Major Histocompatibility Complex Class I Shedding and Programmed Cell Death Stimulated Through the Proinflammatory $P2X_7$ Receptor

A Candidate Susceptibility Gene for NOD Diabetes

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It has been hypothesized that type 1 diabetes is initiated by neonatal physiological pancreatic β-cell death, indicating that the early stages of this autoimmune response may reflect a dysregulated response to immune "danger" signals. One potential danger signal is ATP, high concentrations of which stimulate the purinergic receptor $P2X_7$ on hematopoietic cells. We compared the sensitivity of lymphocytes from model type 1 diabetic (NOD) and control (C57BL/10) mice to activation of this pathway. Stimulation of the $P2X_7$ receptor of NOD mice resulted in more pronounced shedding of the lymphocyte homing receptor CD62L and in increased programmed cell death. Levels of major histocompatibility complex class I molecules, which have previously been reported to be poorly expressed on NOD lymphocytes, were initially normal, but the molecules were shed preferentially from NOD cells after $P2X_7$ receptor stimulation. Thus, although NOD lymphocytes have been considered resistant to programmed cell death, they are highly sensitive to that stimulated through the $P2X_7$ receptor. Because NOD mice express a low activation threshold allele of the $P2X_7$ receptor and the $P2X_7$ gene maps to a locus associated with disease, $P2X_7$ is a good candidate susceptibility gene for NOD diabetes. ***Diabetes*** **53:2012–2017, 2004**

The concept that the immune system has evolved not to simply discriminate self from nonself, but also to respond to signals perceived as dangerous (1), is now widely accepted. These adjuvant signals can be exogenous, such as bacterial cell walls, or endogenous, such as mediators released from dying cells at the site of tissue damage (2–4). In this context, the finding that either a physiological wave of pancreatic β-cell apoptosis or viral or chemical damage to the same cells precedes and may initiate autoimmune responses in type 1 diabetes (5–9) suggests that disease may reflect an aberrant response to endogenous "danger" signals. We have recently suggested that ATP is a danger signal for T-cells, promoting extravasation (J.I.E., F. Marelli-Berg, J. Cooper, S. Jarmin, D. Scott, R. Cassady-Cain, D. Alexander, C.F.H., unpublished observations). This candidate endogenous immune adjuvant is present at a high level in normal cells, but is released upon cell lysis and is thereafter rapidly degraded to ADP, AMP, and then adenosine by the concerted action of ecto-ATPDase/CD39 and ecto-5′-nucleotidase/CD73 (10). Although ATP is proinflammatory, genetic deficiency of the ATP receptor $P2X_7$, expressed on hematopoietic cells activated by high levels of extracellular ATP, results in diminished inflammation (11).

The ATP-dependent, $P2X_7$ receptor–mediated events include three of particular significance to type 1 diabetes: *1*) the rapid release by macrophages of interleukin-1β (IL-1β) within microvesicles (12); *2*) the shedding of the homing receptor, CD62L (13), a process required for lymphocyte migration to inflammatory sites and one involved in diabetes pathogenesis (14,15); and *3*) programmed cell death. Prolonged $P2X_7$ receptor stimulation results in programmed cell death that has many characteristics of "classic" caspase-dependent apoptosis, but also other characteristics more commonly associated with necrosis (16). Such programmed cell death has sometimes been termed "aponecrosis" (17). Whether aponecrosis is immunologically silent or is itself proinflammatory remains to be established. IL-1β release by macrophages invading the pancreas is not only proinflammatory, but may also trigger pancreatic β-cell death directly by stimulating nitric oxide synthesis (18) or inducing CD95 death receptor expression (19,20).

We hypothesized that the $P2X_7$ receptor might be a candidate susceptibility gene for type 1 diabetes. Common mouse strains have been reported to express one of two allelic forms of $P2X_7$, which differ in their threshold of ATP activation (21). These allelic forms differ in a single amino acid at position 451 of the deduced amino acid sequence, with proline ($P2X_7$-P) or leucine ($P2X_7$-L) at this position, conferring high or low sensitivity to ATP, respectively. Significantly, NOD mice (a standard model for type 1 diabetes) (22) express $P2X_7$-P, the variant of $P2X_7$ stimulated by relatively low concentrations of ATP (21). We

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BzATP, benzoyl ATP; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; IL-1β, interleukin-1β; MHC, major histocompatibility complex; $P2X_7$-L, $P2X_7$ with leucine at position 451; $P2X_7$-P, $P2X_7$ with proline at position 451; PS, phosphatidylserine.

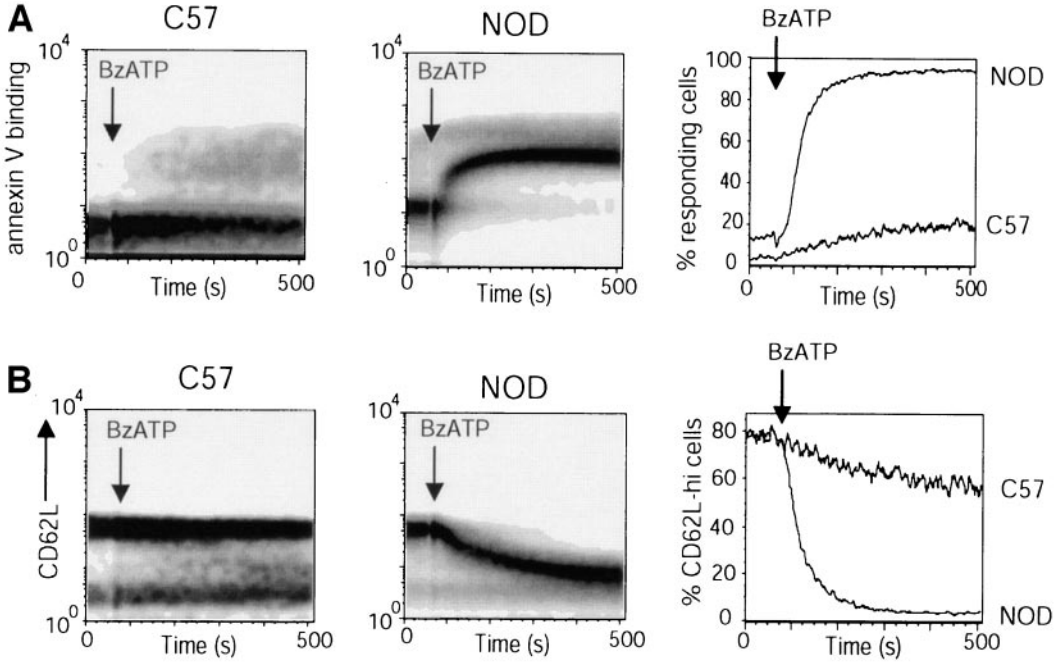

FIG. 1. $P2X_7$-stimulated PS translocation and CD62L shedding by C57BL/10 and NOD.E lymphocytes. Lymphocytes from C57BL/10 or NOD.E mice were labeled with anti-$CD4^{CYCHROME}$ and anti-$CD4^{PE}$ antibodies, respectively, to discriminate between $CD4^+$ cells during subsequent analysis; they were then labeled with anti-$CD62L^{FITC}$. The cells from the two mouse strains were mixed, and annexin V^{CY5} was added to allow simultaneous real-time monitoring in both populations of cells in a single tube. At the time indicated by the arrows, 150 μmol/l BzATP were added to stimulate the $P2X_7$ receptor. *A*: PS translocation, shown as a density plot against time of FL-4 fluorescence, as an indicator of binding of annexin V and therefore PS exposure. The data are also expressed in the far right panel as the percentage of responding cells. *B*: Shedding of CD62L, shown as a density plot against time of FL-1 (FITC) fluorescence. The data are also expressed in the far right panel as the percentage of cells retaining FL-1 fluorescence above a threshold level (35 MFU).

therefore questioned to what extent the expression of this allele is predictive for any given physiological response. We showed that $P2X_7$-stimulated loss of CD62L, exposure of phosphatidylserine (PS), and aponecrosis are all markedly accelerated in NOD lymphocytes compared with lymphocytes from mice not expressing this allele. In addition, although major histocompatibility complex (MHC) class I molecules were initially expressed at normal levels, they were rapidly shed by NOD lymphocytes after stimulation with $P2X_7$. Thus, low MHC class I expression by NOD lymphocytes is likely to be the consequence of in vivo or in vitro exposure of lymphocytes to high levels of ATP released from dying cells. Our data indicate that $P2X_7$ is a strong candidate susceptibility gene for NOD diabetes.

RESEARCH DESIGN AND METHODS

NOD mice carrying an H2-E transgene (NOD.E), as previously described (23), were bred in the Central Biological Services Unit at Hammersmith Hospital and were a generous gift from Prof. E. Simpson. Institute guidelines for the care of laboratory animals were followed. C57BL/10 mice were purchased from Harlan Olac (Bicester, U.K.). All mice used were age 6–14 weeks. All reagents used were obtained from Sigma (Poole, U.K.), unless otherwise stated.

Mesenteric lymph node cells were prepared from adult mice. Cell suspensions (10^7/ml) in phenol red–free Dulbecco's modified Eagle's medium (DMEM) were stained with the indicated combination of $CD4^{APC}$, $CD8^{APC}$, $CD8^{PE}$, $CD62L^{FITC}$ (Becton Dickinson, Sunnyvale, CA), or anti–$D^{b\text{-}FITC}$ (clone B220.249; gift from Dr. M. Millrain) antibodies to permit separation of different cell types during subsequent analysis, washed, and resuspended in DMEM. Cells were then equilibrated with propidium iodide together with annexin V^{FITC} or annexin V^{CY5} (Becton Dickinson), as appropriate, for 4 min and analyzed by flow cytometry on a FACScalibur machine. Data were analyzed using CellQuest (Becton Dickinson) or FlowJo (Treestar) software. Baseline fluorescence was established for ~1 min before benzoyl ATP (BzATP) was added. Debris was excluded from analysis based on forward and side scatter.

RESULTS

NOD T-cells exhibit increased $P2X_7$-dependent PS translocation and CD62L shedding. Stimulation of the $P2X_7$ receptor by ATP, or more potently by its analog BzATP, results in translocation of PS to the outer leaflet of the plasma membrane (12) and rapid shedding of the lymphocyte homing receptor CD62L (13). These responses are absent in lymphocytes from mice lacking $P2X_7$ (11). After brief stimulation of the $P2X_7$ receptor, exposure of PS is reversible and does not initiate programmed cell death (12). To compare the sensitivities of NOD background and C57BL/10 lymphocytes to $P2X_7$-dependent stimulation, we developed real-time flow cytometric assays in which the responses of the two populations could be compared in a single tube. To avoid potential influences resulting from disease, lymphocytes from NOD mice expressing a type 1 diabetes–protective H2-E transgene (NOD.E) (23) were used. Lymphocytes from NOD.E and C57BL/10 mice were labeled with PE- and CYCHROME-conjugated anti-CD4 antibodies, respectively, to allow cells from different mice to be distinguished and then mixed. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD62L antibody to monitor cell surface CD62L and with CY5-conjugated annexin V to monitor cell surface exposure of PS. After stimulation with BzATP, NOD.E lymphocytes exhibited markedly greater rates of PS translocation and CD62L shedding than C57BL/10 lymphocytes (Fig. 1).

$P2X_7$-stimulation results in shedding of MHC class I and cell shrinkage. It has been reported that MHC class I expression is low in NOD mice (24) and autoimmune patients (25–27), although these findings are controversial

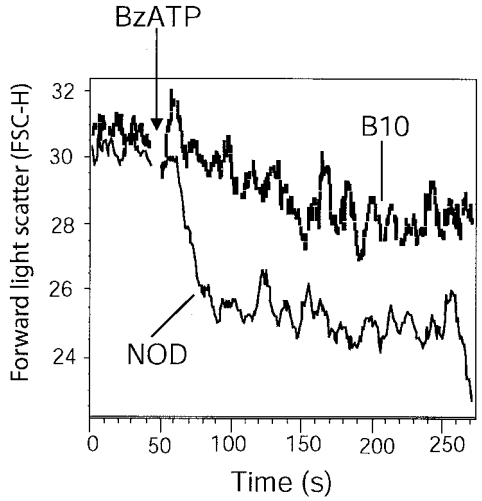

FIG. 2. P2X$_7$-stimulated cell shrinkage of C57BL/10 and NOD.E lymphocytes. Lymphocytes from C57BL/10 or NOD.E mice were labeled with anti-CD4^{CYCHROME} and anti-CD4^{PE} antibodies, respectively, to discriminate between CD4$^+$ cells during subsequent analysis. The cells from each mouse were mixed to allow monitoring of cell shrinkage (forward light scatter, FSC-H) of both populations of cells in a single tube in real time. Then, at the time indicated by the arrows, 150 μmol/l BzATP were added to stimulate the P2X$_7$ receptor. FSC-H is used routinely as a measure of the size of spherical cells (50), with its sensitivity being greatest when light is collected over an angle <10° as in the FACScalibur.

(28–30). Given the increased P2X$_7$ receptor–induced shedding of CD62L in NOD.E mice, we tested the hypothesis that P2X$_7$ stimulation can result in the loss of other cell surface molecules. In particular, we questioned whether low levels of cell surface MHC class I molecules (if apparent) might reflect P2X$_7$-dependent shedding and not defective constitutive expression. Consistent with this hypothesis, P2X$_7$ stimulation resulted in significant shedding of MHC class I (Fig. 2, Table 1) and CD4 and CD8 (data not shown). In each case, although the extent of shedding was less than that observed for CD62L, loss after P2X$_7$ stimulation was greater from NOD.E lymphocytes than from C57BL/10 lymphocytes. Importantly, although NOD.E and C57BL/10 lymphocytes were mixed and stained with anti–MHC class I (anti-D^b) antibody in a single tube to minimize experimental artifacts, initial cell surface expression of MHC class I (D^b) was not significantly different. Given the suggestion that decreased levels of MHC class I might reflect the smaller size of NOD lymphocytes (31), cell volume was also compared. There was little or no initial size difference between lymphocytes from the two strains, although P2X$_7$-stimulation resulted in the marked shrinkage (albeit followed by size recovery) of NOD.E, but not C57BL/10, lymphocytes (Fig. *2*).

TABLE 1
BzATP induces shedding of MHC class I: levels of MHC class I, as indicated by mean anti-D^b binding (FL-1 fluorescence)

	Geometric mean fluorescence		Δ
	$t = 0$	$t = 2$ min	(%)
NOD.E	35.8 ± 4.0	30.5 ± 3.0	14.0
C57BL/10	35.7 ± 7.7	34.1 ± 5.6	4.4

Data are means ± SD unless noted otherwise. Lymphocytes from C57BL/10 or NOD.E mice were labeled with anti-CD8^{APC} or anti-CD8^{PE} antibodies, respectively, to discriminate between CD8$^+$ cells during subsequent analysis, and then were labeled with anti-D$^{b\text{-}FITC}$. The cells from each mouse were mixed to facilitate real-time monitoring of shedding of D^b (MHC class I). At $t = 0$, 150 μmol/l BzATP was added.

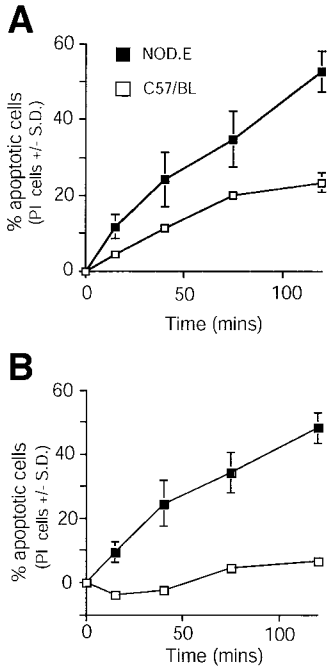

FIG. 3. P2X$_7$-stimulated aponecrosis of C57BL/10 and NOD.E lymphocytes. Lymphocytes from C57BL/10 ($n = 8$) and NOD.E ($n = 8$) mice were labeled with anti-CD4^{APC} and anti-CD8^{FITC} antibodies, equilibrated with propidium iodide (PI) as a measure of membrane permeability and therefore cell death, and analyzed by flow cytometry. At the time indicated by arrows, 150 μmol/l BzATP were added to stimulate the P2X$_7$ receptor. Graphs show the increase in the percentage of late aponecrotic cells at the times indicated compared with the percentage at $t = 0$. Data are shown for CD4$^+$ cells (*A*) and CD8$^+$ T-cells (*B*).

NOD lymphocytes show increased susceptibility to P2X$_7$-dependent aponecrosis. NOD lymphocytes have been reported to be resistant to a range of stimuli that induce programmed cell death (32–35). Because prolonged (though not brief) P2X$_7$ stimulation results in aponecrosis and NOD lymphocytes are relatively sensitive to P2X$_7$ stimulation, NOD lymphocytes might be expected to be sensitive, rather than resistant, to P2X$_7$-dependent cell death. Indeed, NOD lymphocytes (and thymocytes [data not shown]) were found to be significantly more susceptible than those from C57BL/10 mice to P2X$_7$-induced aponecrosis, as indicated by terminal cell membrane rupture and consequent uptake of propidium iodide (Fig. 3). Thus, the relative resistance or sensitivity of NOD lymphocytes to programmed cell death is stimulus dependent.

DISCUSSION

In rodents and humans, neonatal pancreatic β-cells undergo a wave of physiological cell death in a process known as tissue remodeling (5–7,36,37). Although usually benign, this event, at least in the NOD mouse strain, may trigger leukocytic infiltration and, eventually, autoimmune destruction and diabetes (5–7). Similarly, the diabetogenic activity of both coxsackie B4 virus and streptozotocin result from induction of damage to β-cells (8,9). These observations suggest that the early stage of disease may

reflect an aberrant response to immune "danger" signals. Two such signals released at sites of tissue damage are ATP and NAD, both of which activate the proinflammatory $P2X_7$ receptor (11,12,38). Indeed, given the central role of the $P2X_7$ receptor in IL-1β secretion and shedding of the lymphocyte homing receptor, CD62L, both of which are involved in the pathogenesis of NOD diabetes (14,15,18,20, 39), any factors affecting the expression, regulation, or innate sensitivity of this receptor may affect disease susceptibility. This raises the possibility that the gene encoding the NOD $P2X_7$ receptor might be a candidate susceptibility gene in type 1 diabetes.

Different mouse strains express one of two common alleles of the $P2X_7$ receptor (21), with that being expressed by NOD mice having a relatively low activation threshold. Because the expression of a single allelic difference (e.g., $P2X_7$-P) can be a poor predictor of complex physiological events, we compared the sensitivity of NOD and C57BL/10 lymphocytes to $P2X_7$-induced shedding of CD62L and MHC class I, externalization of PS, and aponecrosis. All were greater in NOD than in C57BL/10 lymphocytes. Thus, although the resistance of NOD lymphocytes to a range of stimuli that induce programmed cell death has been implicated in disease pathogenesis (32–35), our data indicate that sensitivity and resistance to programmed cell death is stimulus dependent; both properties may promote disease. It is important to note that we have used programmed cell death as a nonmechanistic term, defined as any one of a range of active processes stimulated through a physiological receptor by a relevant agonist or through the removal of a stimulus required for cell viability. However, there is no generally accepted terminology. Although it is often assumed that apoptosis implies caspase-dependent cell death, many studies on caspase-independent apoptosis exist. Furthermore, whereas apoptosis and programmed cell death are frequently used as synonyms, apoptosis has been used more recently to describe one end of a continuum of active mechanisms called programmed cell death (40). Nevertheless, it is clear that caspase involvement is not a good indicator of the physiological importance, or "programming," of a cell death pathway (40). Indeed, programmed cell death can occur in the complete absence of caspase activity; even within programmed cell death in response to a given stimulus, caspase dependence can be variable, perhaps dependent on cell type or differentiation state. ATP-induced cell death has, for example, been found independent of caspase-1 (41), except after lipopolysaccharide-dependent potentiation of macrophages (42). It is necessary, therefore, to determine to which forms of the spectrum of death pathways known as programmed cell death NOD lymphocytes are resistant or sensitive. Finally, because ATP is released from dying cells, $P2X_7$ receptor–induced cell death may be an important confounding factor in some studies of NOD apoptosis (34,43).

Our data clarify a long-standing controversy over the level of MHC class I molecules on lymphocytes in diabetes. According to one hypothesis, low expression of MHC class I in cells from NOD mice and patients with a variety of autoimmune conditions predisposes the subject toward disease (24–27). By contrast, other reports suggest that NOD MHC class I expression is only slightly reduced (28) or simply reflects a size difference with control cells (29) and that MHC class I levels are normal in patients with type 1 diabetes (30). Consequently, whether or why autoimmune disease is associated with reduced expression of MHC class I molecules is hotly disputed (26,44). We showed here that lymphocytes from type 1 diabetes–resistant NOD mice expressing an H2-E transgene (to minimize experimental results that are the consequence, not cause, of disease) express normal levels of MHC class I molecules, but that they are rapidly shed after $P2X_7$-stimulation. Loss of MHC class I molecules was moderate (15–20%), corresponding with the relative underexpression of MHC class I reported by others (28). Hence, although our data support the notion that lymphocyte MHC class I expression may be decreased in type 1 diabetes, they suggest that this phenotype is the result, not the cause, of disease. This interpretation is also consistent with observations of increased serum levels of CD62L, CD4, CD8, and MHC class I during inflammation (45–47).

One test of this hypothesis that the $P2X_7$-P allele predisposes toward NOD diabetes is whether the region encompassing $P2X_7$ (the distal end of chromosome 5) appears as a susceptibility locus in a backcross between NOD mice and any strain expressing the alternative, high-threshold $P2X_7$ allele. On this point, the data are inconclusive. Thus, although this region (identified by D5Mit43) was associated ($\chi^2 = 4.4$) with disease in the progeny of a backcross of (B10.H2^{g7} x NOD)F_1 with NOD mice (48), at this relatively low level of significance, the association may have been a false-positive result. Perhaps equally likely, however, is the possibility that the backcross analysis used underestimated the potential role of the $P2X_7$-P allele. Thus, given that $P2X_7$-P and -L alleles differ in their stimulation threshold, then depending on the level of extracellular ATP within the inflamed NOD pancreas, a single copy of $P2X_7$-P may have been sufficient to confer sensitivity to activation. If this were the case, $P2X_7$ would not have been detected as a susceptibility gene in the backcross used.

We have shown that, compared with normal lymphocytes, NOD lymphocytes are highly sensitive to $P2X_7$ receptor–stimulated programmed cell death and the shedding of CD62L and other cell surface markers such as MHC class I. These and other data indicate that $P2X_7$ is a good candidate susceptibility gene in murine autoimmune diabetes. It is attractive to speculate that defective clearance of neonatal apoptotic pancreatic β-cells by NOD mice (49) results in sufficient ATP release to stimulate the low-stimulation threshold allele of $P2X_7$ (P2X-P) and consequent secretion of IL-1β from macrophages and CD62L shedding and extravasation of T-cells. Subsequent T-cell–mediated killing of pancreatic β-cells and consequent release of ATP might then exacerbate the $P2X_7$-dependent inflammatory cascade. Although the $P2X_7$ receptor is encoded by a gene in a region weakly (and thus potentially falsely) associated with susceptibility to diabetes in a backcross analysis, its effects may be underestimated by such studies (48). Establishing a congenic NOD strain bearing the distal region of C57BL chromosome 5 will help determine the role that $P2X_7$ allelic variation plays in type 1 diabetes.

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