thymic rudiment of nude mouse.

The findings described above are concerned with the representative thymus induced by the procedure of this study. Hypoplasia of the thymus induced by dis-diamine varied in morphology. Severely hypoplastic thymus showed less lobulation, a greater number of epithelial cells, a smaller population of lymphocytes, poor development of small vessels and poor cortico-medullary differentiation.

3. Distribution of thymocytes bearing the surface antigens in the thymus

The indirect immunoperoxidase and PAP techniques provided clear staining of thymocytes reacting with all of the monoclonal anti-rat T cell antibodies used in rat thymus. These cells were stained along the cell periphery, usually marging the entire cell membrane. None of thymic epithelial cells were labelled with these antibodies.

Table 4. shows the distribution of thymocytes labelled with these antibodies ac-

<table>
<thead>
<tr>
<th>Day observed</th>
<th>W3/13</th>
<th>W3/25</th>
<th>MRC OX 8</th>
<th>MRC OX 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Medulla</td>
<td>Cortex</td>
<td>Medulla</td>
</tr>
<tr>
<td>Gestation day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16*</td>
<td>39 ± 6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18*</td>
<td>84 ± 8</td>
<td>22 ± 8</td>
<td>37 ± 9</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>20</td>
<td>89 ± 4</td>
<td>96 ± 3</td>
<td>73 ± 9</td>
<td>63 ± 6</td>
</tr>
<tr>
<td>At birth</td>
<td>94 ± 2</td>
<td>97 ± 2</td>
<td>83 ± 5</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>Gestation day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16*</td>
<td>10 ± 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18*</td>
<td>41 ± 15</td>
<td>0</td>
<td>4 ± 6</td>
<td>0</td>
</tr>
<tr>
<td>20*</td>
<td>86 ± 6</td>
<td>26 ± 7</td>
<td>38 ± 7</td>
<td>56 ± 13</td>
</tr>
<tr>
<td>At birth</td>
<td>88 ± 5</td>
<td>95 ± 2</td>
<td>70 ± 10</td>
<td>61 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cortex and medulla could not be distinguished.
According to their localization in the thymus of control and treated rats.

Control rats: On day 16 of gestation, only W 3/13 antibody lightly stained about 40% of thymocytes (Fig. 3a). However, only a few W3/25 positive (W3/25+) macrophages were found sporadically inside and outside the organ. In the surrounding connective tissue, W 3/13+ neutrophils were occasionally found, however none of these antibodies virtually stained any large lymphocyte.

On day 18 of gestation, thymocytes were intensely labelled with W 3/13 antibody and positive cells increased to twice as much compared with that on day 16 (Fig. 3b), while thymocytes labelled lightly with W 3/25, MRC OX8 and MRC OX7 antibodies appeared in varying frequency. Particularly W3/25+ thymocytes showed a minimal staining, often only on a portion of the membrane at the stage.

On day 20 of gestation, all of these antibodies stained preferentially either cortical or medullary thymocytes. W 3/13 antibody labelled almost all thymocytes, but heavier staining was observed in the medulla than in the cortex. W3/25 and MRC OX8 antibodies labelled most of cortical thymocytes and more than half of medullary thymocytes (Fig. 3c and d). MRC OX7 antibody labelled heavily most of cortical thymocytes, and faintly about 40% of medullary thymocytes. At this stage the staining of these antibodies increased in intensity compared with that on gestation day 18. However, it was noted that subcapsular thymocytes consisted mainly of negative cells for these antibodies.

At birth, the distribution of every type of thymocytes labelled with these antibodies was fundamentally similar with that on gestation day 20 (Fig. 3e). Positive cortical thymocytes for these antibodies increased in number compared with that on gestation day 20, and this was correlated well with a decrease of the proportion of subcapsular thymocytes. In the medulla, the percentage of positive thymocytes for these antibodies was hardly changed.

The postnatal thymus from 7 to 84 days old healthy untreated rats showed similar findings with those of newborns (Fig. 3f) and the distribution of positive thymocytes for these antibodies was hardly changed with increasing age at all ages examined.

Treated rats: On day 16 of gestation, only W 3/13 antibody faintly stained a small number of thymocytes.

On day 18 of gestation, about 40% of thymocytes were lightly labelled with W 3/13 antibody. Furthermore, a small number of MRC OX8+ thymocytes were seen in two of six thymuses examined, however no thymocyte was labelled with W 3/25 and MRC OX7 antibodies. In general, the thymus corresponded immunohistochemically as well as histologically to that of the control on gestation day 16.

On day 20 of gestation, W 3/13 antibody intensely stained most of thymocytes (Fig. 3g), whereas W 3/25, MRC OX8 and MRC OX7 antibodies lightly stained thymocytes in varying number (Fig. 3h). From this result, the thymus resembled immunohistochemically that of the control on gestation day 18.

At birth, the preference of staining either cortical or medullary thymocytes with these antibodies became evident, and the distribution of positive cells corresponded to that
As is expected, severely hypoplastic thymuses tended to exhibit serious immuno-histochemical findings such as a smaller population of positive thymocytes, weaker staining and less preference of staining either cortical or medullary thymocytes.

4. Distribution of thymocytes possessing the lysosomal enzyme activities in the thymus

Activities of AcP, B-G and ANAE were clearly demonstrated in rat thymocytes by the procedure employed. Reaction product of AcP activity was expressed as several reddish coarse granules, while that of B-G and ANAE activities were as multiple brownish small granules and one or a few red–brown blocks respectively, in the cytoplasm of thymocytes. These staining patterns were similar in all ages examined in this study.

Activities of these enzymes in the thymus were not limited to thymocytes, i.e., AcP activity was observed in thymic epithelial cells, fibroblasts, macrophages and neutrophils, B-G activity in thymic epithelial cells and macrophages, and ANAE activity in macrophages.

Table 5. shows the distribution of thymocytes possessing the enzyme activities in the thymus.

Control rats: On day 16 of gestation, AcP activity was detected in most of thymocytes (Fig. 4a), whereas B-G and ANAE activities were demonstrated in about 1/3 and 1/10 of thymocytes respectively (Fig. 4b and c). Large lymphocytes scattered in the surrounding connective tissue were almost always positive for AcP, and occasionally positive for B-G and/or ANAE.

On day 18 of gestation, the frequency of AcP positive (AcP+) thymocytes was slightly reduced, and that of B-G+ thymocytes was hardly changed, while the fraction of ANAE+ thymocytes was slightly increased.

On day 20 of gestation, AcP, B-G and ANAE activities were found in a varying but considerable number of cortical and medullary thymocytes (Fig. 4d).

At birth, the frequency of positive thymocytes for AcP, B-G and ANAE in the cortex decreased in comparison with those on day 20 of gestation, while that in the

Table 5. Distribution (mean % ±SD) of thymocytes with the enzyme activities in the thymuses from untreated and bis-diamine treated rats

<table>
<thead>
<tr>
<th>Day observed</th>
<th>AcP</th>
<th>B-G</th>
<th>ANAE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Medulla</td>
<td>Cortex</td>
</tr>
<tr>
<td>Gestation day 16*</td>
<td>89 ± 2</td>
<td>33 ± 7</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>18*</td>
<td>72 ± 7</td>
<td>31 ± 9</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>20</td>
<td>63±8</td>
<td>32±8</td>
<td>25±7</td>
</tr>
<tr>
<td>At birth</td>
<td>52±6</td>
<td>84±9</td>
<td>13±5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day observed</th>
<th>AcP</th>
<th>B-G</th>
<th>ANAE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>Medulla</td>
<td>Cortex</td>
</tr>
<tr>
<td>Gestation day 16*</td>
<td>91 ± 4</td>
<td>29 ± 11</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>18*</td>
<td>78 ± 8</td>
<td>31 ± 6</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>20*</td>
<td>68 ± 8</td>
<td>31 ± 8</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>At birth</td>
<td>65±9</td>
<td>32±5</td>
<td>21±4</td>
</tr>
</tbody>
</table>

* Cortex and medulla could not be distinguished.
medulla increased (Fig. 4e and f). The decrease in number of positive cortical thymocytes for these enzymes correlated with an increase of the proportion of small cortical thymocytes which showed a weak or negative AcP activity and often negative B-G and/or ANAE activities. Thus, the preference of distribution of thymocytes possessing these enzymes in either cortex or medulla became evident.

The postnatal thymus from 7 to 84 days old healthy untreated rats showed similar findings to those of newborns and the distribution of thymocytes with AcP, B-G and ANAE activities was scarcely altered with increasing age at all ages examined.

Treated rats: On day 16 of gestation, there was no significant difference in the distribution of positive cells and staining patterns of these enzymes in thymocytes between the treated and control rats. As seen in the control, large lymphocytes scattered in the surrounding connective tissue were almost always positive for AcP, and occasionally positive for B-G and/or ANAE.

On day 18 of gestation, the percentage of AcP+ thymocytes was slightly reduced, that of B-G+ thymocytes showed no change, and the fraction of ANAE+ thymocytes somewhat increased compared with those on day 16.

On day 20 of gestation, the distribution of positive thymocytes for these enzymes was comparable to that of the control on gestation day 18 (Fig. 4g).

At birth, the percentages of AcP and B-G positive thymocytes were similar both in the cortex and medulla to those on day 20 of gestation (Fig. 4h), while the fraction of ANAE+ thymocytes was slightly increased both in the cortex and medulla. Even at this stage, the preference of distribution of thymocytes possessing these enzymes in either cortex or medulla was not evident. Thus, the thymus enzymohistochemically corresponded to that of the control on gestation day 20.

Furthermore, in general, severely hypoplastic thymuses tended to show a smaller population of ANAE+ thymocytes.

5. Spleen

Spleens from newborn rats with or without treatment by bis-diamine were examined. The spleens from treated rats were also hypoplastic. The average weight of them (5.02 mg ± 1.03 mg) was significantly smaller (p<0.001, Student’s t-test) than that of the control (10.94 mg ± 1.70 mg).

Control rats: The spleen consisted mainly of reticulum cells, lymphoid cells and hemopoietic cells including megakaryocytes. At this stage the white pulp was not yet fully developed which had only several layers of lymphocytes surrounding a central arteriole (periarteriolar lymphocyte sheath; PALS) but no lymphoid follicles (Fig. 2g). Immunohistochemically, the PALS was composed of a mixture of positive and negative lymphocytes for W 3/13, W 3/25 and MRC OX8 antibodies, but consisted of negative lymphocytes for MRC OX7 antibody. Enzymohistochemically, the PALS was intermingled with positive and negative lymphocytes for AcP, B-G and ANAE activities.

Treated rats: The spleen, as seen in the control, consisted largely of reticulum cells, lymphoid cells and hemopoietic cells including megakaryocytes. Compared with the
Fig. 2. Light microscopic findings of the thymuses and spleens from untreated and bis-diamine treated rats (hematoxylin and eosin stain, ×200)

a: Thymus, gestation day 16, control rat. The thymus consists mainly of reticular epithelial cells and large lymphocytes.
b: Thymus, gestation day 18, control rat. Narrow light zones which consist mainly of epithelial cells are noted in the innermost part of the organ.
c: Thymus, gestation day 20, control rat. The differentiation of cortex (C) and medulla (M) is evident.
d: Thymus, at birth, control rat. Both areas of cortex and medulla are expanded with an increase of lymphocytes compared with those on gestation day 20.
e: Thymus, gestation day 18, treated rat. The thymus shows severe hypoplasia.
f: Thymus, gestation day 20, treated rat. Small light areas are noted in the innermost part of the organ.
g: Spleen, at birth, control rat. The white pulp is not yet fully developed, but which has several layers of lymphocytes around a central arteriole (arrow).
h: Spleen, at birth, treated rat. The white pulp has only a few layers of lymphocytes surrounding a central arteriole (arrow).

Fig. 3. Immunohistochemical findings of the thymuses from untreated and bis-diamine treated rats (×200)

a: Gestation day 16, control rat. Some thymocytes are lightly labelled with W 3/13 antibody.
b: Gestation day 18, control rat. The majority of thymocytes are intensely labelled with W 3/13 antibody.
c: Gestation day 20, control rat. W 3/25 positive thymocytes are preferentially noted in the cortex than in the medulla.
d: Gestation day 20, control rat. MRC OX8 positive thymocytes are preferentially noted in the cortex than in the medulla.
e: At birth, control rat. Many cortical thymocytes and a few medullary thymocytes are labelled with MRC OX7 antibody.
f: 7 days old, control rat. Almost all cortical and medullary thymocytes are labelled with W 3/13 antibody with heavier staining in the latter.
g: Gestation day 20, treated rat. The majority of thymocytes are intensely labelled with W 3/13 antibody.
h: Gestation day 20, treated rat. A considerable number of thymocytes are lightly labelled with MRC OX8 antibody.

Fig. 4. Enzymohistochemical findings of the thymuses from untreated and bis-diamine treated rats

a: Gestation day 16, control rat. Most of thymocytes are AcP positive. (×400)
b: Gestation day 16, control rat. Positive and negative thymocytes for B-G are intermingled. (×1,000)
c: Gestation day 16, control rat. ANAE positive thymocytes are sporadically seen. (×800)
d: Gestation day 20, control rat. ANAE positive thymocytes are scattered in both cortex and medulla. (×400)
e: At birth, control rat. Most of medullary thymocytes are AcP positive, whereas cortical thymocytes consist of a mixture of positive and negative cells. (×400)
f: At birth, control rat. B-G positive thymocytes are preferentially noted in the medulla than in the cortex. (×400)
g: Gestation day 20, treated rat. Note the scattering ANAE positive thymocytes. (×400)
h: At birth, treated rat. The majority of cortical and medullary thymocytes are AcP positive. (×400)
control, however, it showed less cellularity and less development of the white pulp which had only a few layers of lymphocytes surrounding a central arteriole and no lymphoid follicles (Fig. 2h). Furthermore, in comparison with the control, the PALS had a smaller number of lymphocytes which were labelled immunohistochemically with W 3/13, W 3/25 and MRC OX8 antibodies, and stained enzymohistochemically for AcP, B-G and ANAE activities. Like in the control, the PALS virtually had no MRC OX7+ lymphocytes.

DISCUSSION

The present study deals with the ontogenic development of rat thymocytes and the results are discussed in the following three articles.

1. Ontogenic development of thymocyte surface antigens

The present immunohistochemical study demonstrated clearly a sequence of cell surface antigen expression on thymocytes in the rat during the ontogenic development. Of the antigens studied, W 3/13 antigen was expressed first on thymocytes on gestation day 16, and thereafter the frequency of thymocytes bearing this antigen was always higher in comparison with other antigens. On day 16 of gestation, no surface antigen was detected on any large lymphocyte in the surrounding connective tissue, which has been suggested to be the precursor of thymocyte to be migrating to the thymic anlage9). This suggests that thymocytes bearing only W 3/13 antigen among antigens examined may be the earliest form of thymocytes differentiated from the precursor cells. On day 18 of gestation when the cortex and medulla were not yet differentiated, W 3/25, MRC OX 8 and MRC OX 7 antigens appeared on thymocytes simultaneously. This suggests that early differentiation of the surface antigens does not require the process of migration of thymocytes to the medulla. The thymocytes bearing these antigens, thereafter, increased rapidly in number during the late fetal life. On and after day 20 of gestation when the cortex and medulla could clearly be distinguished, the distribution of thymocytes bearing these antigens became similar to that in normal young adults. The majority of cortical thymocytes except for some subcapsular thymocytes were W 3/13+, W3/25+, MRC OX 8+ and MRC OX 7+. Whereas medullary thymocytes, almost all W3/13+, were composed of a intermingling of both positive and negative cells for W3/25, MRC OX 8 and MRC OX 7 antigens. Subcapsular thymocytes contained a distinctive population of cells which were negative for all these antigens.

As to the differentiation of T cells, the previous studies using cell suspensions from young adult rat thymuses and peripheral lymphoid tissues have demonstrated that most thymocytes were W3/13+, W3/25+ and MRC OX 8+, while peripheral T cells were, all W3/13+, divided into two non-overlapping subsets which were W3/25+ • MRC OX 8− (helper/inducer) and W3/25− • MRC OX 8+ (cytotoxic/suppressor) cells31,17), and that most thymocytes were MRC OX 7+, but most peripheral T cells were MRC OX 7−18). Besides, it has been thought that the precursors of thymocytes settle and proliferate in the subcapsular cortex of the thymus, and then the derivatives are processed during migration from the
cortex to medulla, consequently emigrating from the medulla to the peripheral lymphoid tissue\textsuperscript{(60)}. Based on these data and the present immunohistochemical study, a possible maturation pathway of T cell surface antigens within the thymus is suggested as follows: W3/13\textsuperscript{−} • W3/25\textsuperscript{−} • MRC OX 8\textsuperscript{−} • MRC OX 7\textsuperscript{−} (subcapsular cortex) → W3/13\textsuperscript{+} • W3/25\textsuperscript{−} • MRC OX 8\textsuperscript{−} • MRC OX 7\textsuperscript{−} (subcapsular cortex) → W3/13\textsuperscript{+} • W3/25\textsuperscript{+} • MRC OX 8\textsuperscript{+} • MRC OX 7\textsuperscript{+} (cortex) → W3/13\textsuperscript{+} • W3/25\textsuperscript{+} • MRC OX 8\textsuperscript{+} • MRC OX 7\textsuperscript{+} (medulla) → W3/13\textsuperscript{+} • W3/25\textsuperscript{−} • MRC OX 8\textsuperscript{+} • MRC OX 7\textsuperscript{−} (medulla). The last two populations can be considered to migrate into the peripheral T cell areas, since they possess the phenotypes of mature peripheral T cells. Accordingly, the thymocytes with the same antigenic profiles as mature peripheral T cells seem to occur within the medulla from day 20 of gestation.

However, there is a striking problem to be solved in this possible maturation pathway. SHORTMAN and JACKSON\textsuperscript{(22)} examined the proliferation kinetics of mouse thymocyte suspensions and demonstrated that the high $\theta$, TL positive small sized thymocytes which were thought to be the cortisone sensitive and short-lived cortical thymocytes appeared to die in situ. If so, most cortical thymocytes observed in the present study may die, since the majority of them are small sized and positive for MRC OX 7 which is $\theta$ homologue in rat\textsuperscript{(60)}. However, the techniques used in the study did not produce any direct evidence to show that MRC OX 7 positive cortical thymocytes would die, nor they demonstrated as to which cortical cells should migrate to the medulla. Accordingly, the decision of the definitive maturation sequence must await studies employing techniques which allow the surface antigen, mobility and vitality of single cell to be demonstrated simultaneously in situ.

The signals which drive thymocytes into proliferation and differentiation are still unknown. There is, however, accumulating evidence that the microenvironment consisting of thymic epithelial cells exerts a major regulatory role on the differentiation of thymocytes\textsuperscript{(103,30)}. The electron microscopical observations of the thymuses from Wistar rat fetuses demonstrated that some thymic epithelial cells were evidently differentiating toward squamous epithelial cells on day 16, and toward endocrine-like cells on day 20, of gestation\textsuperscript{(14)}. These gestation ages coincided respectively with the days in the present study when the surface antigen probably began to occur and when the thymocytes with the same antigenic profiles as mature T cells began to appear. However, the precise role of thymic epithelial cells on the thymocyte differentiation remains unclear.

2. AcP, B-G and ANAE activities in thymocytes during ontogenic development

Previous enzymohistochemical studies have demonstrated that the activities of several lysosomal enzymes of lymphocytes were different depending on their tissue localization. In particular, AcP, B-G and ANAE activities were observed predominantly in the lymphocytes of thymus and peripheral T cell areas of man and rodents\textsuperscript{(18,324)}. Furthermore, it has been suggested that the capability of T lymphocytes to display some of these enzyme activities might be related to their stage of differentiation or activation\textsuperscript{(18)}. However, little is known so far about the activities of such enzymes in thymocytes during their ontogenic development.
The present enzymohistochemical study revealed the variable expressions of such enzyme activities in thymocytes with their ontogenic differentiation in the rat, and the distribution patterns of thymocytes possessing these enzyme activities within the postnatal rat thymus were largely parallel with those previously reported in man and rodents (18, 20, 24). The present enzymohistochemical observations indicate that at least in the rat the presence \textit{per se} of AcP, B-G and ANAE activities is independent of ontogenic thymocyte differentiation. However, the high percentages of AcP, B-G and ANAE positive cells characterize the medullary thymocytes, and the highest frequency of AcP positive cells is observed in the ontogenically early thymocytes and thereafter in the medullary thymocyte. These indications may be supported by a previous report of DAVEY et al. (4) who demonstrated that after blastoid transformation by phytohemagglutinin, the number of human T lymphocytes containing B-G and ANAE activities was reduced, but no significant change was observed in AcP activity. However, the present data on ANAE activity seem to be incompatible with the concept proposed by MUELLER et al. (8), who suggested that in the young adult mouse thymus ANAE activity did not pertain to early presursors of T cell population but was acquired by T lymphocytes at the time of peripheralization in the cortico–medullary junction and medula. This discrepancy may not be due to the difference in species but in age examined, since the distribution of ANAE positive thymocytes within the young adult thymus was little different among rat (the present study), mouse (18) and man (80). As already noted, if most cortical thymocytes die in situ, the finding that cortical thymocytes often showed no detectable ANAE activity by the enzymohistochemistry used may reflect their vital loss rather than their differentiation stage. The same account may be available for the findings that cortical thymocytes showed weak or negative AcP activity and often no B-G activity.

Little is known about the functional significance of AcP, B-G and ANAE activities associated with T lymphocytes. GROSSI et al. (8) examined the human T cell subpopulations from peripheral blood and suggested that T cells with strong globular ANAE activity represented the existence of receptors for IgM and a helper function for B cell proliferation and differentiation to plasma cells. However, in the present enzymo-and immuno-histochemical studies, no definite correlation could be found as far as the thymus was concerned.

3. Effect of bis-diamine on ontogenic thymocyte development

The DIGEORGE's syndrome is a rare congenital immune deficiency disorder in association with reduction anomalies of the III and IV pharyngeal pouches, such as absence or hypoplasia of the thymus and/or parathyroid glands. This syndrome is often accompanied with cardiovascular anomalies and abnormal facies (21, 25). These developmental anomalies lead to the characteristic clinical features such as a peculiar face, neonatal hypocalcemic tetany, cardiac decompensation and increased susceptibility to infection (21). The immunologic characteristic of the DIGEORGE's syndrome is functionally a reduction in cell–mediated immunity and morphologically a decrease in number of T cells in the spleen and lymph node, due to thymus aplasia or hypoplasia (19, 21).

Recently, it has been indicated that bis-diamine could produce multiorgan anomalies
in rat which were quite similar to the anomalies in the DiGeorge's syndrome in man.\textsuperscript{13,14} The bis-diamine-induced anomalies in rat involve aplasia or hypoplasia of thymus, parathyroid, thyroid and spleen\textsuperscript{13,14}, and anomalies of heart, great vessels and face\textsuperscript{13}.

The present study showed that the thymus weight and the ratio of thymus to body weight in the treated rats were significantly lower than those in the control. Most of these hypoplastic thymuses were accompanied by insufficient descent. Histologically, the hypoplastic thymus showed a slight retardation in morphologic development, but there was neither glandular nor cystic formation of thymic epithelial cells which were seen in the thymic rudiment of nude mouse\textsuperscript{29}. These observations were similar to the histologic findings of the thymus in the partial DiGeorge's syndrome in man that the thymus, if present, was hypoplastic but histologically normal\textsuperscript{21}. The present immunohistochemical study demonstrated that the ontogenic development of thymocytes in the treated rats was delayed by about two days with respects to the occurrence and distribution of thymocytes bearing every surface antigen examined in the study. In addition, the present enzymo-histochemical study revealed that the distribution of AcP, B-G and ANAE positive thymocytes in all ages of the treated rats was largely comparable with that in the control of the two days younger age. Accordingly, the degree of retardation in ontogenic thymocyte differentiation well corresponded with the extent of delay in morphologic development of the thymus. These findings suggest that the thymocyte differentiation in the bis-diamine-induced hypoplastic thymus was retarded but underwent an essentially normal maturation process.

As to the peripheral lymphoid organ in the present study, the spleen of the treated newborn rats showed a slight but significant retardation in morphology. The immunohistochemical studies also confirmed that, in comparison with the control, the PALS in the treated spleen contained a smaller number of T lymphocytes. These findings were comparable with the histologic findings of spleen in the DiGeorge's syndrome in man\textsuperscript{21}, and further, seemed to support a report by Okishima et al.\textsuperscript{19} who examined the immune functions of spleen cells from bis-diamine treated newborn rats and demonstrated a reduction of T cell number and of response to PHA and Co A.

As described above, these immunopathological findings in the bis-diamine-induced malformation complex in rat are strikingly similar to those in the DiGeorge's syndrome in man. These results indicate that the bis-diamine-induced malformation complex in rat can also immunologically be a suitable experimental model of the DiGeorge's syndrome in man.

The primary developmental genesis of the bis-diamine-induced anomalies is scarcely known but is postulated to be the disturbance of proliferation of mesenchymal cells or that of the synthesis of extracellular substance before the formation of the primordia of branchial apparatuses, spleen and cardiovascular system\textsuperscript{14,23}.

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