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High Frequency Apoptosis of Recent Thymic Emigrants in the Liver of Lympohpic Diabetes-Prone BioBreeding Rats

Neal N. Ikawoshi, Irving Goldschneider, Frances Tausche, John P. Mordes, Aldo A. Rossini, and Dale L. Greiner

Diabetes-prone (DP) BioBreeding (BB) rats develop spontaneous autoimmune diabetes. DP-BB thymocyte export is reduced, and most thymic emigrants disappear rapidly from peripheral lymphoid tissues. DP-BB rats are consequently lymphopenic and circulate severely reduced numbers of T cells. Peripheral T cells present are phenotypically immature (Thy1−) and appear activated. We hypothesized that DP-BB recent thymic emigrants have a shortened life span and disappear by apoptosis. The percentage of T cells with an αβTCRlow/CD220−CD4+CD8+ phenotype was increased in DP peripheral lymphoid tissues when compared with normal, nonlymphopenic diabetes-resistant (DR) BB rat tissues. There was no evidence of DNA fragmentation in freshly isolated DP- or DR-BB rat cells, but, after 24 h of culture, a higher proportion of DP- than DR-BB splenic T cells underwent apoptosis. We then tested the hypothesis that BB rat T cells with the αβTCRhigh/CD220+CD4+CD8− phenotype accumulate and undergo apoptosis in the liver. Such cells were observed undergoing apoptosis in both DP- and DR-BB rats, but comprised ~80% of intrahepatic T cells in DP vs ~20% in DR-BB rats. Most αβTCRlow/CD220−CD4+CD8− cells in the liver were also Thy1+. The data suggest that T cell apoptosis in the DP-BB rat is underway in peripheral lymphoid tissues and is completed in the liver. Increased intrahepatic apoptosis of recent thymic emigrants appears in part responsible for lymphopenia in DP-BB rats and the concomitant predisposition of these animals to autoimmunity.

Materials and Methods

Animals

Viral Ab-free DP-BB and DR-BB rats were obtained from the National Institutes of Health sponsored colony at the University of Massachusetts Medical Center, Worcester, MA. They were certified as free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan’s virus), GD7, Reo-3, Mycoplasma pulmonis, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, andENCEPHALITOZÖON cuniculi. Animals of either sex were used when 3 to 5 wk old. They were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical Center.
Antibodies

mAbs directed against αβTCR (clone R7.3), CD8α (clone OX-8), CD4 (clone OX-33), B220 (CD45R, clone HIS24), CD90 (Thy1.1, clone OX-7), and CD25 (IL-2 receptor α-chain, clone OX-39) were obtained from PharMingen (San Diego, CA). The DS4.23 hybridoma that secretes an anti-RT6.1 mAb is maintained in our laboratory (11). Anti-HSA (clone HIS50) mAb was obtained from Dr. Jan Rozing, University of Groningen, The Netherlands. PE-, FITC-, and biotinylated mouse IgG1 and IgG2a isotype controls, and Red 670-conjugated streptavidin (SA) were obtained from PharMingen.

Cell preparation

Rat donors were killed in an atmosphere of 100% CO2. Cervical and mesenteric lymph nodes, spleens, and thy were removed, and single cell suspensions were prepared by gentle extrusion of tissues through stainless steel sieves into cold medium (17). Erythrocytes were lysed with hypotonic NH4Cl.

Liver mononuclear cell suspensions were prepared using a minor modification of a technique previously described for use in the mouse (21). Briefly, livers were perfused in situ via the portal vein with 30 ml of PBS and then excised. Single cell suspensions were obtained by gentle extrusion of the organ through a 50-mesh metal cell strainer, washed once with 40 ml of RPMI 1640, and then incubated for 30 min at 37°C in 20 ml of RPMI 1640 containing 50 U/ml of collagenase IV (Sigma, St. Louis, MO) and 0.04% DNase I (Sigma). The digested cell suspensions were washed once in RPMI 1640 medium and centrifuged at 30 × g for 3 min to remove parenchymal hepatocytes and cell aggregates. After centrifugation at 400 × g for 10 min, the cell pellet was suspended in 2 ml of RPMI 1640 medium, and 1 ml of the suspension was then mixed with 4 ml of 30% metrizamide (Sigma) in PBS. The mixture was overlaid with 1 ml of PBS and centrifuged at 400 × g for 20 min at 4°C in a 15-ml washed tube. Cells at the metrizamide/PBS interface were collected, washed twice with 20 ml of PBS, and counted manually using a hemocytometer and 1% trypan blue as previously described (21).

Standard flow microfluorometry

Two- and three-color flow cytometric analyses were performed as previously described (17). Briefly, 1 × 106 viable lymph node, splenic, or intrahepatic T cells were reacted with a mixture of FITC-, biotin-, and/or PE-conjugated mAbs for 20 min at 4°C. Cells were then washed, reacted with Red 670-conjugated SA to visualize biotinylated mAbs, washed again, and fixed with 2% paraformaldehyde. Controls for background staining were FITC- or PE-conjugated mouse IgG1 or IgG2a, or Red 670-conjugated mouse IgG1 or IgG2a, or Red 670-conjugated SA alone. Labeled cells were analyzed using a FACScan instrument (Becton Dickinson, Sunnyvale, CA). T cells were identified by their forward and side light scatter profiles and the detection of αβTCR staining. The composite phenotypes of T cell subsets for up to four antigens markers in addition to αβTCR were determined by combining the results of multiple two- and three-color analyses of replicate samples of lymphocytes, each incubated with a different combination of Abs. In all instances, a minimum of 30,000 events was acquired for both DR and DP animals.

Four-color flow microfluorometry with intravital dyes

Four color analyses were performed on intrahepatic T cells to quantify the relative percentages of live, apoptotic, and dead cells using a combination of two mAbs and two intravital dyes as described (24). Briefly, liver mononuclear cells were isolated, reacted with anti-αβTCR and anti-B220 mAbs, and suspended in 1 ml of PBS-1% BSA that had been warmed to 37°C. Hoechst 33342 (HO342, Molecular Probes, Eugene, OR) was then added to a final concentration of 1 μg/ml, and the cells were incubated at 37°C for 7 min. The second dye, 7-aminoactinomycin-D (7-AAD, Calbiochem, San Diego, CA.), was then added to the cells at a final concentration of 1 μg/ml, and the cell suspension was immediately placed on ice in the dark. Cells were reacted with the 7-AAD for exactly 10 min, washed in PBS, and analyzed within 1 h using a FACScan instrument.

Detection of DNA fragmentation by flow microfluorometry

Suspensions of thyocytes or splenocytes (20 × 106 cells/ml) were incubated in RPMI 1640 containing 10% lot-selected FBS for 24 h at 37°C in 5% CO2. Replicate cultures were harvested at intervals, stained with FITC-conjugated anti-αβTCR mAb as described above, fixed in 70% cold ethanol, and pelleted. Cells were suspended in a staining solution containing propidium iodide (PI, 50 μg/ml) and RNase (100 μU/ml), as previously described (17). Cell surface (FITC) and intranuclear (PI) fluorescence intensity were analyzed simultaneously using a FACScan instrument.

Purification of TCRlowB220+ liver cells

Intrahepatic cells were prepared and reacted with PE-conjugated anti-αβTCR and FITC-conjugated anti-B220 mAbs as described above. The TCRlowB220+ cell subset was sorted on a Mo Flo cell sorter (Cytometry, Fort Collins, CO). Debris, dead cells, and nonlymphoid cells were excluded by appropriate gating based on forward and side scatter profiles.

RT-PCR

RNA was prepared from cell suspensions by the guanidine thiocyanate-phenol, chloroform method and used for cDNA synthesis by reverse transcription (RT) as previously described (25, 26). TCRβ and β-actin primers were synthesized by the Oligonucleotide Synthesis Core Facility at our institution based on published sequences as previously described (26). PCR were performed using a total volume of 50 μl PCR buffer (Promega, Madison, WI); 2 μl of cDNA were incubated with 1.25 U of Taq DNA polymerase (Promega), 0.5 mM deoxynucleotide triphosphates, and 1 μM sense and antisense primers. The linear range of amplification for each primer was established in independent preliminary studies. PCR reaction products were separated on an ethidium bromide gel and visualized in ultraviolet light.

Statistical analysis

Parametric data are presented as arithmetic means ± 1 SD. Pairs of means were compared by two-tailed t test using separate variance estimates and the Bonferroni adjustment for multiple comparisons, as required (27).

Results

Atypical TCRlowCD4low or TCRlowCD8low lymph node cells that express B220 and HSA are abundant among recent thymic emigrants in DP-BB rats

Many lymph node T cells in the DP-BB rat were found to exhibit an atypical phenotype that was Thy1+ (~56% of total T cells) and TCRlow (~24% of total T cells, Fig. 1). In rats, the TCR+ Thy1+ phenotype is characteristic of cells that are recent thymic emigrants (17, 18). Three-color flow cytometric analysis of DP-BB rat lymph node cells for expression of αβTCR, Thy1, and either CD4, CD8, B220, or HSA consistently revealed that 20% to 30% of total T cells exhibited TCRlow CD4low, CD8low, B220+, or HSA+ phenotypes (Fig. 2, Table I). In addition, >65% of these unusual T cells expressed the Thy1 Ag (Fig. 2). In contrast, analysis of DR-BB rat lymph node cells revealed that the T cell phenotype of these animals to be normal, with very low percentages of T cells expressing the atypical phenotypes found in the DP-BB rat (Table I, Figs. 1 and 2). The ratio of TCRlow to TCRhigh lymph node cells was comparable in all single, two-color, and three-color flow cytometric analyses (Table I, Figs. 1 and 2). In their aggregate, the data document the existence of a previously unrecognized minor population of TCRlow lymph node cells in the DP-BB/Wor rat.

Additional three-color flow cytometric analysis of the DP-BB rat for Thy1, TCR, and either CD4, CD8, B220, or HSA consistently revealed that most Thy1+ lymph node T cells that exhibited the atypical CD4low, CD8low, B220+, or HSA+ phenotypes were also TCRlow (Fig. 3). Again, very low percentages of T cells expressing the atypical phenotypes were found in the DR-BB rat.

Calculations based on these data indicate that a minimum of ~8% and as many as ~16% of immature (Thy1+) peripheral T cells in DP-BB rats express an αβTCRlow+, CD4low, (B220 or HSA)+ phenotype. Mouse T cells undergoing apoptosis have previously been associated with this phenotype (21).

Increased frequency of apoptosis in cultured DP-BB rat splenic T cells

Suspensions of freshly isolated and cultured thyocytes and splenic cells from DR-BB and DP-BB rats were stained with R7.3 anti-TCR mAb, permeabilized, and reacted with propidium iodide. Cells were analyzed by flow cytometry for the presence of the
In both lymphopenic DP-BB and nonlymphopenic DR-BB rats, intrahepatic T cells were observed to express two distinct phenotypes, one TCRhighB220+ (R2 gate) and the other TCRlowB220+ (R3 gate, Fig. 5, upper panels). The percentage of TCRhighB220+ intrahepatic lymphocytes in the DR-BB rat was ~sevenfold greater than in the DP-BB rat (Table III). Conversely, the percentage of TCRlowB220+ intrahepatic lymphocytes in the DP-BB rat was ~threefold greater than in the DR-BB rat. When the data are presented as the calculated percentage of intrahepatic TCR+ T cells, only 18% of all intrahepatic T cells in the DR-BB rat were TCRlowB220+ T cells whereas, in the DP-BB rat, 81% of all intrahepatic T cells expressed this unusual phenotype (Table III).

To characterize these intrahepatic T cell populations, we first determined relative cell size of TCRhigh and TCRlow cells by flow cytometric measurement of forward light scatter (Fig. 6). As expected, TCRhigh T cells from the normal DR rat exhibited a narrow range of cell sizes that was comparable to that of small resting lymphocytes found in peripheral lymphoid tissues. In contrast, TCRhigh T cells from the autoimmune DP-BB rat exhibited a broad range of cell sizes; the great majority of the cells were larger than those observed in the DR rat and consistent with the expression of an activated phenotype. The TCRlow cells in both rat strains also exhibited a broad range of cell sizes, consistent with the presence of activated cells.

To characterize further the intrahepatic T cell populations, twocolor flow cytometry was performed for the expression of CD4, CD8, Thy1.1, RT6.1, or CD25 on cells that also expressed either the αβTCRhigh (Fig. 7) or B220+ (Fig. 8) phenotype. In DR-BB
FIGURE 2. Flow cytometric profiles of total lymph node T cell populations. Lymph node cells were obtained from DR-BB/Wor (left column) and DP-BB/Wor (right column) rats 3 to 5 wk of age, reacted with the reagents indicated, and processed for flow cytometry. Each profile was determined in three to four separate experiments (Table I). Shown are representative three-color flow cytometric profiles of surface expression of αβTCR (row 1), B220 (row 4), and HSA (row 5) Abs plotted against Thy1 Ag expression on total lymph node T cells (TCR⁺). Also shown are representative profiles of total CD4⁺ (row 2) and CD8⁺ (row 3) cells plotted against Thy1 expression. Horizontal and vertical marker cutoffs define positive and negative cell populations (B220, HSA, Thy1) or strongly positive (hi) and weakly positive (lo) cells (αβTCR, CD4, and CD8). The percentage of cells localized to each quadrant is indicated. The percentages shown in the various quadrants exclude the populations of TCR-negative cells that are shown in Figure 1.
rats, the TCR\textsuperscript{high} intrahepatic T cell population exhibited a phenotype consistent with that previously reported (9, 10, 12, 17) for peripheral T lymphocytes (Fig. 7, left column). These cells exhibited a CD4\textsuperscript{CD4\textsuperscript{8}} ratio of \( \sim 2.1 \) and included a large number of RT6\textsuperscript{+} cells, only a small number of Thy1.1\textsuperscript{+} cells, and a very small number of cells expressing the IL-2 receptor (CD25) (Fig. 7, Table IV). In DP-BB rats, the TCR\textsuperscript{high} intrahepatic T cell population also exhibited a phenotype consistent with that previously reported (9, 10, 12, 17) for DP peripheral T lymphocytes (Fig. 7, right column). They were predominantly CD4\textsuperscript{+}CD8\textsuperscript{–}. Approximately 50% were Thy1.1\textsuperscript{+} and very few were RT6\textsuperscript{+}. A substantial number expressed the IL-2 receptor (CD25) (Fig. 7, Table IV).

In both DR- and DP-BB rats, the B220\textsuperscript{+} intrahepatic T cell populations exhibited a phenotype consistent with that previously described (21) for apoptotic T lymphocytes (Fig. 8). These cells expressed a CD4\textsuperscript{low}/\textsuperscript{–}, CD8\textsuperscript{low}/\textsuperscript{–}, RT6\textsuperscript{low}/\textsuperscript{–}, and CD25\textsuperscript{low}/\textsuperscript{–} phenotype, and \( \sim 90\% \) expressed Thy1.1 (Fig. 8, Table IV).  

**TCR\beta gene expression in αβTCR\textsuperscript{low}B220\textsuperscript{–}CD4\textsuperscript{+}CD8\textsuperscript{–} intrahepatic cells**

We next established that the TCR\textsuperscript{low}B220\textsuperscript{–} subset observed in Figure 5 was, in fact, T cells expressing B220. cDNA was synthesized from total RNA extracted from sorted TCR\textsuperscript{low}B220\textsuperscript{–} DP-BB intrahepatic cells and from control DP-BB TCR\textsuperscript{low}B220\textsuperscript{–} sorted lymph node T cells. RT-PCR analysis using β-actin-specific primers was performed to control for the integrity of the extracted mRNA and for the amounts of mRNA assayed. As shown in Figure 9, TCR\β mRNA was readily detected in cDNA prepared from both TCR\textsuperscript{low}B220\textsuperscript{–} lymph node cells and from TCR\textsuperscript{low}B220\textsuperscript{–} intrahepatic cells.

Because most TCR\textsuperscript{high} T cells are B220\textsuperscript{–} and most B220\textsuperscript{+} T cells are TCR\textsuperscript{low} in both strains (Fig. 5, upper panels), these results, together with the data in Figures 7 and 8, indicate that a population of TCR\textsuperscript{low}B220\textsuperscript{–}CD4\textsuperscript{+}CD8\textsuperscript{–} T cells accumulate in the liver of both DP-BB and DR-BB rats.  

**Intrahepatic αβTCR\textsuperscript{low}B220\textsuperscript{–}CD4\textsuperscript{+}CD8\textsuperscript{–} T cells are apoptotic in both DP- and DR-BB rats**

To test the hypothesis that the αβTCR\textsuperscript{low}B220\textsuperscript{–}CD4\textsuperscript{+}CD8\textsuperscript{–} T cells in the livers of DR-BB and DP-BB rats (Fig. 5, upper panels) are undergoing apoptosis, we used four-color flow cytometry. We combined two-color analysis on the TCR\textsuperscript{high}B220\textsuperscript{–} (R2 gate, shown in red) and the TCR\textsuperscript{low}B220\textsuperscript{–} (R3 gate, shown in blue, Fig. 5) populations of intrahepatic T cells with HO342 and 7-AAD staining.

The vital dye HO342 stains apoptotic cells with greater intensity than nonapoptotic cells. The dye 7-AAD stains dead cells that lack membrane integrity but is excluded from live cells (24). The results obtained using this dye combination were analyzed as follows: HO342\textsuperscript{low}, 7-AAD\textsuperscript{–} populations were categorized as live cells; HO342\textsuperscript{intermediate}, 7-AAD\textsuperscript{–} populations were categorized as apoptotic, and HO342\textsuperscript{high}, 7-AAD\textsuperscript{–} cells were categorized as dead. The method does not permit discrimination between cells that died as a result of apoptosis and those that died as the result of damage and necrosis.

The results of the four-color flow cytometric analysis of DP- and DR-BB intrahepatic T cells are shown in the lower panels of Figure 5. The TCR\textsuperscript{low}B220\textsuperscript{–} intrahepatic T cell subset in both DR-BB and DP-BB rats (R2 gate, red) exhibited predominantly the living cell phenotype (HO342\textsuperscript{low}, 7-AAD\textsuperscript{–}). The TCR\textsuperscript{low}B220\textsuperscript{–} intrahepatic T cell subset in both the DR-BB and DP-BB rats (R3 gate, blue) exhibited predominantly the apoptotic (HO342\textsuperscript{intermediate}, 7-AAD\textsuperscript{–}) and dead cell phenotypes (HO342\textsuperscript{high}, 7-AAD\textsuperscript{–}).

In the case of the DR-BB rat, the data in Table III had demonstrated that the majority (\( \sim 82\% \)) of intrahepatic DR-BB T cells exhibited a TCR\textsuperscript{high}B220\textsuperscript{low} phenotype, whereas most (\( \sim 81\% \)) intrahepatic DP-BB T cells exhibited a TCR\textsuperscript{low}B220\textsuperscript{–} phenotype. The results shown in lower panels of Figure 5 recapitulate these proportions. The majority of intrahepatic DR-BB T cells were live cells whereas most DP-BB intrahepatic T cells were either undergoing apoptosis or dead.

**Discussion**

The present experiments have generated two main findings. 1) As in the mouse, a significant fraction of peripheral T cells in a phenotypically normal rat undergo apoptosis in the liver. 2) This apoptotic process is greatly amplified in the lymphopenic, autoimmune DP-BB rat. The latter finding suggests possible mechanisms by which the lyp gene leads to lymphopenia in these animals and links abnormalities in the regulation of apoptosis to the generation of autoimmunity.

**Apoptosis of intrahepatic T cells in normal rats and mice**

Our first finding is based on studies of the intrahepatic T cell population in DR-BB rats. Although this rat strain is known to possess a genetic predisposition to the generation of autoreactive T cells (2, 3), they are phenotypically normal. In the absence of experimental manipulation they never develop spontaneous autoimmune disease (2, 3). As expected, very few peripheral T cells in the normal DR-BB rat expressed an apoptotic phenotype. In contrast, we observed that \( \sim 20\% \) of the total intrahepatic T cell population in these animals expressed an αβTCR\textsuperscript{low}B220\textsuperscript{–}CD4\textsuperscript{+}CD8\textsuperscript{–} phenotype and were undergoing apoptosis. This phenotype is similar to that of apoptotic intrahepatic T lymphocytes in the mouse (20).

The present studies extend previous observations made in the mouse by identifying the phenotype of the intrahepatic apoptotic T cell population in the rat as Thy1\textsuperscript{+}RT6\textsuperscript{–}. Previous reports have provided strong evidence that rat T cells expressing this phenotype are predominantly immature recent thymic emigrants (19, 28, 29). Our inference that the Thy1\textsuperscript{+}RT6\textsuperscript{–} intrahepatic T cell population undergoing apoptosis in the rat is comprised of recent thymic emigrants was confirmed in a preliminary study that showed that, 24 h after an intrathymic injection of FITC, \( \sim 10\% \) of TCR\textsuperscript{low} intrahepatic T cells undergoing apoptosis in the rat were FITC\textsuperscript{+} (D. L. Greiner, unpublished observations). Both CD4\textsuperscript{+}αβTCR\textsuperscript{+} and CD8\textsuperscript{+}αβTCR\textsuperscript{+} recent thymic emigrants in the rat express an immature Thy1\textsuperscript{+}RT6\textsuperscript{–} phenotype (18, 19). Between 3 to 7 days of their release from the thymus, the majority of these cells become Thy1\textsuperscript{–}RT6\textsuperscript{+}, the phenotype associated with mature peripheral T cells in the rat (12, 17, 18). More than 90% of the intrahepatic

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**Table I. Phenotype of lymph node T cells in DR- and DP-BB rats**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>DR-BB</th>
<th>DP-BB</th>
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<tbody>
<tr>
<td>TCR\textsuperscript{low}</td>
<td>5.6 ± 1.7</td>
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<tr>
<td>CD4\textsuperscript{low}</td>
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<td>CD8\textsuperscript{low}</td>
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</tr>
<tr>
<td>B220\textsuperscript{+}</td>
<td>4.0 ± 1.9</td>
<td>28.0 ± 6.5</td>
</tr>
<tr>
<td>HSA\textsuperscript{+}</td>
<td>2.6 ± 0.9</td>
<td>23.9 ± 4.8</td>
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</tbody>
</table>

* Lymph node cells were isolated from DR-BB and DP-BB rats 3 to 5 wk of age and reacted with the R7.3 anti-TCR and the second mAb as indicated. Cells from each rat were individually analyzed by two- or three-color flow cytometry. Data are presented as means ± 1 SD of analysis of three to seven individual animals. *Each comparison between the DR-BB and DP-BB rat is statistically significant (p < 0.05).
FIGURE 3. Flow cytometric profiles of total Thy1+ lymph node T cell populations. Lymph node cells were obtained from DR-BB/Wor (left column) and DP-BB/Wor (right column) rats 3 to 5 wk of age, reacted with the reagents indicated, and processed for flow cytometry. Each profile was determined in three to four separate experiments. Shown are representative three-color flow cytometric profiles of surface expression of CD4, CD8, B220, and HSA Ag plotted against αβTCR Ag expression on total Thy1+ T cells. Horizontal and vertical markers define positive and negative cell populations (B220, HSA) or strongly positive (hi) and weakly positive (lo) cell populations (CD4, CD8). The percentage of cells localized to each quadrant is indicated.
apoptotic T cell population in the normal DR-BB rat expressed the phenotype of recent thymic emigrants.

Our data do not permit us to identify the coreceptor that was expressed on any given apoptotic intrahepatic T cell. Both CD4 and CD8, in addition to βTCR, are down-regulated in cells that up-regulate B220 and undergo apoptosis (20). Whether the CD4 \(^2\)CD8 \(^2\) cells being destroyed in the rat liver originally expressed CD4 or CD8 cannot be determined.

In the mouse, it was initially hypothesized that apoptotic peripheral T cells were taken up and rapidly eliminated by local tissue macrophages (30). This hypothesis predicted that apoptotic cells should not be readily detectable unless the salvage system was saturated. This prediction was tested in the mouse and found to be valid in the case of apoptotic peripheral CD4 \(^+\) T cells. After the administration of superantigens, which leads to extensive, rapid depletion of peripheral CD4 \(^+\) T cells, apoptotic cells become readily detectable among freshly isolated spleen cells and in situ in peripheral lymphoid tissues (23, 31, 32).

In contrast, apoptosis of peripheral mouse CD8 \(^+\) T cells has not been observed either in situ in the spleen or in freshly isolated spleen cells (21). To account for the behavior of the CD8 \(^+\) T cells, a second mechanism for elimination of apoptotic peripheral T cells was recently proposed. It was hypothesized that apoptotic peripheral CD8 \(^+\) T cells home to nonlymphoid tissues such as the liver for elimination (20–22). This prediction was tested and confirmed using MHC class I-restricted anti-SV40 large T Ag TCR-transgenic mice (21). Peptide-induced peripheral deletion of most peripheral CD8 \(^+\) TCR-transgenic T cells resulted in their rapid disappearance from the lymph node and spleen and their accumulation in the liver. This mechanism appears not to apply to CD4 \(^+\) T cells (W.Z. Mehal and I.N. Crispe, unpublished observations).

Based on these results, we interpret our results as demonstrating that the majority of rat intrahepatic T cells undergoing apoptosis are naive, immature peripheral T cells. As a corollary, we would also predict that a subset of the CD8 \(^+\) cells undergoing intrahepatic apoptosis in the mouse may be recent thymic emigrants. The liver, in this view, would function as a site of activation-induced cell death (as shown in the mouse) and programmed cell death (as shown in the present study) of newly released naive T cells.

Massive apoptosis of intrahepatic lymphocytes in the DP-BB rat

In normal DR-BB rats, 4 to 6% of peripheral T cells, but ~20% of intrahepatic T cells, expressed an apoptotic phenotype. In contrast, in the DP-BB rat an αβTCR \(^{low}\)/B220 \(^{low}\)/CD4 \(^+\)/CD8 \(^+\) phenotype was observed in ~25% of peripheral T cells and ~80% of intrahepatic T cells. In addition, although the apoptotic phenotype

Table II. Percentage of fresh and cultured BB rat lymphoid cells with evidence of DNA fragmentation

<table>
<thead>
<tr>
<th>Duration of Culture (h)</th>
<th>Thymocytes</th>
<th>Splenic T Cells</th>
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<tr>
<td></td>
<td>DR-BB</td>
<td>DP-BB</td>
</tr>
<tr>
<td>0</td>
<td>1.3 ± 0.7</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>5.3 ± 4.1</td>
<td>4.9 ± 3.2</td>
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<td>20.2</td>
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<tr>
<td>24</td>
<td>32.8 ± 8.1</td>
<td>33.2 ± 8.1</td>
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</table>

*Thymocytes and spleen cells were obtained from DR-BB and DP-BB rats three to four animals per group) 3 to 5 wk of age, pooled, and reacted with the R7.3 anti-TCR mAb and PI either immediately or after various periods of incubation. TCR \(^+\) cells were then analyzed by flow cytometry for determination of DNA content. Each data point represents the mean ± SD of three to four separate experiments, except for 14-h time point, which represents a single experiment. *p < 0.05 vs DR-BB spleen cells. No other paired comparisons are statistically significant.

FIGURE 4. DNA content of BB rat spleen cells. Spleen cells were obtained from DR-BB (left column) and DP-BB (right column) rats 3 to 5 wk of age and reacted with the R7.3 anti-TCR mAb and PI either immediately (upper row) or after incubation for 24 h (lower row). TCR \(^+\) cells were then analyzed by flow cytometry for determination of DNA content. Each analysis was performed in three or four separate experiments. “M1” indicates the “subG1” peak indicative of the presence of DNA fragmentation and apoptosis.

In normal DR-BB rats, 4 to 6% of peripheral T cells, but ~20% of intrahepatic T cells, expressed an apoptotic phenotype. In contrast, in the DP-BB rat an αβTCR \(^{low}\)/B220 \(^{low}\)/CD4 \(^+\)/CD8 \(^+\) phenotype was observed in ~25% of peripheral T cells and ~80% of intrahepatic T cells. In addition, although the apoptotic phenotype...
TCR low B220-1 was present in both the periphery and in the liver, apoptotic cell death (evidenced by the presence of a "subG1" peak indicative of the DNA fragmentation present in cells that are in the final stages of apoptotic cell death) could be documented only in freshly isolated intrahepatic T cells. We interpret our findings as evidence that the process of T cell apoptosis is greatly amplified in the lymphopenic, autoimmune DP-BB rat where it is underway in the periphery and completed in the liver.

The present data derived from the DR-BB rat suggest that intrahepatic T cells undergoing apoptosis are both immature (Thy1+ RT6+) and activated. In the DP-BB rat, 30 to 50% of peripheral T cells are known to express an immature phenotype (12, 17, 33). In addition, ~15% of Thy1+ peripheral T cells in BB rats are in the S/G2/M phase of cell cycle, indicative of an activated phenotype and proliferation (17). The presence of such cells in abundance in the periphery is consistent with the large number of such cells observed to be undergoing apoptosis in the liver in the present study.

It has been argued that only mature or effector T cells are capable of homing to nonlymphoid tissues (12), but our observation that most apoptotic intrahepatic T cells in DP-BB rats are Thy1+ immature T cells suggests that there are exceptions to that rule. The present data do not permit us to determine whether the increase in intrahepatic T cell apoptosis in the DP-BB rat is the consequence of premature death of recent thymic emigrants or the result of an abnormal intrahepatic process that is eliminating such cells. Previous analyses of irradiated BB rats reconstituted with normal rat bone marrow have, however, demonstrated normal donor T cell development in the chimeric animal (34). This observation would argue against an intrinsic abnormality of the DP-BB rat liver leading to T cell elimination.

As was the case with the DR-BB rat, our data do not permit us to determine whether the CD4+ CD8- intrahepatic T cells undergoing apoptosis were derived from CD4+ or CD8- peripheral T cells. As in the mouse, DP-BB rat CD4+ T cells (which are the

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**Table III. Percentage of Intrahepatic BB rat T cells with the TCR<sup>low</sup> B220<sup>low</sup> apoptotic phenotype**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Total Intrahepatic Lymphocytes</th>
<th>Total Intrahepatic T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DR-B</td>
<td>DP-B</td>
</tr>
<tr>
<td>TCR&lt;sup&gt;low&lt;/sup&gt;B220&lt;sup&gt;low&lt;/sup&gt;</td>
<td>27.3 ± 2.9</td>
<td>3.7 ± 0.9*</td>
</tr>
<tr>
<td>TCR&lt;sup&gt;low&lt;/sup&gt;B220&lt;sup&gt;low&lt;/sup&gt;</td>
<td>5.9 ± 3.1</td>
<td>15.8 ± 3.2†</td>
</tr>
</tbody>
</table>

*Intrahepatic lymphocytes were isolated from DR-BB (N = 3) and DP-BB (N = 3) rats 3 to 5 wk of age and reacted with the R7.3 anti-TCR and HIS 24 anti-B220 mAbs; cells from each rat were individually analyzed by dual channel flow cytometry. The percentage of B220<sup>-</sup> and B220<sup>+</sup> intrahepatic T cells was calculated from the total percentage of intrahepatic lymphocytes that were either TcR<sup>low</sup> or TcR<sup>low</sup>. Data are presented as means ± 1 SD of analysis of six individual animals. *p < 0.02 vs. DR, †p < 0.005 vs DR.
predominant peripheral T cell subset in these animals) may be eliminated in the periphery by resident macrophages (23, 31, 32) whereas DP-BB CD8^+ T cells may be eliminated in the liver (21). It will be important to determine whether the intrahepatic T cells undergoing apoptosis in the DP-BB rat include both immature CD4^+ and immature CD8^+ T cells.

It is known that severe lymphopenia in the DP-BB rat (9, 10, 12, 17) results from homozygosity for a gene of unknown function designated lyp (13). The high frequency of T cells undergoing apoptosis in the livers of these animals suggests that intrahepatic apoptosis contributes to the lymphopenia and may be a direct consequence of lyp. How the lyp gene might lead to the amplification of intrahepatic T cell apoptosis in the rat is not known, but interference with positive selection is an attractive hypothesis.

It is clear that the immature T cells in DP-BB peripheral tissues have undergone positive selection, a process involving TCR signaling, because the RAG-1 and RAG-2 transcripts are down-regulated in these cells (35). We hypothesize that the lyp gene may induce abnormal TCR signaling during positive selection in the thymus, resulting in a chronic state of T cell activation, apoptosis, and a deficiency of single positive thymocytes. Consistent with this hypothesis, intrathymic maturation of CD8^+ T cells is decreased (9, 10, 12), thymocyte export is reduced (17), and the thymic emigrants that do reach the periphery are abnormal. Most of the peripheral T cells are proliferating, activated, immature cells that fail to develop into mature Thy1^+ T cells; the few Thy1^+ T cells that do develop appear normal (17).

Apoptosis and autoimmunity in the DP-BB rat
The DP-BB rat is both susceptible to autoimmune diabetes and severely lymphopenic (2, 3). The majority of its T cells are short-lived and disappear from the peripheral lymphoid tissues soon after their release from the thymus (12, 17, 18). These observations lead to an intriguing question as to the identity and life span of the autoreactive T cell population that mediates the autoimmune diabetes.

It has been demonstrated that a population of CD4^+ peripheral T cells in the DP-BB rat resists apoptosis in the presence of a superantigen (36). It has also been observed that the induction of insulin-dependent diabetes mellitus in both BB rats (37–39) and nonobese diabetic (NOD) mice (40–43) appears to depend on the presence of both CD4^+ and CD8^+ T cell populations. In the DP-BB rat, a minor population of CD8^+ T cells has been detected and hypothesized to include the autoreactive effector cell (10, 12). The present data suggest that a small subset of either CD8^+ T cells, CD4^+ T cells, or both must be resistant to the apoptotic effects of the lyp gene and that these apoptosis-resistant T cells are likely to be the mediators of DP-BB rat diabetes. Understanding the basis for the resistance of these minor populations of T cells to lyp-induced apoptosis should provide insight into the biology of the autoreactive effector T cell population active in autoimmune diabetes.

**FIGURE 6.** Cell size determination. Intrahepatic lymphocytes were obtained from DR-BB/Wor (left panels) and DP-BB/Wor (right panels) rats 3 to 5 wk of age, reacted with anti-αβ TCR, and analyzed for cell size by flow cytometry. Shown are representative flow cytometric profiles of the distribution of TCR^hi and TCR^lo T cell populations according to their degree of forward light scatter. Comparable results were obtained in three individual animals (Table IV).
FIGURE 7. Flow cytometric profiles of TCR$^{\text{hi}}$ intrahepatic lymphocytes. Intrahepatic lymphocytes were obtained from DR-BB/Wor (left panels) and DP-BB/Wor (right panels) rats 3 to 5 wk of age and reacted with an mAb directed against the TCR and a second mAb directed against the surface Ags indicated on the left. Cells were analyzed by flow microfluorometry and gated to display the fluorescence profile of only the TCR$^{\text{hi}}$ T lymphocyte population. Shown are representative histograms depicting fluorescence intensity plotted against cell number for each of the five cell surface Ags indicated. The insets show fluorescence profiles of the same TCR$^{\text{hi}}$ T cell populations reacted with FITC- or PE-conjugated isotype control Ig. Comparable results were obtained in three individual animals (Table IV).
FIGURE 8. Flow cytometric profiles of B220+ intrahepatic T cell populations. Intrahepatic lymphocytes were obtained from DR-BB/Wor (left panels) and DP-BB/Wor (right panels) rats 3 to 5 wk of age and reacted with an mAb directed against B220 and a second mAb directed against the surface Ags indicated on the left. Cells were analyzed by flow microfluorometry and gated to display the fluorescence profile of only the B220+ T lymphocyte population. Shown are representative histograms depicting fluorescence intensity plotted against cell number for each of the five cell surface Ags indicated. The insets show fluorescence profiles of the same B220+ T cell populations reacted with FITC- or PE-conjugated isotype control Ig. Comparable results were obtained in three individual animals (Table IV).
Table IV. Phenotype of intrahepatic T cells in DR- and DP-BB rats

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>DR-BB</th>
<th>DP-BB</th>
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<tbody>
<tr>
<td>CD4⁺</td>
<td>66.5 ± 0.2</td>
<td>63.7 ± 1.3</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>15.2 ± 6.1*</td>
<td>21.6 ± 1.8*</td>
</tr>
<tr>
<td>Thy1⁺</td>
<td>28.6 ± 3.0</td>
<td>14.8 ± 4.6</td>
</tr>
<tr>
<td>RT6⁺</td>
<td>45.2 ± 3.4</td>
<td>76.5 ± 4.4</td>
</tr>
<tr>
<td>CD25⁺</td>
<td>60.1 ± 7.5</td>
<td>18.4 ± 2.6</td>
</tr>
<tr>
<td>CD4⁻</td>
<td>10.1 ± 1.8</td>
<td>20.8 ± 3.3</td>
</tr>
<tr>
<td>CD8⁻</td>
<td>21.2 ± 1.2²</td>
<td>3.3 ± 0.3*</td>
</tr>
</tbody>
</table>

* Intrahepatic lymphocytes were isolated from 3 to 5 wk-old DR- (n = 3) and DP-BB/Wor (n = 3) rats as described in Materials and Methods. Results represent the mean ± SD of percentage of positive cells of three individual rats as determined by flow cytometry. p < 0.02 vs TCR⁺⁺⁺B220⁻ ; p = NS vs TCR⁺⁺⁺B220⁻.

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References


