T-Lymphopenia and T-Cell Imbalance in Diabetic *db/db* Mice

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SUMMARY

The diabetic *db/db* mice of the C57 BL/KsJ strain display anti-islet immunity, thymic dysfunction, and lymphopenia. In the present work, lymphocytes, T-cells, and T-cell subsets were enumerated in thymus and spleen from diabetic *db/db* mice and their *db/+* heterozygote littermates from the 10th day to the 10th month of life. A significant lymphopenia was detected in thymus and spleen from the second month on, involving specifically the T-cell compartment, as assessed by use of a monoclonal anti-Thy1 antibody in indirect fluorescence. The study of T-cell subsets by monoclonal anti-Lyt1 and anti-Lyt2 antibodies revealed a significant increase in Lyt1$^+$ cells and a decrease in Lyt2$^+$ cells, with a corresponding increase of the Lyt1$^+$/Lyt2$^+$ ratio. These anomalies appeared early in life, and were apparently linked neither with the degree of hyperglycemia nor with weight loss or infection. The T-cell depletion in thymus was more pronounced in young male (<3 mo) than in young female *db/db* mice. These alterations may correspond to an increase in the helper/suppressor-cytotoxic ratio and could be linked with the thymic anomalies present in these mice, contributing to the development of anti-islet autoimmunity. DIABETES 1986; 35:198–203.

Several recent studies suggest that the immune system plays a significant role in the pathogenesis of type I (insulin-dependent) diabetes mellitus and some related animal models.[1–3] The thymus-dependent lymphocytes could be of decisive importance for the development of anti-islet immunity.[4,5] A T-lymphocyte imbalance, associated with a decrease in T-cell suppressor function, could contribute to the anti-islet immunity[2] in a way similar to that described in some other autoimmune diseases.[6–8] However, conflicting results have been obtained regarding the T-suppressor function[2,9–12] as well as the number of circulating T-lymphocytes and their subsets.[13–21] This is due to a variety of reasons. Surface markers and lymphocyte function are not constantly superimposable.[22] Demonstration of a deficient suppressor function in vitro is not easy experimentally[2,7] and a defect in suppressive function specifically directed to islet antigen(s) remains to be demonstrated. Furthermore, while a thymic microenvironment is necessary for the differentiation of T-cells,[8] poor metabolic control of diabetes can influence the lymphocyte functions and the presence of immune anomalies.[23]

The *db/db* mouse appears a privileged model with regard to the above considerations, since it associates (1) anti-islet immunity[24,25] associated with a progressive loss of beta cells[26] and the presence of immunoglobulin deposits[27] and macrophages[28] in the islets; (2) a thymic dysfunction with decreased levels of thymic hormone (FTS) in plasma and decreased numbers of FTS-positive cells in the thymus;[29] (3) a T-cell lymphopenia in peripheral blood;[30] and (4) diabetes mellitus, with two successive metabolic patterns: initial obesity, hyperinsulinemia, and insulin resistance followed by insulinopenia associated with marked hyperglycemia. It was therefore of interest to evaluate the time course of lymphocytes, T-cells, and their subsets, in the thymus and spleen of these mice, during the two successive hyperinsulinemic and hypoinsulinemic phases of their diabetic disease.

MATERIALS AND METHODS

Characterization of *db/db* mice. C57 BL/KsJ mice of the *db/db* and *db/+* strains, obtained from the C.E.S.A.L. (C.N.R.S., Orléans, France), were bred in the laboratory and followed up, weekly, for body weight, blood glucose, and plasma insulin until the 10th month of life. Because of the sterility of homozygous *db/db* mice, heterozygous *db/+* mice were used, providing litters including *db/db*, *db/+*, and *+/+* mice. Distinguishing *db/db* from other strains was easy from the first month on, using body weight, blood glucose level, and fur color for the *+/+* (misty) mice. Before the 30th day of life, insulinemia, which is precociously ele-

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vated, was the first biochemically detectable marker of *db/db* genotype.[31] The number of *db/db* and *db/+* mice tested was 12–26 in each group from the first month on. Four to six mice were studied for the earliest stages tested, i.e., 10th, 15th, and 21st days. All mice were fed ad libitum. Insulin treatment was never given. Overt skin infections were noted in most *db/db* homozygote mice and in 50% of heterozygote controls from the 10th month of life.

Total cell counts. Mice were anesthetized with ether and killed. Thymus and spleen were rapidly dissected and put into chilled Hanks' medium (Eurobio, Paris, France). Cell suspensions were prepared from each individual organ by gentle disruption in a Potter-Elvehjen grinder in the presence of 5 ml medium. Homogenates were individually filtered through a nylon grid and centrifuged (400 × *g*, for 10 min at 4°C). Supernatants were discarded and cells were resuspended in 5 ml chilled RPMI medium (RPMI 1640, Flobio, Courbevoie, France) containing 2% (vol/vol) molar Hepes buffer (Flobio), 0.2% (wt/vol) sodium azide, and 4% (vol/vol) fetal calf serum. Cells were then counted in Malassez hemocytometers (Polylabo, Strasbourg, France).

T-cell counts. The cell suspensions were adjusted to 10^7 cells/ml in RPMI medium modified as described above. Ten microliters of anti-Thy1 monoclonal antibody (NEI 0.01, New England Nuclear, Boston, Massachusetts) were added to 0.1 ml cell suspension. After a 40-min incubation at 4°C, 20 μl of fluorescein-conjugated goat anti-mouse antiserum (GAM/Fab/FITC, Nordic, Tilburg, The Netherlands) diluted 1:15 was added for a further 45-min incubation at 4°C. Suspensions were then rinsed twice in RPMI medium. Fluorescent cells were enumerated with a Leitz Orthoplan microscope equipped for epiluminescence (Wild Leitz, Heerbrugg, Switzerland). Membrane immunoglobulin-bearing cells were counted on identical samples, after incubation with GAM/Fab/FITC alone, and the number of Thy1+ cells was deduced by subtracting Ig+ cells from the total number of cells labeled with the anti-Thy1 antibody.

T-lymphocyte subset evaluation. The T-lymphocyte subsets bearing the Lyt1 and Lyt2 antigens were determined using an immunofluorescence technique.[32] Double labeling with two different fluorochromes (fluorescein and rhodamine) enabled the direct determination of the three major subsets: $Lyt1^+2^-$, $Lyt1^-2^+$, and $Lyt1^+2^+$. Monoclonal antibodies were purchased from Becton Dickinson (Sunnyvale, California). The anti-Lyt1 monoclonal was conjugated to fluorescein, the anti-Lyt2 to biotin, which was revealed by avidin coupled to rhodamine. Both anti-Lyt1 and anti-Lyt2 were of the IgG 2a class.

To thymus and spleen cells (10^6 cells in 100 μl, prepared as described above) were added 5 μl (5 μg antibody) of the anti-Lyt1-fluorescein conjugate and incubated at 4°C for 30 min. Cells were washed and labeled for 30 min with 2.5 μl (2.5 μg antibody) of the anti-Lyt2-biotin conjugate. Cells were washed and 1 μl (1 μg protein) of the avidin-rhodamine conjugate was added to the cell pellet for 30 min at 4°C. The cells were then washed twice. The $Lyt1^+$, $Lyt2^+$, and doubly labeled $Lyt1^+2^+$ cells were enumerated with a Leitz Orthoplan microscope. The three different Lyt lymphocyte subsets were counted on the whole cell population, and results are expressed as the percentage of each subset within the total Lyt population.

Characterization of macrophages and B-lymphocytes. Macrophages were characterized by latex incorporation. Fifty microliters of 2% (vol/vol) latex bead suspension (Sigma, St. Louis, Missouri; diameter: 1091 nm) was added to 10^6 cells in 0.1 ml medium complemented with 0.05 ml fetal calf serum. After 60 min of incubation at 37°C, cells were washed with modified RPMI, then anti-Thy1 and GAM were added as described above. Cells containing three or more latex beads were considered as macrophages. To evaluate B-cells, cell suspensions were incubated at 37°C for 40 min to exclude macrophages. B-lymphocytes were then identified by incubating the cell suspensions (10^6 cells) for 45 min with 20 μl of a 1/20 dilution of fluorescein-conjugated rabbit anti-mouse (RAM) immunoglobulin-F (ab')2 fragment (Zymed, San Francisco, California).

Expression of results. Results are presented as mean values ± SEM in each group. Statistical significance of differences was evaluated by Student's *t*-test for unpaired values. Since a sex difference was recently noted with regard to the phenotypic expression of anti-islet antibodies in *db/db* mice,[33] results were also analyzed separately for each sex subgroup.

RESULTS

CHARACTERIZATION OF *db/db* MICE

The respective courses of body weight, plasma glucose, and insulin levels were within the range of previously published values.[24,25,31] In brief, the *db/db* mice displayed a significant hyperinsulinemia from the 10th day of life onward: 52 ± 5 μU/ml (versus 27 ± 2 μU/ml in controls, $P < 0.01$), and a significant hyperglycemia from the 30th day: 18 ± 2 mmol/L (versus 7 ± 2 mmol/L in controls, $P < 0.01$). After the fifth month, dramatic changes occurred in blood glucose levels, which rose to a mean of 33 ± 3 mmol/L and plasma insulin concentrations, which dropped from 850 ± 50 (fifth month) to a mean of 100 ± 10 μU/ml (10th month).

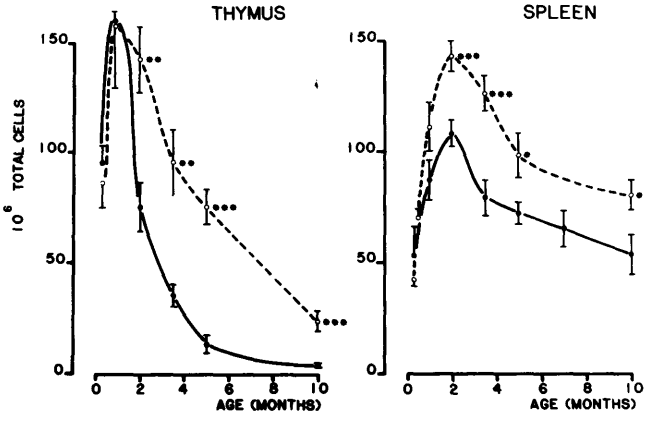

FIGURE 1. Time course of cell counts in thymus (left panel) and spleen (right panel) from *db/db* (●—●) and *db/+* (○–○) mice. Results are presented as mean values ± SEM. Number of animals was 12–26 in each test group, except for the 10–21-day-old mice, where it was 4–6. Statistical significance of differences between *db/db* and *db/+* at same time is denoted by: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

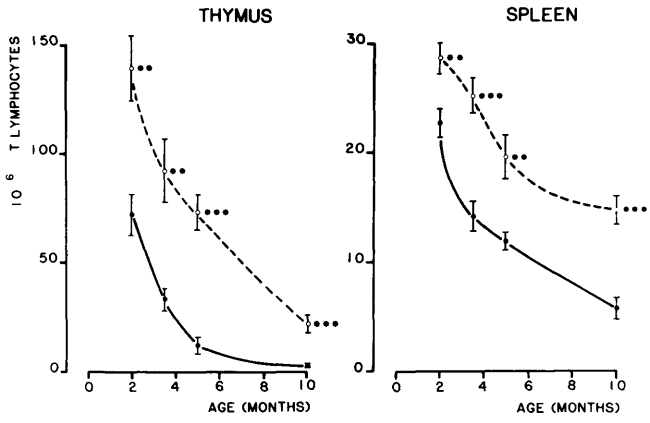

FIGURE 2. Time course of T-lymphocyte counts in thymus (left panel) and spleen (right panel) from *db/db* (●—●) and *db/+* (○--○) mice. Number of animals was 6–12 in each test group. Same presentation as in Figure 1. Note the different scales for thymus and spleen T-cell numbers.

TOTAL CELL COUNTS (FIGURE 1)

Thymus. The cells counts increased sharply in *db/+* mouse thymus from the 10th day of life to the 30th day (Figure 1, left panel) and declined later on.

In the *db/db* mice, values comparable to the controls were obtained on the 10th and 30th days. Conversely, from the 60th day on, all values were significantly lower in *db/db* thymuses than in those from *db/+* controls ($P < 0.01$).

Spleen. The time course of spleen cells (Figure 1, right panel) was marked in both strains by a sharp increase in number from the 10th to the 60th day of life followed, later, by a progressive decrease. The values in *db/db* mice were not significantly lower than in *db/+* controls, neither on the 10th nor the 30th days; they only became significantly lower in *db/db* mice from the 60th day onward.

T-CELL COUNTS (FIGURE 2)

Thymus. Most lymphocytes in the thymus are T-cells (Figure 2, left panel). In the *db/+* strain, the T-cell counts declined from the second month onward being $140 \pm 15 \times 10^6$ cells at 2 mo and $20 \pm 2 \times 10^6$ cells at 10 mo. In the *db/db* mouse thymus, a similar decrease occurred with age, all values being lower than those of the corresponding *db/+* controls: $70 \pm 11 \times 10^6$ cells at 2 mo and $2.0 \pm 0.1 \times 10^6$ cells at 10 mo. Interestingly, the percentage of non-T-cells in the thymus increased in *db/db* mice from 4% at 2 mo to 19% at 10 mo, while it remained relatively constant in *db/+* mice: 2% at 2 mo and 3% at 10 mo.

Spleen. In the *db/+* mice, the total number of T-cells declined from $28 \pm 2 \times 10^6$ cells at 2 mo to $15 \pm 2 \times 10^6$ cells at 10 mo. The T-cell counts were significantly lower in *db/db* than in *db/+* mice for all time samples, being $23 \pm 2 \times 10^6$ cells at 2 mo and $5 \pm 1 \times 10^6$ cells at 10 mo (Figure 2, right panel). Furthermore, in the *db/+* mice the percentage of T-cells among total spleen lymphocytes did not vary significantly, being 20% at 2 mo and 18.5% at 10 mo. In the *db/db* mice, by contrast, this percentage declined from 21% at 2 mo to 11% at 10 mo.

T-CELL SUBSETS

The T-cell subsets were enumerated in mice aged 3 and 5 mo (Tables 1 and 2, respectively). Two major differences appeared between the *db/db* and *db/+* mice: (1) The percentage of $Lyt1^+$ cells was higher in the *db/db* mice than in the *db/+* mice and that of $Lyt2^+$ cells was lower, so that the $Lyt1^+/Lyt2^+$ ratio was increased; these differences were more pronounced in 5-mo-old than in 3-mo-old mice and were also more clearly detectable in the spleen than in the thymus. (2) The percentage of $Lyt1^+2^+$ cells was decreased in the *db/db* mice. This pattern is particularly illustrated by Table 2.

MACROPHAGES AND B-LYMPHOCYTES

As noted previously, the percentage of cells not stained by the anti-Thy1 serum was initially higher in the *db/db* mouse thymus than in control mice and increased with age, up to 19% at 10 mo. A mean of 14% of mononuclear cells phagocytized three or more latex beads (versus 3% in *db/+* preparations) and were considered macrophages. A further 5% were identified as B-lymphocytes by the anti-F (ab')2 RAM fluorescein-conjugated serum. No B-lymphocytes were detected in the *db/+* preparation, and no germinal centers were observed in thymic histologic sections of either *db/db* or *db/+* mice.

SEX DIFFERENCES IN T-CELL NUMBERS

Because of differences in phenotype expression of anti-islet immunity (as developed hereafter), the results were analyzed separately according to sex. Interestingly, the T-cell depletion in the thymus was significantly more pronounced in the male than in the female *db/db* mice at 2 mo: $37 \pm 6 \times 10^6$ versus $92 \pm 23 \times 10^6$ cells ($P < 0.01$). This difference became insignificant at 3.5 mo ($25 \pm 8 \times 10^6$ versus

TABLE 1
T-cell subsets and helper-suppressor cytotoxic ratio in 3-mo-old *db/db* and *db/+* mice

		% $Lyt1^+$ cells	Percent $Lyt2^+$ cells	$Lyt1^+/Lyt2^+$ ratio	Percent $Lyt1^+2^+$ cells
Spleen	*db/db*	46.1 ± 1.8 †	10.2 ± 0.4	4.5 ± 0.1 *	43.7 ± 2.3 †
	db/+	36.4 ± 1.3	9.8 ± 0.4	3.7 ± 0.2	53.8 ± 1.4
Thymus	*db/db*	14.3 ± 3.3	4.6 ± 1.7	4.7 ± 1.1	81.1 ± 4.8
	db/+	7.5 ± 0.3	1.9 ± 0.3	4.1 ± 0.7	90.5 ± 0.3

Results are presented as mean values ± SEM.
Number of animals tested in each group was three; 500 cells were read in each instance.
Statistical significance of differences between the corresponding groups is denoted by: *$P < 0.05$ and †$P < 0.02$.

TABLE 2
T-cell subsets and helper-suppressor cytotoxic ratio in 5-mo-old *db/db* and *db/+* mice

		% Lyt1$^+$ cells	Percent Lyt2$^+$ cells	Lyt1$^+$/Lyt2$^+$ ratio	Percent Lyt1$^+$2$^+$ cells
Spleen	*db/db*	56.1 ± 3.4 ‡	6.0 ± 0.8 †	10.3 ± 2.2 †	37.9 ± 2.8
	db/+	36.7 ± 3.1	13.1 ± 2.1	3.0 ± 0.5	50.1 ± 4.3
Thymus	*db/db*	15.9 ± 2.4 *	4.8 ± 1.4	4.5 ± 1.6	79.4 ± 3.7
	db/+	7.0 ± 1.7	3.8 ± 1.4	2.1 ± 0.3	89.2 ± 3.0

Results are presented as mean values ± SEM.
Number of animals tested in each group was four; 500 cells were read in each instance.
Statistical significance of differences between the corresponding groups is denoted by: *$P < 0.05$, †$P < 0.02$, and ‡$P < 0.01$.

34 ± 8 × 10^6 cells) and disappeared at 10 mo. No difference was detectable in the *db/+* mice and no sex difference appeared in spleen T-lymphocyte numbers.

DISCUSSION

Results presented above show that *db/db* mice display a significant T-cell lymphopenia from the second month of life onward associated with an imbalance of the T-cell subset phenotype manifested by an increase in Lyt1$^+$/Lyt2$^+$ ratio. An excessive number of macrophages was noted in the thymus of the eldest *db/db* mice, with neither B-lymphocyte excess nor the appearance of germinal centers. This sequential and comparative study confirms and expands previous observations. The weight of thymus and spleen is reduced in 14-wk-old mice[30,34] and lymphopenia, affecting particularly the T-cells, has been detected in thymus and spleen.[30] DNA synthesis is decreased in the thymus, but not in the spleen.[34] These observations in a strain with anti-islet immunity and diabetes raise two questions: (1) are the lymphocyte modifications primary or secondary to the diabetic state and (2) can they contribute to the anti-islet autoimmunity?

The anti-islet cellular and humoral immunities in *db/db* mice were, until recently,[24,25] unrecognized because of the initial obesity and hyperinsulinemia and the absence of typical insulitis.[26] These immunities are supported, however, by recent converging observations. Immunoglobulin deposits are present in *db/db* mice islet cells,[27] and antibodies reacting with normal mouse islets have been detected by indirect fluorescence in 83% of male and 22% of female *db/db* mice.[33] Sera from 17% of these mice also contain antibodies to gastric cells.[33] Murine hybridoma cells prepared with *db/db* splenocytes secrete anti-islet antibodies, and these cells, when injected into normal mice, are specifically trapped by the pancreas.[35] Bone marrow transplantation from nondiabetic, H^2-compatible normal mice to *db/db* mice induce a decrease in the recipient blood glucose levels;[36] as well, macrophages infiltrate the *db/db* islets and macrophages from *db/db* mice are toxic to islet cells in vitro.[24] The specific suppression of insulin release from normal islet cells in vitro by *db/db* lymphocytes is a T-cell phenomenon.[25]

Other immune anomalies are present in these mice: impairment of graft rejection,[37,38] depression of mixed lymphocyte reactions and cell-mediated cytotoxicity in response to alloantigens,[39] reduction of the production of interleukin-2 and of the phagocytosis by macrophages,[40] and increased susceptibility to Candida and Coxsackie virus infections.[40] Conversely, antibody synthesis in vitro is not altered.[30]

The early and sustained T-lymphopenia in *db/db* mice is reminiscent of that in NOD mice,[41] BB rats,[42] and diabetic dogs.[43] The increase in the Lyt1$^+$/Lyt2$^+$ ratio is also a reminder of the subset imbalance detected in BB rats[44,45] and in recent-onset diabetic subjects,[13–21] although this imbalance is transient[16,18] and not constantly detected[14,18,19] in human subjects. Inasmuch as phenotype markers correspond to lymphocyte functions,[8,32] this imbalance could reflect an increase in the helper-suppressor cytotoxic ratio. These anomalies in lymphocyte subsets (and/or functions) could be related to the thymic anomalies present in these mice, namely, a marked decrease in the serum thymulin level and a decrease in the number of thymulin-positive cells in the thymus together with the presence, in serum, of an antithymulin autoantibody.[29] These anomalies become significant at about the 30th day of life, then worsen with time. Thymulin controls the expression of T-lymphocyte markers and T-cell functional competence,[46] and the thymic defect could contribute to the lymphopenia and T-cell imbalance described in this study. Treatment of *db/db* mice with thymosin fraction V, which contains thymulin as well as other thymic hormones, restores the immune cell number and function to normal.[47] These data strongly suggest that the T-lymphopenia is linked with the thymic hormone deficiency.

Taken together, these observations are consistent with the attractive hypothesis of a thymic deficiency contributing to an excessive helper-suppressor cytotoxic ratio and the triggering of anti-islet immunity. A decrease in suppressor cell function has indeed been detected in recent-onset diabetic subjects independent of their metabolic control[9–12,14,15] and in long-standing diabetic subjects.[21] Deficient plasma levels of thymic hormone (FTS) were detected in only a minority of juvenile, recent-onset diabetic subjects.[18]

These observations, however, are far from constant. No reduction in rosette-forming lymphocytes has been detected,[13,28,48] and the helper-suppressor ratio has sometimes been found either decreased[20] or minimally altered.[19] Phenotypic markers, as used to define helper and suppressor T-lymphocyte subsets, may not be specific. Borel and Lafferty have demonstrated Lyt1$^+$ cells with suppressor activity and Lyt1$^-$, 2$^+$, 3$^+$ cells with helper function.[22] Furthermore, there is no way of discriminating between cytotoxic and suppressor T-cells among Lyt2$^+$ cells. Functional identification of the T-cell depletion and subsets is therefore necessary.

The lymphopenia and T-cell imbalance could also depend on the metabolic environment. The presence of insulin and its binding to lymphocytes are necessary to the blastogenic reaction.[23,30,49] Appropriate insulin levels could be necessary for the expression of lymphocyte phenotypes and functions. Mice rendered diabetic by alloxan or streptozocin display a marked lymphopenia in thymus and spleen[30,37,50] associated with immunodeficiency;[50–52] this immunodeficiency is not fully corrected by insulin treatment.[50,51] Large doses of streptozocin or alloxan exert prolonged toxic effects on the thymus, spleen, and bone marrow lymphocytes,[50,51] which make these models irrelevant for the analysis of metabolic/immunologic relationships. Poorly controlled human diabetic subjects are, indeed, immunodeficient.[53] Their lymphopenia, when observed, was either unaffected[15,18] or accentuated[54] by poor diabetic control. The phenotype subset imbalance, when observed, was unaffected[15–17] by metabolic control. The activation by phytohemagglutinin in vitro was impaired in lymphocytes from ketoacidotic patients,[55] but the suppressive lymphocyte activity in vitro, when found decreased in recent-onset diabetic subjects, was progressively restored independent of the metabolic status.[2] Interleukin-2 production in vitro by lymphocytes from recent-onset diabetic subjects was reduced[56] or in the upper-normal range.[57]

In *db/db* mice, the regular progression in T-cell lymphopenia and subset imbalance contrasted with the sharply opposite metabolic patterns with, initially, a moderate hyperglycemia with hyperinsulinemia, and then an abrupt insulin decline with extreme hyperglycemia. These patterns do not support the concept of metabolism-dependent changes in lymphocytes. One may argue that the insulin resistance that is present in hyperinsulinic *db/db* mice[26] contributes to impaired immunity as it does in *ob/ob* mice.[31] Overall, however, if the environmental hypothesis can account for some functional impairment of the immune defense in vivo, it can hardly account for the expression of the T-cell phenotypes, the lymphopenia, and the T-cell imbalance observed in *db/db* mice. Additionally, many diabetic syndromes in humans and animals are not associated with any detectable anomaly in lymphocyte counts, despite very abnormal insulin and glucose levels. Involution of the thymus due to stress states in *db/db* mice, which are often infected mice, could be an important issue. No correlation was found between lymphocyte counts and the presence of skin infection or the variation in body weight.

Thus, the present straightforward observations based on marker analysis still await a pathophysiologic explanation. Further studies, including the parallel analysis of markers and functions (particularly the suppressive function in response to pancreatic antigens) and their variations after metabolic control and immunosuppression treatment, are required.

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