Research article

The P2X\textsubscript{7} receptor is a candidate product of murine and human lupus susceptibility loci: a hypothesis and comparison of murine allelic products

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Abstract

Systemic lupus erythematosus and its murine equivalent, modelled in the New Zealand Black and New Zealand White (NZB × NZW)\textsubscript{F1} hybrid strain, are polygenic inflammatory diseases, probably reflecting an autoimmune response to debris from cells undergoing programmed cell death. Several human and murine loci contributing to disease have been defined. The present study asks whether the proinflammatory purinergic receptor P2X\textsubscript{7}, an initiator of a form of programmed cell death known as aponecrosis, is a candidate product of murine and human lupus susceptibility loci. One such locus in (NZB × NZW)\textsubscript{F1} mice is \textit{lbw3}, which is situated at the distal end of NZW chromosome 5. We first assess whether NZB mice and NZW mice carry distinct alleles of the \textit{P2RX}\textsubscript{7} gene as expressed by common laboratory strains, which differ in sensitivity to ATP stimulation. We then compare the responses of NZB lymphocytes, NZW lymphocytes and (NZB × NZW)\textsubscript{F1} lymphocytes to P2X\textsubscript{7} stimulation. NZB and NZW parental strains express the distinct P2X\textsubscript{7}-L and P2X\textsubscript{7}-P alleles of \textit{P2RX}\textsubscript{7}, respectively, while lymphocytes from these and (NZB × NZW)\textsubscript{F1} mice differ markedly in their responses to P2X\textsubscript{7} receptor stimulation. NZB mice and NZW mice express functionally distinct alleles of the proinflammatory receptor, P2X\textsubscript{7}. We show that current mapping suggests that murine and human \textit{P2RX}\textsubscript{7} receptor genes lie within lupus susceptibility loci \textit{lbw3} and SLEB4, and we argue that these encode a product with the functional characteristics consistent with a role in lupus. Furthermore, we argue that aponecrosis as induced by P2X\textsubscript{7} is a cell death mechanism with characteristics that potentially have particular relevance to disease pathogenesis.

Introduction

Systemic lupus erythematosus (SLE) is a polygenic disease, although the genes contributing towards the disease are unknown. Several human susceptibility loci have been identified, with eight of the strongest candidates mapping to 1q23, 1q25-31, 1q41-42, 2q35-37, 4p16-15.2, 6p11-21, 12q24 and 16q12 [1]. Of the murine models, the New Zealand Black and New Zealand White (NZB × NZW)\textsubscript{F1} hybrid strain is widely studied due to its similarity to human disease and its female preponderance. In a study of (NZB × NZW)\textsubscript{F2} mice, eight susceptibility loci were identified [2]. In the case of the locus \textit{lbw3}, at the distal region of chromosome 5, homozygosity for the NZW-derived locus was associated with increased mortality at 12 months. Although originally mapped to 88 cM on murine chromosome 5 [2], more recent data locate the microsatellite used to define \textit{lbw3} at 81 cM (discussed later).

We have studied the properties of the proinflammatory purinergic receptor P2X\textsubscript{7}, encoded by a gene within the human SLE locus SLEB4 [3] at 12q24 (Ensembl Genome Browser: http://www.ensembl.org/Homo_sapiens/contigview?chr=12&vc_start=119982631&vc_end=120098041) and by the murine \textit{lbw3} region (Ensembl Genome Browser: http://www.ensembl.org/Mus_musculus/contigview?chr=12&vc_start=38090&vc_end=38108&highlight=ENSG00000004668)

BzATP = 2′,3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; DMEM = Dulbecco’s modified Eagle’s medium; ELISA = enzyme-linked immunosorbent assay; FCS = foetal calf serum; FITC = fluorescein isothiocyanate; IL = interleukin; NZB = New Zealand Black; NZW = New Zealand White; PCD = programmed cell death; PCR = polymerase chain reaction; PS = phosphatidylserine; SLE = systemic lupus erythematosus.
ceptibility. Moreover, PCD stimulated through the P2X
phisms in this gene would be predicted to affect lupus sus-
proinflammatory and induces PCD, functional polymor-
An allelic variatio n (P451L) of the cytoplasmic domain of
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In this respect, SLE is of particular interest. Not only is SLE
an inflammatory disorder, but it probably reflects, at least in
part, an immune response to debris of cells undergoing
programmed cell death (PCD). As P2X7 stimulation is
proinflammatory and induces PCD, functional polymor-
phisms in this gene would be predicted to affect lupus sus-
cceptibility. Moreover, PCD stimulated through the P2X7
receptor belongs to a category that bears many of the hall-
marks of ‘classic’ caspase-dependent apoptosis, but also to
other categories such as cytoplasmic vacuolization more
often associated with necrosis. Such cell death has some-
times been termed ‘aponecrosis’ [6]. Whereas removal of
‘classic’ apoptotic cells is believed to be immunologically
silent, necrotic cell debris is proinflammatory [7]. The effect
of intermediate forms of PCD such as aponecrosis, for
which clearance mechanisms have not been defined, is
unknown, yet such material potentially plays a significant
role in the pathogenesis of SLE (discussed later). Finally,
P2X7 stimulation results in rapid translocation of phosphati-
dyserine (PS) from the inner to the outer leaflet of the
plasma membrane, which is reversible if stimulation is brief
(and thus independent of cell death). As PS and associated
proteins are major targets of autoantibodies in SLE [8],
cells stimulated via the P2X7 receptor may be a significant
source of autoantigen in this disease.

An allelic variation (P451L) of the cytoplasmic domain of
the P2X7 receptor in commonly used mouse strains is asso-
ciated with significant differences in its sensitivity to the
ATP ligand [9]. These allelic forms with proline (P2X7-P)
and leucine (P2X7-L) at position 451 confer high sensitivity
and low sensitivity to stimulation by ATP, respectively.
While the NZW strain has been shown to express the more
responsive allele of P2X7 (P2X7-P) [9], that expressed by
the NZB strain is unknown. We show in the present article
that NZB mice and NZW mice express different alleles of the
proinflammatory receptor P2X7, and furthermore that NZW
lymphocytes are markedly more responsive to P2X7 stimu-
lation than those from NZB mice. Lymphocytes from (NZB
× NZW)F1 mice exhibited intermediate sensitivities to
P2X7-induced PS translocation and to PCD, but were as
sensitive to induction of CD62L shedding as those from
NZW mice, indicating a comparatively complex phenotypic
penetration. The results indicate that P2X7 is a strong can-
didate for being the product of the murine Ibw3 locus. As
the human P2RX7 gene maps close to SLEB4, we hypo-
thesize that similar polymorphisms may also contribute
towards human disease.

Methods

Mice
Male mice were purchased from Harlan-Olac (Bicester,
UK) and used at between 8 and 14 weeks. Institute guide-
lines for care of laboratory animals were followed. All stud-
ies received ethical review approval.

P2X7, PCRs
PCR amplification of the NZB mouse genomic sequence
encompassing the T1352C polymorphism [9] was per-
formed using the forward and reverse primers CCTGTCATGCTGTCCCTAT
and GCTTATGGAAGAGGCTTGAG for 30 cycles. PCR prod-
ucts were cloned using the TOPO TA cloning system (Inv-
trogen, Paisley, UK). Forty-three independent clones were
sequenced using an ABI PRISM Big Dye terminator ready
reaction kit (Applied Biosystems, Warrington, UK) and
were analysed on a 3700 DNA Analyser (Applied Biosys-
tems). Nucleotide and amino acid substitutions were num-
bered using the cDNA sequence accession number NM-
011027.

Reagents
Matrix metalloproteinase inhibitor III was from Calbiochem
(Nottingham, UK). Other reagents were from Sigma (Poole,
UK), unless stated. Diluents had no effect in any assay
used.

Flow cytometry
Mesenteric lymphocyte cells (10^7/ml) in phenol red-free
DMEM were stained with a combination of CD4APC,
CD4CYCHROME, CD4PE, CD8APC, CD8CYCHROME, CD8PE,
CD8RTC and CD62LRTC antibodies (Becton Dickinson,
Oxford, UK) as indicated, washed and resuspended in
DMEM. Cells were equilibrated with annexin VRTC or
annexin VCTS (Becton Dickinson) to assess cell surface PS
exposure, or with propidium iodide for 4 min to assess cell
death, and were analysed by flow cytometry on a FACScal-
ibur machine using CellQuest (Becton Dickinson) or Flowjo
(Tree Star, Ashland, OR, USA) software. Baseline fluores-
cence was established for approximately 1 min prior to
addition of 150 µM (unless otherwise stated) 2′,3′-O-(4-
benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP). Cells
were monitored for PS exposure or CD62L shedding con-
tinuously in real time for up to 9 min or were monitored for
uptake of propidium iodide, as indicated. All results are re-
presentative of at least three independent experiments.
IL-1β secretion

Spleens from NZW mice and NZB mice were disaggregated and erythrocytes were lysed (Puregene RBC lysis solution; Gentra Ltd, Minneapolis, MN, USA). Splenocytes (5 × 10⁶/ml) were then resuspended in DMEM supplemented with 10% FCS (Helena Biosciences, Sunderland, UK), and were stimulated with 10% FCS (Helena Biosciences, Sunderland, UK), and were stimulated with 2 µg/ml lipopolysaccharide. After 6 hours at 37°C the medium was removed and replaced with DMEM. Cells were then incubated at 37°C for 30 min with BzATP added as indicated. The supernatant was then collected and the cells and particulate matter were removed by centrifugation. Supernatants were then frozen at -20°C. IL-1β was quantified by ELISA (Quantikine mouse IL-1β kit; R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer’s instructions. Statistical significance was measured by Student’s t test.

PS translocation

PS is largely confined to the inner leaflet of the plasma membrane in healthy cells. Loss of lipid asymmetry, as evidenced by surface exposure of PS occurring prior to membrane breakdown, is generally assumed to be a marker of PCD. To enable the direct comparison of responses of cells from NZB mice, NZW mice and (NZB × NZW)F₁ mice in a single tube, lymphocytes from these three strains were stained with anti-CD4 CYCHROME, anti-CD4 PE and anti-CD4 APC, respectively, mixed and equilibrated with annexin V FITC. Thus labelled, cells could subsequently be distinguished by flow cytometric gating. Cells were stimulated with the P2X₇ agonist 2’-3’-O-(4-benzoylbenzoyl)-adenosine 5’-triphosphate (BzATP) at the time indicated by the arrow in (a). (a) Density plots of the rate of extracellular PS exposure in each cell population, as indicated by increased binding of annexin V FITC. (b) Corresponding percentage of cells bearing exposed PS in each population at a single timepoint (indicated by boxes in (a)).

Results

Real-time comparison of P2X₇-stimulated PS translocation and CD62L shedding by NZW, (NZB × NZW)F₁ and NZB lymphocytes

We initially confirmed that NZW mice are homozygous for the P2X-P allele of P2RX₇ [9] associated with high sensitivity to stimulation, and we showed that NZB mice are homozygous for the low sensitivity allele P2X-L (data not shown). These forms differ at a single amino acid (451). P2X₇ activation, stimulated by BzATP (Fig. 1), results in rapid externalization of PS and shedding of CD62L. We therefore developed real-time flow cytometric assays to directly compare the responses to P2X₇ simulation of NZB lymphocytes, NZW lymphocytes and (NZB × NZW)F₁ lymphocytes.

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CD62L shedding

Shedding of CD62L from T cells is a key event in lymphocyte migration to inflammatory sites [10] and is known to be induced by P2X₇ stimulation. Lymphocytes from NZB mice, NZW mice and (NZB × NZW)F₁ mice were differentially stained as already stated but were labelled with FITC-conjugated anti-CD62L in place of annexin V FITC to allow direct comparison of the rate of CD62L shedding in a single tube. P2X₇-stimulated CD62L shedding was apparent as a decrease in fluorescence in the FL-1 channel. While the rates of P2X₇-stimulated CD62L shedding were high and low in NZW lymphocytes and NZB lymphocytes, respectively (Fig. 2), interestingly the rate of CD62L shedding by (NZB × NZW)F₁ lymphocytes was indistinguisha-
P2X7-stimulated shedding of CD62L by lymphocytes. To enable the direct comparison of responses of cells from New Zealand Black (NZB) mice, New Zealand White (NZW) mice and (NZB × NZW)F1 (NZB/W) mice in a single tube, lymphocytes from these strains were stained with anti-CD4PE, anti-CD4CYCHROME and anti-CD4APC, respectively, mixed and stained with anti-CD62LFITC. Thus labelled, cells could subsequently be distinguished by flow cytometric gating. Cells were stimulated with the P2X7 agonist 2′3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP) at the time indicated by the arrow in (a). (a) Density plots of the rate of CD62L shedding in each cell population, as indicated by decreased binding of anti-CD62LFITC. (b) Corresponding levels of cell surface CD62L in each population (NZB, red line; NZW, green line; NZB/W, black line) immediately preceding P2X7 stimulation (indicated by left hand gates in (a)) or 7 min after P2X7 stimulation (indicated by right hand gates in (a)). (c) Effect of a broad inhibitor of metalloproteinases on loss of CD62L. Lymphocytes from NZW mice were stained with anti-CD4CYCHROME and anti-CD62LPE, and the rate of loss of CD62L was assessed by flow cytometry. Cells were stimulated with BzATP in the presence or absence of 10 µM metalloproteinase inhibitor at the time indicated by an arrow. Shedding of CD62L is indicated by decreased binding of anti-CD62LPE. MMP, matrix metalloproteinase.

P2X7-stimulated shedding of CD62L thus appears dominant with respect to CD62L shedding, indicating that factors downstream of P2X7 stimulation contribute to this phenotype. That loss of CD62L reflects shedding and not decreased cell surface expression through other mechanisms is evidenced by its blockade by an inhibitor of matrix metalloproteinase [11] (Fig. 2c).
P2X<sub>7</sub>-induced secretion of IL-1β

Stimulation of the P2X<sub>7</sub> receptor on lipopolysaccharide-stimulated monocytes and macrophages promotes secretion of the proinflammatory cytokine IL-1β [5,12], which may therefore be expected to differ between mice bearing P2X<sub>7</sub>-L or P2X<sub>7</sub>-P receptors. Indeed IL-1β secretion by NZW splenocytes stimulated in vitro with lipopolysaccharide and BzATP exceeded that by cells from NZB mice (P < 0.05 at 50, 100 and 150 µM BzATP; Fig. 3). Elevated IL-1β secretion by NZW splenocytes was apparent even in the absence of BzATP (although slightly below statistical significance), suggesting that inadvertent stimulation of the high (but not low) sensitivity P2X7 receptor may have occurred through cell death and the consequent release of ATP during cell preparation (spleen disaggregation and erythrocyte lysis). In one NZW splenocyte preparation exhibiting particularly high IL-1β secretion, cells were refractory to further stimulation of the P2X7 receptor in vitro.

P2X<sub>7</sub>-induced PCD of NZW and NZB lymphocytes

Several lines of evidence indicate that lupus reflects an autoimmune response to debris from cells undergoing PCD. Although stimulation of P2X<sub>7</sub> results in rapid PS translocation, the effects are reversible if exposure to the agonist is brief [4]. Only prolonged treatment with agonist results in PCD. Translocation of PS following P2X<sub>7</sub> activation cannot therefore be used as a direct measure of irreversible commitment to PCD. To measure PCD following prolonged P2X<sub>7</sub> stimulation, we therefore compared the rate of terminal membrane breakdown (indicated by propidium iodide (PI)) in NZW lymphocytes, NZB lymphocytes and (NZB × NZW)<sub>F1</sub> lymphocytes (Fig. 4). P2X<sub>7</sub> stimulation resulted in significant PCD in all populations tested, with the order of sensitivity NZW > (NZB × NZW)<sub>F1</sub> > NZB, consistent with the high responder status of the NZW cells and the dominance of the NZW-derived P2RX<sub>7</sub> allele in this response.
Discussion

Stimulation of the proinflammatory haematopoietic P2X7 receptor [5] results in IL-1β secretion, in high rates of PCD [4] and in CD62L shedding [13], each of which is associated with human SLE [14-16]. The P2X7 receptor therefore has the characteristics of a candidate lupus susceptibility gene product. Moreover, the gene encoding human P2X7 is located within a region (12q24; Ensembl Genome Browser: http://www.ensembl.org/Homo_sapiens/contigview?chr=12&vc_start=119982631&vc_end=12009&highlight=ENSG00000089041) recently identified and confirmed in Hispanic and European-American Families as a lupus susceptibility locus, designated SLEB4 [3].

A polymorphism in the cytoplasmic domain of the P2X7 receptor of common mouse strains is associated with differential responsiveness [9]. While most strains, including NZW mice [9], possess proline in amino acid position 451, we showed that NZB mice express P2X7 with lysine at this position and that the variant confers markedly decreased sensitivity to P2X7 stimulation. Notably, the murine P2RX7 gene is encoded by a gene on chromosome 5 within a region designated lbw3 due to the identification of a NZW-derived susceptibility locus conferring increased mortality at 12 months [2]. While susceptibility regions are broad, the microsatellite marker D5Mit101 (defining lbw3 [2]) was in the original study mapped to 88 cM on chromosome 5, which may have discouraged identification of P2RX7 as a candidate susceptibility gene. Current mapping data, however, show this marker located at 81 cM – Mouse Genome Informatics http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=6077a and Ensembl Genome Browser http://www.ensembl.org/Mus_musculus/markerview?marker=D5Mit101. As the marker D5Mit118 that is adjacent to P2RX7 is located at 67 cM – Ensembl Genome Browser http://www.ensembl.org/Mus_musculus/contigview?&chr=5&vc_start=119870152&vc_end=11987 and Mouse Genome Informatics http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=mark erl&key=6095 – the two are approximately 14 cM (or 19 Mb) apart (120 Mb versus 139 Mb), easily within the 20 cM distance used by Kono and colleagues [2] to define coverage by markers in their study.

Although gene polymorphisms may have unpredicted effects, other than P2RX7, there appear to be few candidate susceptibility genes (based on lymphoid expression and protein activity) in the region described by lbw3. However, other candidates might include those encoding: Ink, an adaptor protein in T-cell signalling (65.0 cM) [17]; P2X4, a purinergic receptor (65.0 cM) whose activity is assumed primarily to be neuronal, but which is also expressed (at least at the level of mRNA) in lymphocytes [18]; shp2, a tyrosine phosphatase [19] (~66 cM [118.6 Mb]); and FLT3 (CD135, 82.0 cM), a tyrosine kinase expressed in haematopoietic cells [20]. None of these has been reported to be polymorphic between NZB mice and NZW mice.

It is widely thought that lupus reflects an autoimmune response to cells undergoing PCD. Aberrant responses to such debris may reflect qualitative or quantitative abnormalities; for example, if its handling is defective and/or following exposure to increased levels of 'apoptotic' material. Both have been reported to contribute to human SLE [15,16]. That prolonged stimulation of the P2X7 receptor induces PCD is therefore of particular note. However, multiple pathways of PCD exist. While 'apoptosis' and 'PCD' are frequently used as synonyms, 'apoptosis' is often used to imply caspase-dependent cell death. Nevertheless, caspase involvement is not a good indicator of the physiologic importance, or 'programming', of a cell death pathway, and consequently classic 'apoptosis' may describe one end of a continuum of active PCD mechanisms [21]. Hence, in principle, a defect in one of many PCD pathways, rather than increased susceptibility to PCD per se, may be sufficient to increased the burden of cellular debris and hence the susceptibility to lupus. Indeed, that SLE may reflect an autoimmune response to debris from 'apoptotic' cells, despite clearance of such material being thought generally immunologically silent [7], has been a conundrum.

To reconcile these findings it has been suggested that, in SLE, mechanisms for removing apoptotic debris are overloaded, with remaining cells undergoing secondary necrosis, and/or that apoptotic cells have some immunostimulatory properties [7]. We suggest the additional possibility that different forms of PCD may give rise to debris with different degrees of immunogenicity. It is therefore necessary to dissect distinct PCD pathways to assess the potential effects that defects have on the disease process. It is attractive to speculate that P2X7-induced aponecrotic debris, perhaps due to the catastrophic nature of its generation or the apparent differences in cell dismantling, may be more necrotic than apoptotic in character and thus be immunostimulatory. Such material may either promote responses to surrounding 'apoptotic' cells and/or directly stimulate autoimmune responses to itself (if lupus autoantigens are appropriately packaged in P2X7-induced PCD). P2X7-receptor-induced PCD is therefore potentially a source of lupus autoantigens or may represent a catastrophic form of cell death that overwhelms the host's ability to clear such material.

We therefore suggest the following involvement of the P2X7 receptor in SLE. ATP exists at very high concentrations in normal cells (5–10 mM), and is released upon cell death before its rapid breakdown by ATPases. Consequently, extracellular concentrations of ATP, although normally low, are transiently increased at sites of tissue...
damage. Stimulation of P2X<sub>7</sub> occurs at sufficient concentrations of ATP, resulting in secretion of IL-1β and in CD62L shedding within minutes. P2X<sub>7</sub> stimulation thus acts to promote the inflammatory response. The resulting lymphoid infiltration leads to additional lymphocyte-mediated cell death, and to consequent ATP release, exacerbating the P2X<sub>7</sub>-driven inflammatory cycle. Indeed, given sufficient tissue damage, prolonged stimulation of P2X<sub>7</sub> itself induces PCD, further adding to the cycle of ATP release and destruction. Release of autoantigens within P2X<sub>7</sub>-stimulated aponecrotic debris may also contribute to a breakdown in self-tolerance and initiation of autoimmunity.

While one must make the proviso that little is known at the moment about the level of ATP released at sites of tissue damage, its rate of decay and how these may vary between pathological conditions including SLE, we suggest it is reasonable to hypothesize that polymorphisms within P2X<sub>7</sub> can influence the pathogenesis of lupus. Importantly, there are a number of polymorphisms within P2X<sub>7</sub> that affect its activity. The Ile-568 to Asn [22], Arg307 to Gln [23], and Glu496 to Ala [24] polymorphisms therefore all result in reduced function of human P2X<sub>7</sub>, and might each be hypothesized to result in decreased severity of SLE.

Conclusions
In summary, we have shown that polymorphism of the P2X<sub>7</sub> receptor between NZW and NZB strains is associated with marked differences in P2X<sub>7</sub>-stimulated proinflammatory responses, consistent with high responsiveness and low responsiveness previously reported for the two alleles. We also show that current genetic mapping indicates that the P2RX<sub>7</sub> gene is located within the region defined as lbw3 and is a therefore a strong candidate for being the product of this lupus susceptibility locus. Furthermore, as the human gene maps very close to SLEB4, we hypothesize that polymorphisms within P2RX<sub>7</sub> may also contribute to human disease. Stimulation of the P2X<sub>7</sub> receptor is proinflammatory and induces a form of cell death known as apoptosis, which exhibits several characteristics of apoptosis. We therefore suggest that the P2X<sub>7</sub> receptor and gene have the functional and positional characteristics suggestive of a role in the pathogenesis in SLE, and that the potential of the cell death mechanism aponecrosis to contribute to disease warrants study.

Competing interests
The author(s) declare there are no competing interests.

Authors’ contributions
JIE conceived the study, carried out the flow cytometric and IL-1β secretion experiments, and wrote the first draft of the manuscript. JHM designed allele-specific primers and typed the P2RX<sub>7</sub> genes of NZB mice and NZW mice, and contributed to drafting of the manuscript. CFH contributed to the design of experiments and drafting of the manuscript. All authors read and approved the final manuscript.

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