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Early and Quantal (by Litter) Expression of Insulin Autoantibodies in the Nonobese Diabetic Mice Predict Early Diabetes Onset¹

Evie Melanitou,^{2,3} Devasenan Devendra, Edwin Liu, Dongmei Miao, and George S. Eisenbarth

Aiming to study the early stages of type 1 diabetes phenotype, before insulinitis appears, we measured insulin autoantibodies (IAA) between 3 and 5 wk of age in the NOD mouse (early-IAA (E-IAA)). We report that IAA are found as early as at 3 wk of age, at weaning, and their expression is a quantal phenotype. Maternal autoantibody status influences this early phenotype, because animals of litters issued from IAA-positive ante partum mothers develop E-IAA with a significantly higher incidence than animals issued from IAA-negative mothers. These E-IAA represent synthesized rather than transplacental autoantibodies, as evidenced by higher levels in many offspring compared with maternal IAA, and negative as well as positive offspring in the same litters and it correlates with early diabetes onset, defining the first autoimmune window in diabetes pathogenesis. Therefore, autoimmune processes leading to type 1 diabetes initiate early in life, are influenced by maternal autoantibody status, and can be revealed by the presence of IAA. Our data suggest that the mechanisms responsible for the breakdown of self-tolerance are subjected not only to genetic predisposition, but also to the physiological status of the mother. Pathological progression to autoimmunity is marked by the presence of immunological windows relating early steps with final disease onset. *The Journal of Immunology*, 2004, 173: 6603–6610.

Low penetrance of genes implicated in autoimmune disorders renders more difficult the study of the early molecular mechanisms involved. This is the case not only in human, but also in animal models. In addition, heterogeneity is characteristic of autoimmune phenotypes and is usually associated with disorders controlled by genetic and environmental factors. This heterogeneity renders difficult the uniform selection of an experimental population, destined to the study of the end disease phenotype.

Type 1 diabetes (T1D)⁴ is an organ-specific autoimmune disease resulting from the self-destruction of the insulin-producing β cells in the islets of Langerhans in the pancreas. It is a heterogeneous disorder where genetic susceptibility and environmental factors play a role. The time to disease onset, up to 30 years in humans and several months in animal models renders difficult studies to identify early disease-related mechanisms. Therefore, establishing an early, diabetes-related subphenotype represents an interest not only

for disease prevention in humans, but also for selecting susceptible individuals before autoimmune destruction takes place in the experimental animal. The nonobese diabetic (NOD) mouse is one of the best-studied animal models for T1D as it spontaneously develops the disease and has similarities with the human disorder. Although NOD mice are inbred, not all develop the disease with an incidence of 40–90% in female mice, depending on the colony (1). With the aim to establish an early subphenotype related to later diabetes onset, we evaluated insulin autoantibodies (IAA) in the NOD mouse, at weaning and between 3 and 5 wk of age.

The presence of anti-islet autoantibodies (2) characterizes insulin-dependent diabetes. In particular, IAA appear to be unique, as it has been shown that their presence, between 8 and 16 wk of age, is predictive of the later appearance of disease in NOD mice and their levels are dramatically and inversely correlated with the age at which T1D develops in humans (3).

It has been proposed that in the NOD mouse around 3 wk of age, autoimmune-prone molecular changes might take place before any insulinitis is evident (4). Several hypotheses have been advanced concerning the early steps of autoimmune islet destruction. It has been suggested that a local insult renders the pancreatic endothelium to release sequestered pancreatic entries attracting T cells whose homing potential might be groomed in the periphery with early priming of T cells in the pancreatic lymph nodes (4). β cell Ags thereafter are thought to trigger reactive T cells to invade the islets (5). Yet, the first steps of the autoimmune process remain unclear, hampered by the inability to select individuals who will develop the final stages of disease with certainty.

The aim of our study has been 1) to identify early phenotypic markers allowing the selection of individual mice as autoimmune prone, before any detectable autoimmune destruction appears and 2) to establish subphenotypes that correlate with the final disease phenotype to simplify studies aiming to unravel the successive steps in T1D pathogenesis. We speculated that, as early as at weaning (3 wk of age), IAA might already develop in a subset of mice. In this study, we report on the presence of IAA in 30–38% of the

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⁴ Abbreviations used in this paper: T1D, type 1 diabetes; NOD, nonobese diabetic; IAA, insulin autoantibody; E-IAA, early-IAA; matIAA, maternal IAA.

NOD mice, at 3–5 wk of age. Although these autoantibodies preceded any autoimmune destruction (insulinitis), they correlated with early diabetes onset. Interestingly, maternal ante partum IAA status influences this subphenotype in the litters after weaning, indicating that the immune response transmitted by the mother might trigger the existing autoimmune mechanisms in the litters, with the consequence of increasing predisposition by accelerating disease onset.

Finally, the physiopathological processes leading to T1D are marked by distinct autoimmune windows characterized by the presence of IAA as an early marker of diabetes autoimmunity.

Materials and Methods

Mice

NOD mice were purchased from Taconic Farms (Germantown, NY). Mice were housed in specific pathogen-free barrier facilities at the Barbara Davis Center, University of Colorado Health Sciences Center (Denver, CO). Experimental protocols were under conditions approved by the Institutional Animal Care and Use Committee. Pregnant females were bled ~1 wk before delivery. Litters were bled at 3 wk (weaning), 4 and 5 wk, and at 8 and 16 wk of age, by retro orbital vein.

For the identification of the presence of early-IAA (E-IAA), a total of 127 mice corresponding to 15 different litters were monitored for IAA weekly at 3–5 wk of age. Twenty pregnant females were also accessed for IAA at ~1-wk ante partum, and at 3 and 4 wk post partum. For correlation of E-IAA with T1D, five different litters (total number of mice = 22) were followed for IAA at 3, 4, 5, 8, and 16 wk of age.

Ninety-six-well filtration plate MicroIAA assay

IAA was measured in the serum using a 96-well filtration plate MicroIAA assay as described (3, 6). In brief, ^{125}I -labeled insulin (Amersham Biosciences, Piscataway, NJ) of 20,000 cpm was incubated with 5 μl of serum with and without cold human insulin, respectively, at a 1/5 dilution of serum for 3 days at 4°C in buffer A (20 mM Tris-HCL buffer (pH 7.4) containing 150 mM NaCl, 1% BSA, 0.15% Tween 20, and 0.1% sodium azide). Fifty microliters of 50% protein A/8% protein G-Sepharose (Pharmacia, Peapack, NJ) were added to the incubation in a MultiScreen-NOB 96-well filtration plate (Millipore, Bedford, MA) which was precoated with buffer A, overnight, at room temperature. The plate was shaken at low speed for 45 min at 4°C followed by two cycles of four washes per each cycle with cold buffer B (buffer A with 0.1% BSA added) by using the Millipore vacuum-operated 96-well plate washer. After washing, 40 μl of scintillation liquid (Microscint-20; Packard Instrument, Meriden, CT) was added to each well and radioactivity was determined directly in the 96-well plate with a Top-Count beta counter (Packard Instrument). The IAA level was calculated based on the difference (Δ) in cpm between the well without cold insulin and the well with cold insulin and expressed as an index: $\text{index} = (\text{sample } \Delta \text{ cpm} - \text{human negative control } \Delta \text{ cpm} \pm \text{assay calibration control}) / (\text{human positive control } \Delta \text{ cpm} - \text{human negative control } \Delta \text{ cpm} \pm \text{assay calibration control})$. The limit of normal (0.010) was chosen as the 99th percentile from a previous analysis of nondiabetic strains of mice including 23 BALB/c and C57BL/6 mice (3).

Diabetes assessment

Mice (22 from which 15 females and 7 males) were monitored after 12 wk of age, on a weekly basis, for the development of spontaneous diabetes. Blood glucose measurements were made using an Elite glucometer (Bayer, Elkhart, IN). Two consecutive nonfasting glucose measurements, with values greater than 250 mg/dl, constituted diagnosis of diabetes. For T1D, animals were monitored for glycemia from 12 up to 40 wk of age.

Histology

Histological and immunohistochemical analysis of pancreas was performed as previously described (6) by fixation in 10% formalin, and by being paraffin-embedded, sectioned, and stained with H&E. A minimum of 20 islets from each animal was observed.

Statistical analysis

Statistical analysis was performed using the regression analysis and Fisher exact test with EPISTAT (Round Rock, Richardson, TX), or INSTAT (GraphPad, San Diego, CA), χ^2 Fisher's exact test, and Mann-Whitney *U* test. The survival curve analysis was performed by estimation using the

Kaplan-Meier method and compared by the long rank test with PRIZM software (GraphPad).

Results

IAA can be detected as early as at 3 wk of age in NOD/Tac mice

We speculated that IAA might be present as early as at 3 wk in a subset of animals. Therefore, we assessed for the presence of E-IAA starting at weaning (3 wk) and at 4 and 5 wk of age, in a total of 127 animals from 15 different litters (Fig. 1a). At 3 wk, 30.7% of the animals were IAA positive, and subsequently 34.6 and 38.5% at 4 and 5 wk, respectively, were positive for IAA (Table I). Although values for IAA positive at 3 wk were relatively low (Fig. 1b), ranging from 0.010 to 0.026, they were above the 99th percentile cut-off value (mean \pm SD: 0.01011 \pm 0.01623). These values increased at 4 wk (0.01425 \pm 0.02238) and by 5 wk reached an IAA index of 0.04015 \pm 0.1943 (Fig. 1b). Differences between ages were significant (Fig. 1a) indicating that new IAA synthesis occurs along with age.

Histological examination of the pancreas from E-IAA-positive or -negative offspring at 5 wk of age showed no correlation of E-IAA with insulinitis (Fig. 2) in that peri-insulinitis has been only occasionally observed in IAA-positive litters while most of the animals, positive or negative for IAA, were insulinitis-free at this age. This argues in favor of IAA being present early before insulinitis appearance in the NOD mouse.

E-IAA is a quantal phenotype

Although NOD mice are inbred and therefore share identical genotypes, at 3 wk of age only ~30% of the animals were found to be positive for IAA (Table I). As it has been mentioned above, the 127 animals used in this study were issued from 15 different litters and thus we were able to access IAA distribution according to the litter (Table I). Interestingly, IAA showed a quantal by litter distribution, with litters developing IAA after 4 wk and litters having a high percentage of animals already IAA positive by 3 wk. Differences between litters for the same age were significant at 3 and 4 wk ($p = 0.0008$ and $p = 0.0236$, respectively) (Table I). However, at 5 wk of age differences between litters were not significant ($p = 0.0904$), indicating that at around this age, in the majority of the animals, IAA production is taking place, independently of the

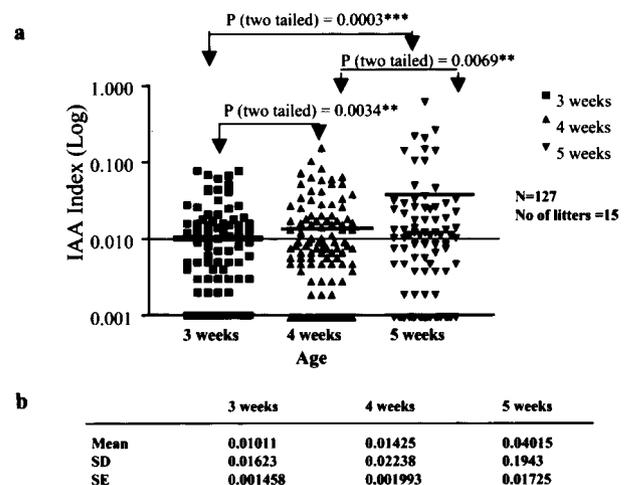


FIGURE 1. Early appearance of IAA. *a*, IAA index in sera from litters of 3-, 4-, and 5-wk-old mice. Total number of animals: 127 from 15 different litters. A value of *p* (two-tailed test) indicates differences of IAA between ages. *b*, Mean values of IAA within each age.

Table I. Early appearance of IAA in NOD mice is quantal by litter^a

Litters/Age	IAA ⁻ Ante Partum							IAA ⁺ Ante Partum							Total (%)	χ ² (p Value)	
	A1	A12	A36	A37	A44	A45	A8	A5	A7	A9	A10	A15	A39	A41			A40
3 wk	0/8	0/5	0/10	0/4	0/8	0/8	0/10	0/10	0/5	<u>5/10</u>	<u>5/8</u>	<u>7/11</u>	<u>4/7</u>	<u>11/12</u>	<u>7/11</u>	39/127 (30.7)	36.65 (0.0008)
4 wk	1/8	1/5	0/10	1/4	0/8	0/8	3/10	1/10	<u>4/5</u>	<u>4/10</u>	<u>5/8</u>	<u>11/11</u>	<u>5/7</u>	<u>7/12</u>	1/11	44/127 (34.6)	26.31 (0.0236)
5 wk	3/8	2/5	2/10	<u>3/4</u>	1/8	3/8	0/10	1/10	<u>3/5</u>	1/10	<u>5/8</u>	<u>10/11</u>	<u>5/7</u>	<u>9/12</u>	1/11	49/127 (38.5)	21.46 (0.0904 NS)

^a Litter distribution of IAA in NOD/Tac mice (from three different experiments). Differences between phenotypes observed in each litter are shown by the p values calculated at each age. Numbers of E-IAA-positive animals exceeding half of the total number of offspring in each litter are underlined.

litter. No gender differences for IAA have been observed (Table II) at all three ages studied, however a slight male/female distortion of the IAA-positive ratio was observed at 3 wk of age, with males being more frequently positive than females (33.8 vs 27.7%, respectively), (Table II). Five of 18 females IAA positive at 3 wk (28%) and 7 of 21 males (33%) showed transient expression of IAA, at 3 wk, with their subsequent disappearance by 5 wk. These data taken together indicate that, similarly to the more dramatic phenotypes such as insulinitis as well as the end phenotype T1D, the mechanisms of autoimmune destruction put in place are not only dependent upon genetic factors, but also upon other factors inherent to the individual animal/litter.

Maternal autoimmune environment influences the IAA levels of the litters

Because the first measurements of IAA have been performed at an early age (3 wk), corresponding to weaning, and in addition, as the presence of E-IAA shows a quantal distribution between litters, we addressed the possibility of a maternal transplacental origin of these E-IAA. Therefore, we systematically measured IAA in the sera of the mothers within 1 wk before delivery (ante partum) and at 3 and 4 wk post partum. From 15 mothers, 7 were IAA-negative (46%) (Fig. 3a) and 8 IAA-positive (53%) ante partum (Fig. 3b), while 9 remained or became positive at 3 wk post partum (Fig. 3). Furthermore, the distribution of the litters according to the maternal IAA (matIAA) status shows elevated IAA within litters from ante partum-positive mothers (Fig. 3b). These differences between maternal ante partum-negative and -positive litters were significant with a higher incidence of IAA in animals issued from IAA-positive mothers for all ages studied (Fig. 4, a–c). In particular, at 5

wk of age, although offspring IAA positive at 3 and 4 wk remained positive, newly developed IAA were also observed in animals issued from IAA-negative ante partum mothers (8 additional mice) (Fig. 4c). Mean IAA values increased also in the maternal negative group at this age (Fig. 4d) (mean ± SEM were: for matIAA positive: 0.03174 ± 0.01062 and for matIAA negative: 0.06395 ± 0.04782), even though still a higher number of animals were IAA positive in the maternal-positive group rather than in the negative group (47.3 vs 26.4%, respectively) (Fig. 4c). Differences between the two groups were significant at all ages (Fig. 4, a–c). Because IAA observed at 3 wk of age remained more frequently present at 4 and at 5 wk and their levels increased as seen from individual values (Fig. 3) and the mean values (Fig. 4d), these data suggest that E-IAA are an inherent characteristic of the individual animals rather than due to transplacental transmission. Moreover and in agreement with a non transplacental origin of IAA, the levels were often higher in the offspring than in the maternal circulation (Fig. 3b, e.g., litter A15), and did not correlate with matIAA levels (Fig. 3b). Finally, the observation that within the same litters with highest IAA-positive offspring, negative mice were also present (Table I), as well as the intralitter heterogeneity of IAA levels, make unlikely attribution of the offspring E-IAA to transplacental transmission. On the contrary, it is noteworthy that some IAA-positive animals at 3 wk showed transient IAA expression, because they became negative at 5 wk (Table III, litters A9, A41, and A40). Indeed, 12 (5 females and 7 males) of 39 (30.7%) IAA-positive mice at 3 wk showed transient IAA expression. This concerned the matIAA-positive group as no positive animals at 3 wk were observed in the matIAA-negative ante partum group (Table IV). Therefore, even though several observations argue against a solely transplacental origin of the E-IAA, as mentioned above, it might be that the autoimmune-prone environment transmitted from the mother includes transplacental transmission of E-IAA.

No effect from IAA transmission through the maternal milk has been observed in these experiments, as seen by the independent distribution of IAA within the litters of post partum-positive mothers (Fig. 3). Indeed, no significant differences have been observed in litters from matIAA post partum-positive or -negative mothers, neither within the matIAA-positive ante partum group nor within the matIAA-negative ante partum group (Table V). In addition, numbers of IAA-positive offspring remained similar in litters from

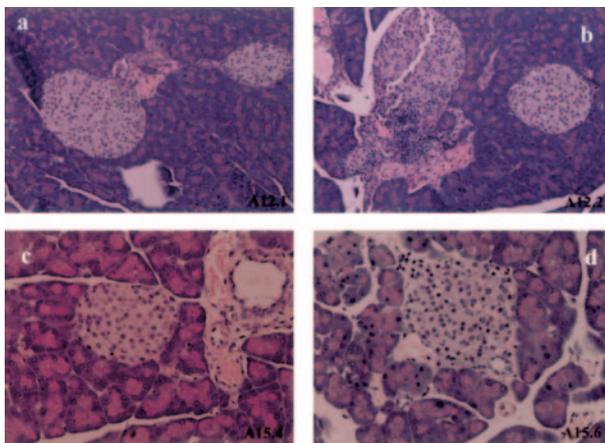


FIGURE 2. E-IAA precedes insulinitis in NOD mice. Photomicrographs of pancreatic sections from four individual mice at 5 wk of age. a, E-IAA negative, no sign of lymphocytic infiltration. b, E-IAA positive (IAA index: 0.023), arrow shows an islet inflammatory infiltrate. c and d, E-IAA-positive animals: (IAA index: 0.035 and 0.031, respectively), showing no lymphocytic infiltration.

Table II. Gender distribution of IAA in NOD/Tac litters^a

Age (wk)	IAA (%) Females	IAA (%) Males	Total (%)	p Value (for F vs M Differences)
3	18/65 (27.7)	21/62 (33.8)	39/127 (30.7)	0.5641 NS
4	24/65 (36.9)	20/62 (32.8)	44/127 (34.6)	0.7093 NS
5	26/65 (40)	23/62 (37.1)	49/127 (38.6)	0.8555 NS

^a Differences between males (M) and females (F) as shown by the p values were not significant (NS).

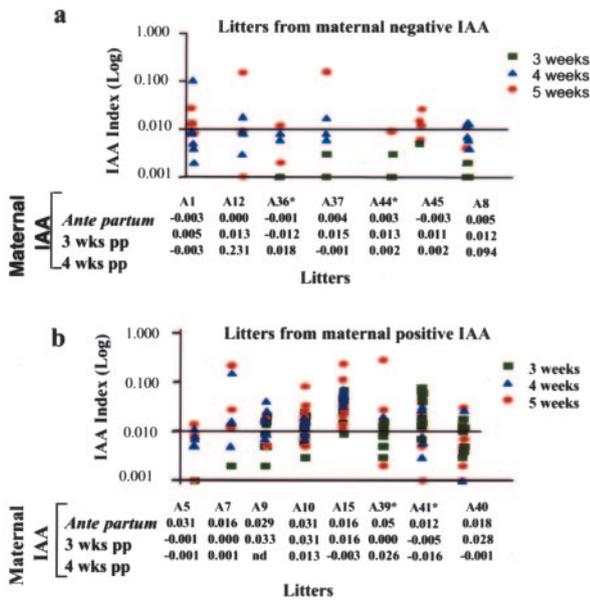


FIGURE 3. Correlation between E-IAA phenotype in the litters and maternal IAA ante partum status. Values under the figures represent matIAA for each litter as measured at ante partum and at 3 and 4 wk post partum. *a*, Litters from negative matIAA. *b*, Litters from matIAA positive.

matIAA-positive ante partum and remained positive at 3 wk post partum (see for example A15, comparing with A41 Fig. 3*b* and Table V).

Collectively, these results indicate that there is no effect of the milk-transmitted IAA through breastfeeding, but in the contrary the maternal autoimmune-prone environment, as reflected by IAA, during pregnancy (ante partum) plays a role in the appearance of early autoimmunity in the pups.

Biological significance of E-IAA

To assess the biological role of E-IAA to the final disease onset, we used a new set of 22 animals, 15 females and 7 males, issued

from five different litters. IAA time measurements were conducted as described in the previous sections at ante partum and at 3 and 4 wk post partum for the mothers, and at 3, 4, 5, 8, and 16 wk of age for the offspring. Animals were followed for glycemia weekly from 16 wk until 40 wk of age.

At first, we correlated the presence of matIAA ante partum with IAA in the pups at 3 wk of age, with the aim to evaluate in this group of animals the influence in the offspring of the maternal autoimmune-prone environment. A total of 66.6% of the animals from IAA-positive ante partum mothers were also IAA positive at 3 wk, while none of the animals from IAA-negative mothers were positive at this age (Table VI). This indicates that as expected from our previous experiments, matIAA status influences the presence of E-IAA in the litters.

Evolution of E-IAA phenotype

It has been previously described that the presence of autoantibodies at 8 wk of age in the NOD mouse strongly correlated with early development of diabetes (2). Therefore, we evaluated for a correlation between IAA at 3 wk and IAA at 8 wk of age for these mice. Indeed 62.5% of animals IAA positive at 3 wk were found to be positive also at 8 wk of age (Table VII), while 64% of IAA-negative animals at 3 wk remained negative at 8 wk of age (Table VII). Therefore, the early presence of IAA does not reflect a transient phenomenon due to the only passage of transplacental autoantibodies, but rather a maternal effect in early autoimmune predisposition. These data strongly argue for a biological role, etiologic or correlative, of E-IAA in later disease phenotype.

Correlation of E-IAA with type 1 diabetes

We subsequently followed these 22 mice for diabetes incidence. Comparison of survival curves between IAA-positive and IAA-negative animals at 3 to 5 wk of age showed a delay in diabetes onset in the IAA-negative animals and this difference is statistically significant ($p < 0.01$, for sex combined) (Fig. 5*a*). Similarly, when females and males were examined separately for diabetes onset, differences between IAA positive and IAA negative were significant for females ($p < 0.001$) (Fig. 5*b*) and approximated

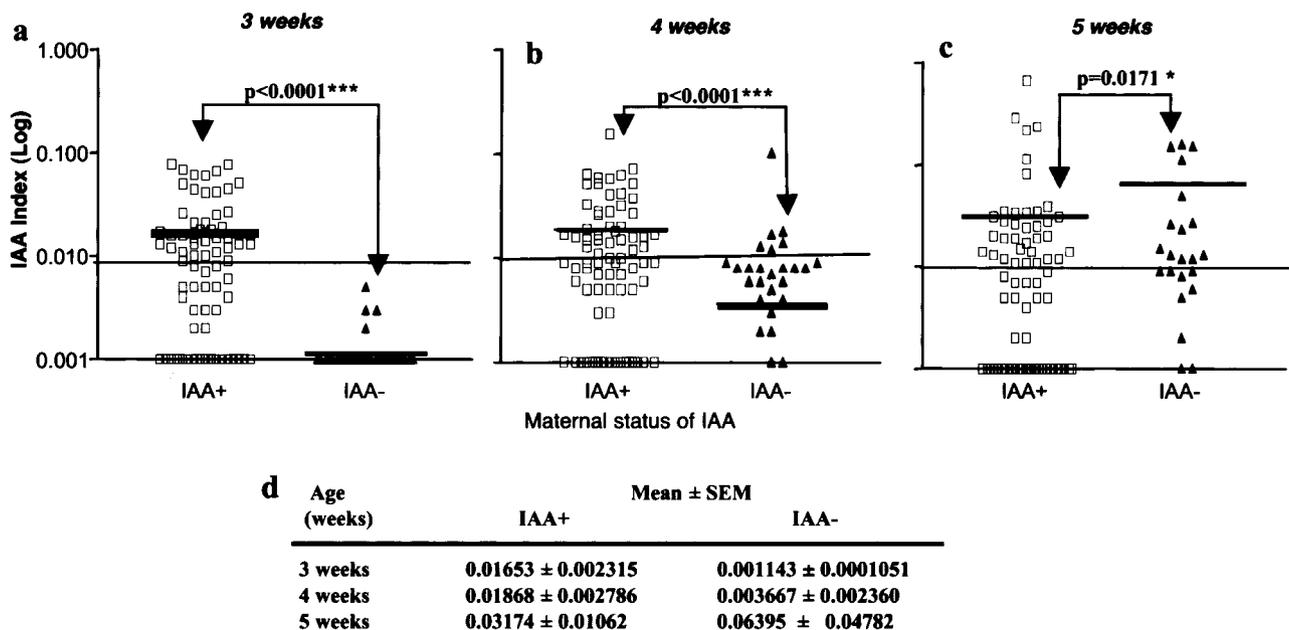


FIGURE 4. Comparison of IAA levels between matIAA-positive and -negative litters. IAA index (*a*) at 3 wk of age, (*b*) at 4 wk, (*c*) at 5 wk. *d*, Mean values of IAA index in offspring from matIAA positive (IAA⁺) and negative (IAA⁻; p values are as noted and measured by unpaired two-tailed t test).

Table III. Females and males from IAA⁺ ante partum litters

Litters/Age	A5	A7	A9	A10	A15	A39	A41	A40	Total (%)	<i>p</i> Value
Females										
3 wk	0/2	0/1	2/6	1/1	3/7	3/4	5/5	4/7	18/33 (54.5)	
4 wk	1/2	1/1	4/6	1/1	7/7	3/4	5/5	0/7	22/33 (66.6)	3 vs 4 wk: 0.4502 NS
5 wk	0/2	1/1	1/6	1/1	6/7	3/4	5/5	0/7	17/33 (51.5)	3 vs 5 wk: 1 NS
Males										
3 wk	0/8	0/4	3/4	4/7	4/4	1/3	6/7	3/4	21/41 (51.2)	
4 wk	0/8	3/4	0/4	4/7	4/4	2/3	2/7	1/4	16/41 (39)	3 vs 4 wk: 0.3749 NS
5 wk	1/8	2/4	0/4	4/7	4/4	2/3	4/7	1/4	18/41 (43.9)	3 vs 5 wk: 0.6586 NS

significance for males, ($p = 0.07$) (Fig. 5c). Furthermore, animals in the IAA positive at 3 wk of age group showed acceleration of diabetes onset in comparison with IAA negative of the same group ($p < 0.01$) (Fig. 5d).

Consequently, we established three phenotypic windows corresponding to final disease phenotype. Window I corresponds to early diabetes onset at 16–20 wk of age, window II to diabetes onset at 21–25 wk, and window III at 25–30 wk of age (Fig. 6). Interestingly, 8 of 13 E-IAA positive, between 3 and 5 wk, developed diabetes also early at window I (diabetes risk 61.5%) and only 3 of 13 E-IAA-positive animals developed diabetes later (window II) at 20–25 wk of age (diabetes risk 23%), while none of these E-IAA⁺ animals developed diabetes at window III (Fig. 6). However, two animals (15%) of this E-IAA-positive group remained diabetes-free (data not shown).

In contrast, of animals that were E-IAA negative, but IAA positive at 8 wk, four of nine developed diabetes at window II (21–25 wk of age), (diabetes risk 44.4%) and two of nine (22.2%) at over 25 wk, window III (total diabetes risk for this group 67%) (Fig. 6).

Finally, from the remaining animals, only one female that has been IAA negative at all ages developed diabetes at 22 wk of age. On the contrary from three females that remained diabetes-free at 30 wk of age, two were IAA positive only after 8 wk, and only one was E-IAA positive. Similarly, from three males remaining diabetes-free at 30 wk of age only one was E-IAA positive; the other two were IAA positive after 8 wk of age (data not shown).

These data, taken together, show that the presence of E-IAA predisposes to early T1D and emphasize the biological significance of the IAA phenotype as an early marker of autoimmune destruction.

Discussion

IAA as an early phenotypic marker for T1D

Autoimmune destruction of target tissues is a slow process marked by a long phase before disease onset. No clinical signs exist to clearly indicate the preautoimmune condition. Prediction of the

disease is one of the prerequisites for accessing therapeutic methods to human patients.

Although T1D is considered as a T cell-mediated disease, several studies have investigated the presence of autoantibodies against islet Ags in the sera of human patients as well as in animal models (7–9). A correlation between the presence of autoantibodies and T1D in human patients has been established (10, 11). In particular, IAA are an important predictor in the diagnosis of T1D (3). IAA are frequently found and are among the first detectable autoantibodies in young children developing the disease (12–16). They precede T1D with reported appearance between 8 and 20 wk of age in the NOD mouse and they correlate with diabetes incidence (3).

In the present study, we evaluated IAA as an early marker of autoimmunity between 3 and 5 wk of age in the NOD mouse. Our data demonstrate the identification of autoantibodies against insulin as early as at 3 wk of age in the NOD mice. This is unexpected because at this age, usually no insulinitis is yet present. Indeed, examination of the pancreas at 5 wk showed no correlation of E-IAA with insulinitis (Fig. 2), arguing in favor of IAA being an early marker of autoimmunity in the NOD mouse. In addition, autoantibodies against insulin were also present at 4 and 5 wk of age, in the majority of the animals (Table I and Figs. 1, 3, and 4); although few mice showed transient IAA expression and predominantly they were males (Table III). The E-IAA presence as shown by the biological significance of autoantibodies in acceleration of T1D represents a predictive factor for early disease onset in the NOD mouse and underlines immune mechanisms that seem to take place early in life (at weaning). Because this E-IAA presence is related to the matIAA ante partum status, it constitutes an exogenous, to the animal, factor of predisposition identified at 3 and 4 wk of age. The inherent to the animal IAA production more clearly observed at 5 wk of age masks the maternal effect, probably due to transmission of an autoimmune-prone environment from the mother. Gender differences of E-IAA expression were not observed at this early age, with the exception of a slight distortion of

Table IV. Females and males from IAA⁻ ante partum litters

Litters/Age	A1	A12	A36	A37	A44	A45	A8	Total (%)	<i>p</i> Value	
Females										
3 wk	0/7	0/2	0/6	0/2	0/4	0/7	0/4	0/32 (0)		
4 wk	1/7	0/2	0/6	0/2	0/4	0/7	1/4	2/32 (6.25)	3 vs 4 wk: 0.4921 NS	
5 wk	2/7	1/2	1/6	1/2	1/4	3/7	0/4	9/32 (28.1)	3 vs 5 wk: 0.0020	
Males										
3 wk	0/1	0/3	0/4	0/2	0/4	0/1	0/6	0/21 (0)		
4 wk	0/1	1/3	0/4	1/2	0/4	0/1	2/6	4/21 (19)	3 vs 4 wk: 0.1069 NS	
5 wk	1/1	1/3	1/4	2/2	0/4	0/1	0/6	5/21 (23.8)	3 vs 5 wk: 0.0478	

Table V. Correlation of mat IAA post partum during breastfeeding (up to 3 wk) with E-IAA presence in the offspring

Ante Partum	Maternal IAA-Negative Post Partum			Maternal IAA-Positive Post Partum		
	3 wk (%)	4 wk (%)	5 wk (%)	3 wk (%)	4 wk (%)	5 wk (%)
IAA Negative	0/10 (0)	2/10 (20)	7/10 (20)	0/26 (0)	4/26 (15.3)	7/26 (27)
IAA Positive	15/34 (44)	17/34 (50)	18/34 (50)	24/40 (60)	21/40 (52.5)	17/40 (42.5)

IAA presence between females and males found at 3 wk, 27.7 and 33.8% IAA positive, respectively (Table II). This might reflect that in the NOD mouse, the mechanisms conferring resistance to autoimmunity, usually found in males for the final diabetes phenotype, are not yet in place at this early age.

We then evaluated the biological role of the presence of E-IAA by following 22 mice for IAA and subsequently for T1D up to 40 wk of age. We found that E-IAA at 3 wk is correlated with the presence of IAA at 8 wk (Table VII) and with early onset of T1D, at 16 to 20 wk of age (Fig. 5d). We propose that this corresponds to the first autoimmune window representing early diabetes onset marked by E-IAA presence. Interestingly, three autoimmune windows for disease onset could be clearly established according to the age of the first appearance of autoantibodies against insulin (Fig. 6). Although a causative effect cannot be with certainty attributed to IAA, our data clearly demonstrate that they precede disease and their early presence is correlated with early T1D onset.

Thus, we show herein that autoimmunity to insulin at an early age affects final diabetes incidence by accelerating its onset.

Maternal autoimmune imprinting predisposes offspring to T1D

Interestingly, the E-IAA phenotype is quantal, by litter, because litters varied between 0 and 91% of IAA-positive mice at 3 wk, and between 0 and 100% at 4 wk of age (Table I). Two possible explanations may account for this quantal characteristic of IAA: first, it might reflect the quantitative nature of autoimmune diabetes i.e., the interaction of environmental or nongenetic factors with the genetic predisposition marked by several responsible genes, as observed also with the final phenotype. Second, it might reflect more precisely the maternal influence to the autoimmune environment of the offspring early in life. Consequently, to clarify the causes of the quantal nature of E-IAA we assessed for matIAA. We show herein that matIAA status influences the presence of IAA in the offspring, early in life, between 3 and 5 wk of age, as litters issued from IAA-positive mothers had a higher incidence of IAA (Figs. 3 and 4). Moreover, the biological significance of matIAA is accentuated by the presence of high diabetes incidence before 20 wk of age in offspring from matIAA-positive litters (Fig. 5, a–c). Consequently, matIAA confer high probability for early diabetes onset, before 20 wk of age. Maternal immune imprinting therefore seems to take place predisposing the litters to early development of autoimmunity and this can be followed by the evolution of autoantibodies against insulin. This is in agreement with a previous study that has demonstrated that matIAA contributed in a significant manner to diabetes onset in the NOD mouse (17). In this

study, the authors have eliminated maternally derived IAA by three ways: 1) by using B cell-deficient NOD mothers to eliminate the transmission of maternal Ig, 2) by the use of Ig transgenic NOD mothers to exclude specificities from the maternal B cell repertoire, and finally 3) by performing implantation experiments of NOD embryos in pseudopregnant females of nonautoimmune strains. Their data showed that in all three cases, NOD progeny was protected from spontaneous diabetes (17). Transient maternally acquired islet autoantibodies have been also associated with autoantibody persistence and T1D risk in humans (18). In another study, it has been reported that offspring of diabetic fathers had significantly higher prevalence of autoantibodies than did offspring of diabetic mothers, but only between 10 and 30 years old in humans (19). This reflects genetic imprinting rather than immune imprinting as our data demonstrate and it has not been related to the presence of autoantibodies in pregnant mothers (19) as reported herein.

Although no evidence has been observed in our data for an influence of IAA from the maternal milk (Table V), it might be of interest, to confirm and further elucidate these observations, by performing cross-suckling experiments in which litters from IAA-positive mothers are nursed by IAA-negative females and vice versa.

Our data show for the first time the presence of IAA early in life, at weaning, in the NOD mouse and correlate this early subphenotype with early T1D onset. Moreover, we demonstrate that E-IAA define three autoimmune windows for final disease onset.

It is tempting to relate the maternal autoimmune imprinting with the maternally transmitted resistance to infectious agents observed in mammals. After Ehrlich's (20) first documentation of the protective function of maternally transferred Abs against microbial infections in the offspring, numerous studies clearly demonstrated that maternal Ab exert immunomodulatory effects which might even be visible at a later age when no longer detectable in the serum (21–23). Maternal Ab may even enhance immune responses in murine offspring (23, 24). Finally, it has been shown that maternal Abs exert an active, immunoregulatory influence on the nascent immune system of the neonate (25). Similarly, concerning T1D, it has been suggested that in infancy, increasing Ab levels most likely reflected de novo synthesis of diabetes-associated autoantibodies (26). Although the autoantibody requirement for T1D onset in NOD mice has been clearly demonstrated, still the causative relationship, if there is any, between insulin autoantibodies and T1D remains unknown. In the present study, we demonstrated a relationship between the maternal autoimmune environment, the early presence of IAA in the offspring, and the later diabetes onset.

Table VI. Correlation between matIAA ante partum and IAA in litters at 3 wk of age^a

matIAA Ante Partum	IAA Positive/Total n of Pups (%)	IAA Negative/Total n of Pups (%)
Maternal positive	8/12 (66.6)	4/12 (33.3)
Maternal negative	0/10 (0)	10/10 (100)

^a Numbers correspond to IAA-positive offspring vs total number of pups tested.

Table VII. Evolution of E-IAA phenotype^a

	IAA Positive at 8 wk (%)	IAA Negative at 8 wk (%)
IAA positive at 3 wk	5/8 (62.5)	3/8 (37.5)
IAA negative at 3 wk	5/14 (35.7)	9/14 (64)

^a Correlation between IAA-positive offspring at 3 wk and subsequently remaining IAA positive at 8 wk of age.

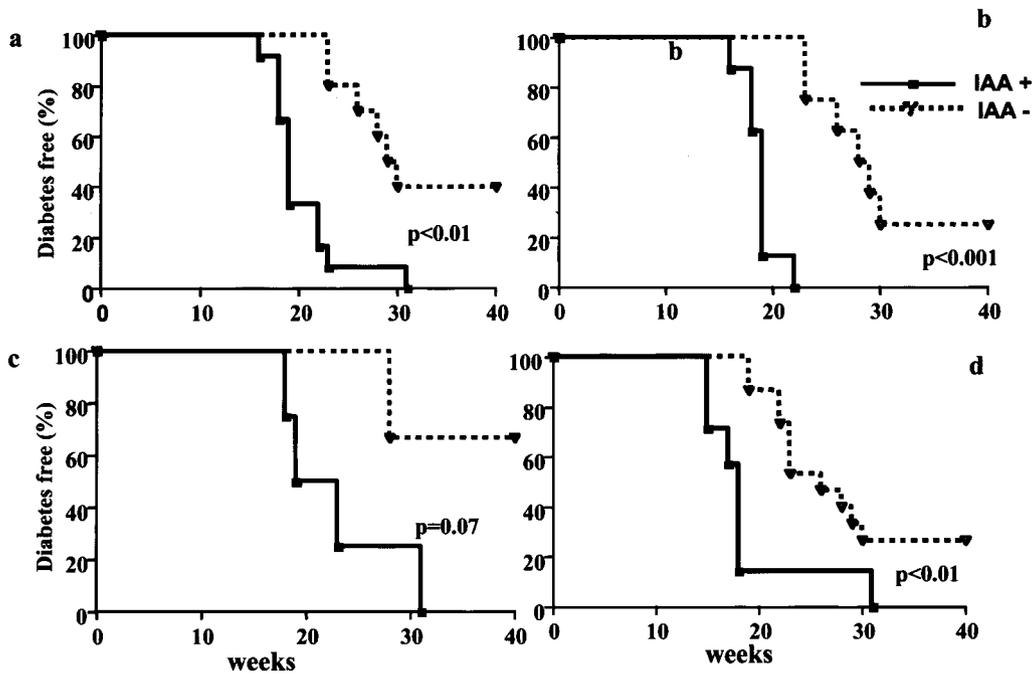


FIGURE 5. Biological significance of E-IAA. Life survival curves for E-IAA-positive and E-IAA-negative offspring. *a*, Sex combined; *b*, females; *c*, males; *d*, diabetes incidence in IAA-positive offspring at 3 wk of age.

The significant acceleration of disease in E-IAA-positive animals (Fig. 6) is shown for the first time herein and constitutes a breakdown of the prediabetes phenotype. Although the exact molecular/biochemical mechanisms involved are not known, this is an important observation because it provides insight to speculate that therapeutic intervention at the appropriate time might be more efficient in disease prevention. It also allows for studies to be designed aiming to better understand the disease pathogenesis. Furthermore, because immune responses are regulated by both positive and negative pathways with the clear result determining final disease onset, disposing markers (E-IAA as described herein) for precise phenotypic windows might help in understanding the factors involved in disease acceleration so that appropriate therapeutic targets can be designed.

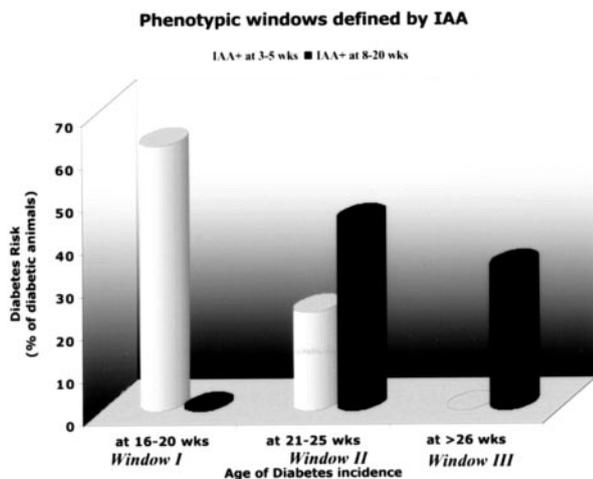


FIGURE 6. Phenotypic windows in T1D pathogenesis, as defined by IAA. Animals have been grouped according to IAA appearance. White: IAA positive at 3–5 wk of age. Black: IAA positive at 8 to 20 wk of age.

We would like to consider, in an analogy to the immunologic memory (27–31), that the period shortly before and after birth might be the key in also understanding autoimmunity. Because at this phase of development the immune system is relatively incompetent, transferable maternal immunologic memory is essential for the survival of the fetus, newborn, and infant (32). Similar to the immune protection provided by the maternal immune system, it is possible that autoimmune transferable maternal memory might also influence susceptibility to disease. Therefore, the pathogenic role of the maternal autoimmune environment tracked by the presence of IAA in T1D is transferable to the offspring and it predisposes to autoimmune diabetes. This might be the essential role attributed to B cells (autoantibodies) in opposition to T cells. Indeed, although T cells transfer disease and might have a causative effect for diabetes, they cannot be maternally transmitted because of differences in tissue Ags (HLA in particular) between the mother and the fetus (32). Thus, they cannot play a role in immune imprinting. Because maternal Abs result mainly from thymus-dependent immune responses that have undergone immune maturation, they represent the highest quality of the collective maternal immunological experience. Therefore, we propose that in analogy to the conferred maternal microbial protection in mammals (33), maternally transmitted IAA exert not only a regulatory influence on the T cell repertoire, but also an affinity enhancement of a proportion of early primary Abs and thus contribute in diabetes onset in a time-dependent fashion. Our data support this hypothesis and underline the presence of maternal autoimmune imprinting for T1D in the NOD mouse. In a recent report it has been shown that T cell restriction and positive selection are affected by B cells and Ig (34). Thus, in autoimmune diseases a similar role might be attributed to B cells. This could also explain the T1D acceleration in E-IAA-positive mice, whereas an early autoimmune environment is established by the presence of B autoreactive cells. To further elucidate the causative relationship of the presence of E-IAA, it will be of interest to assess for the presence of T

autoreactive and regulatory cells early at 3–5 wk of age in NOD mice that are E-IAA positive.

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