BALB/c.CBA/N mice carrying the defective Btk<sup>xid</sup> gene are resistant to pristane-induced plasmacytomagenesis

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Abstract

The X-chromosome from the CBA/N mouse which carries the defective Bruton's tyrosine kinase (Btk) allele (X<sup>xid</sup>) has been introgressively backcrossed onto the plasmacytoma (PCT) induction-susceptible BALB/cAN. Inbred BALB/c.CBA/N-xid/xid (C.CBA/N) mice raised and maintained in our conventional colony were given three 0.5 ml injections of pristane and were highly refractory to PCT induction. Only one PCT was found among 59 mice followed for >300 days. Twenty mice were examined at day 200 for foci of plasma cells in the oil granuloma. Ten mice had small foci of plasma cells, most of which were plasmacytotic, embedded in the inflammatory oil granuloma. In one there were multiple foci, but most of the mice had only one or two foci. F<sub>1</sub> hybrid X<sup>xid</sup>Y males derived from CBA/N females crossed to BALB/cAnPt were also resistant to PCT induction, while heterozygous and homozygous XY males were susceptible. C.CBA/N mice can develop extensive mucosal plasma cells as well as plasma cell accumulations in oil granuloma tissue, but the precursors of these plasma cells do not give rise to PCT in genetically susceptible hosts. The failure of C.CBA/N mice to develop PCT is probably due to the elimination of B cell clones that can be perpetuated by repeated exposure to thymus-independent type 2 antigens.

Introduction

In 1993 the genes for XLA (X-linked agammaglobulinaemia) in humans and xid (X-linked immunodeficiency) in mice were cloned and sequenced, and found to code for a non-receptor protein tyrosine kinase Bruton's agammaglobulinemia tyrosine kinase (Btk) (1–4). The wild-type gene product was in the Btk/Tec subfamily of src kinases that contained pleckstrin homology (PH), TH (Tec), SH2, SH3 and kinase domains (5). The xid mutation in the mouse produces a critical Arg to Cys replacement in the PH domain that blocks the activation of this kinase and its subsequent effector functions, leading to DNA synthesis (6,7), cell cycle completion in B cells and induction of anti-apoptotic proteins (8–11).

In humans the XLA mutations produce a severe immunodeficiency, a developmental arrest in the pre-B stage, and a loss of peripheral B cells and plasma cells (12). In the mouse the xid mutation is associated with defective immune responses to thymus-independent type 2 antigens (TI-2) (see reviews in 13,14). B cells that recognize the polyvalent TI-2 antigens are activated into DNA synthesis and cell division by cross-linking the slgM receptors (B cell receptor). Most of these antigen–antibody interactions are associated with low binding affinities and polyreactivity (15). The epitopes on TI-2 antigens are widely distributed in nature and thus can be of autogenous origin (16), many are polysaccharides (13). Subsets of mature peripheral B cells that react to TI-2 antigens are activated in DNA synthesis and cell division by cross-linking the slgM receptors (B cell receptor). Most of these antigen–antibody interactions are associated with low binding affinities and polyreactivity (15). The epitopes on TI-2 antigens are distributed in nature and thus can be of autogenous origin (16), many are polysaccharides (13). Subsets of mature peripheral B cells that react to TI-2 antigens are characterized by an IgM<sup>high</sup>, IgD<sup>low</sup>, B<sub>220</sub><sup>low</sup>, CD5<sup>+</sup> or CD5<sup>−</sup> phenotype, and are located in the peritoneal cavity (PerC) and spleen (17). These B cells are commonly called B-1 cells, are thought to develop predominantly from fetal hematopoietic stem cells and are produced de novo.

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Plasma cells (28). Over 95% of the PCT have consistent
is usually preceded by the appearance of foci of proliferating
where PCT develop morphologically (27,28). PCT formation
injected into the peritoneal space where it induces the forma-
silicone gels (26). The pristane model of plasmacytoma-
materials: solid plastic objects (23,24), paraffin oils, e.g.
susceptible BALB/cAn mice by several different kinds of
receptor (22).
B-1 cells in the mouse requires signaling through the B cell
marrow (19). The self renewal process and maintenance of
maintained by self-renewal of VβDβJβ/VβLβ rearranged cells
(15,18). Thereafter they are
expressive backcrosses was then converted to a homozygous
previously described (28).
Methods
The BALB/cAn.CBA/N-xid/xid (C.CBA/N) strain was origin-
ated by a breeding strategy that permitted the introduction of the CBA/N X chromosome onto a different inbred back-
ground (31). Essentially, CBA/N-xid/xid females were mated
to BALB/c to obtain F₁ hybrid males X<sup>xid</sup>Y, which were then
mated back to CBA/N to obtain backcross X<sup>xid</sup>X<sup>xid</sup> female
progeny. These homozgyous females were then mated to
BALB/cAn to continue the introgressive backcrossing and the
process repeated for 20 generations. In this scheme
autosomal crossing over occurs, but there is no opportunity
for crossing over between BALB/c and CBA/N X chromo-
somes. This raises the possibility that other PCT resistance
genes of CBA X-chromosomal origin could also be introduced in
these congenics. This congenic strain made by 20 introgres-
sive backcrosses was then converted to a homozygous
strain. C.CBA/N mice were introduced into our AAALAC
approved barrier-protected conventional mouse colony at
PerImmune (Rockville, MD) and continuously inbred. All mice
were maintained on Purina Mouse Chow 5001 (PMI Feeds,
St Louis, MO) and acidified tap water <i>ad libitum</i>. The mice
were housed in shoe-box-type cages, four to five mice per
cage. The bedding used in cages contained cedar shavings
to control for mites. The colony harbors common potential
pathogens such as Sendai virus, Mouse Hepatitis Virus and
pin worms.

Pristane was purchased from Aldrich (Milwaukee, WI). To
induce PCT the mice were injected with three 0.5 ml doses
given i.p. on days 0, 60 and 120 performed under Animal
Use Protocol no. 190. After day 120 ascites or peritoneal
exudate was aspirated with 25 gauge needles every 3 weeks.
Cytofuge preparations were made and the cells were stained
with Wright's Giemsa stain. Plasmacytomas were diagnosed
by finding ≥10 atypical plasma cells per slide. In most cases
two diagnoses per mouse were made. In most fully developed
PCT the cytofuge reparations contained >100 PCT cells.
When the mice were autopsied, the mesenteric and omental
tissues were excised in large blocks after stripping away the
intestines. These were then cut after fixation in Fekete's
modification of Tellyesniczky's fixative (70% ethanol:form-
alin:glacial acetic acid in 20:2:1 parts) into 2–10 mm
fragments. All of the 4 µm sections were stained with
hematoxylin & eosin and scanned for the presence of
plasmacytic foci or PCT. Selected cases were immuno-
stained to determine the heavy chain class by methods
previously described (28).

Results
C.CBA/N mice male and female mice were injected with
pristane on days 0, 60 and 120. Of the 79 mice, 20 were
autopsied on day 201, another 20 on day 303, and the
remaining 39 mice were watched until day 397 for the
development of ascites and the appearance of PCT cells in
ascites, and then examined for evidence of plasmacytoma-
Table 1. Plasmacytoma induction studies in BALB/cAn.CBA/N mice given three 0.5 ml i.p. injections of pristane on days 0, 60 and 120

<table>
<thead>
<tr>
<th>Cross or strain</th>
<th>Sex</th>
<th>XY genotype</th>
<th>No. mice</th>
<th>No. PCT (%)</th>
<th>No. mice with foci</th>
<th>Latent period (days)</th>
<th>No. observed days</th>
</tr>
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<tbody>
<tr>
<td>C.CBA/N</td>
<td>F</td>
<td>X&lt;sup&gt;xid&lt;/sup&gt;x&lt;sup&gt;xid&lt;/sup&gt;</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>201</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>X&lt;sup&gt;xid&lt;/sup&gt;Y</td>
<td>10 (1)</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>X&lt;sup&gt;xid&lt;/sup&gt;x&lt;sup&gt;xid&lt;/sup&gt;</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>C.CBA/N</td>
<td>M</td>
<td>X&lt;sup&gt;xid&lt;/sup&gt;Y</td>
<td>19</td>
<td>1</td>
<td>0</td>
<td>(351)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>397</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>X&lt;sup&gt;xid&lt;/sup&gt;x&lt;sup&gt;xid&lt;/sup&gt;</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>BALB/cAnPt</td>
<td>M</td>
<td>–</td>
<td>148</td>
<td>137 (46)</td>
<td>–</td>
<td>220 (120–300)</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>–</td>
<td>150</td>
<td></td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B/c×C.CBA/N&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
<td>YX</td>
<td>28</td>
<td>18 (64)</td>
<td>–</td>
<td>233 (152–307)</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>XX</td>
<td>23</td>
<td>10 (43)</td>
<td>–</td>
<td>229 (168–307)</td>
<td>337</td>
</tr>
<tr>
<td>C.CBA/N×B/c&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
<td>X&lt;sup&gt;xid&lt;/sup&gt;Y</td>
<td>44</td>
<td>1 (2.3)</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>F</td>
<td>X&lt;sup&gt;xid&lt;/sup&gt;X</td>
<td>25</td>
<td>9 (36)</td>
<td>–</td>
<td>213 (189–277)</td>
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<sup>a</sup>A total of 22 foci were found; most were typical of the one shown in Fig. 2(D). One mouse had seven plasmacytotic foci; one mouse had a plasmacytotic and an atypical focus; one mouse had a single atypical focus.

<sup>b</sup>This mouse had multiple plasmacytotic foci.

<sup>c</sup>Latent period of the single observed tumor.

<sup>d</sup>B/c or C = BALB/cAnPt.

generation (Table 1 and Fig. 1). Tissue sections of the mesenteric and omental oil granuloma were surveyed for the presence of plasma cells, PCT and foci which contained aggregates of >50 plasma cells (Fig. 2). Foci that contained differentiated plasma cells with round nuclei were classified as plasmacytotoc foci (32) (Fig. 2B and D). Some of these were still present when the tissue blocks were recut, stained for IgA. In contrast, the plasma cells in foci that contained atypical plasma cells (atypical foci) were larger in size and hyperchromatic, had more irregular nuclei, and resembled the plasma cells in fully developed PCT (Fig. 2A). The oil granulomatous tissue at 200 days was highly cellular with many macrophages, neutrophils and fibroblasts, and was typical of the inflammatory phase of oil granuloma development in BALB/cAn mice. By day 300 and after, the oil granuloma became progressively more sclerotic and calcified after 300 days. IgA cells were seen scattered through the granulomatous tissues and were abundant in the lamina propria sections of the gut of xid mice (Fig. 2C). IgA immunostained plasma cells in the lamina propria in ileal villi were counted. Only profiles of villi that included the base (crypt) and tip of the villus were used. Forty-two ileal villi from xid mice were counted and found to contain an average of 49 cells (range 30–90) per villus. IgA-producing cells in the lamina propria tissue of villi from pristane-injected wild-type BALB/cAn mice averaged 33.6 cells per villus with a range of 18–64 cells. This suggested that pristane-treated xid mice contained more IgA than wild-type controls. The significance of this difference must await further study.

Among the 20 mice autopsied on day 201, 10 (Table 1) had 22 plasmacytotic foci (each with >50) composed of mature plasma cells (Fig. 2B) and in one, seven plasmacytotic foci were found. Two atypical foci were seen (Fig. 2A): one mouse had a single focus of atypical plasma cells and in the other the atypical focus was associated with a plasmacytotic focus. One mouse in the day 303 sample had multiple foci of differentiated plasma cells. A single advanced PCT was found at day 351; the cells in this tumor were atypical IgA-secreting plasma cells characteristic of PCT and extensively invaded the oil granulomatous tissue. Control BALB/cAnPt mice that were given similar doses of pristane from eight different experiments run during the same period as the C.CBA/N mice developed an overall incidence of 46% PCT (range 30–62.5%) by day 300. The results indicate that C.CBA/N mice were highly refractory to PCT induction by a potent PCT induction regimen but were able to develop typical oil granulomatous tissue that contained plasma cells and focal accumulations of plasma cells.

We previously found that CBA-T6/T6 and (CBA-T6/T6×BALB/c)F<sub>1</sub> mice were resistant to PCT induction by pristane, indicating that CBA-T6/T6 carries strong resistance genes (33). To determine if the C.CBA/N congenic mouse carried PCT resistance genes other than xid, C.CBAN mice were reciprocally crossed with BALB/cAnPt (Table 1 and Fig. 3) and injected with pristane. The X<sup>xid</sup>Y males were resistant and resembled C.CBA/N males. XY males were the most susceptible. The high incidence of PCT in the XY males indicates that autosomal CBA/N resistance genes were not present in the C.CBA/N strain. The XX<sup>xid</sup> and X<sup>xid</sup>X females developed an intermediate incidence of PCT, 43 and 36% respectively. This partial resistance could be due to additional resistance genes on the CBA/N X<sup>xid</sup> chromosome or to the effects of the X<sup>xid</sup> chromosome. Our studies cannot distinguish between these two possibilities.

**Discussion**

BALB/cAnCBA/N (xid/xid) mice are resistant to PCT induction by three 0.5 ml doses of pristane. Only one PCT was found among 59 mice followed for 300–397 days. To determine if the C.CBA/N congenic might carry autosomal resistance genes to PCT induction, BALB/cAnPt males were crossed to C.CBA/N and the F<sub>1</sub> hybrid males (X<sup>xid</sup>Y) were again found to be resistant to PCT induction, while the females (X<sup>xid</sup>X, XX<sup>xid</sup>) showed a substantial but partial susceptibility (36–43%). Overall, the results did not indicate the presence of autosomal resistance genes of CBA/N origin. The possibility of another weak PCT resistance gene on the X chromosome...
of CBA/N origin, however, could not be ruled out. The results indicate the Btk<sup>xid</sup> gene was principally responsible for the resistance to PCT induction.

Histological studies revealed an abundance of plasma cells in the lamina propria of the intestinal villi. Further, the oil granulomatous tissues examined at day 200 contained isolated plasma cells and in 10 of 20 mice at day 200 plasmacytotic foci were found, i.e. aggregates of $\geq 50$ plasma cells. Thus, plasma cell formation can occur in these immunodeficient mice at the very sites where plasma cells undergo progression to neoplasia.

How can the resistance to PCT induction in C.CBA/N mice be explained? The basic explanation is that the B lymphocyte precursors of PCT in wild-type BALB/cAn mice are missing in xid mice because of developmental clonal elimination mechanisms resulting from defective Btk activation.

The defective xid allele has been reported to interfere with the transmission of signals for DNA synthesis and completion of the cell cycle in response to antigen receptor cross-linking by TI-2 antigens (7,22). Although B cells from xid mice do respond weakly to B cell receptor cross-linking and enter the G<sub>1</sub> phase, they fail to activate cyclin/cdk kinases (7) and complete the cell cycle (6). In addition, Btk-deficient cells do not up-regulate the anti-apoptotic proteins Bcl-2 (10) and Bcl-x<sub>L</sub> (6,34). Thus, B cells in xid mice which respond to cross-linking of their BCR via natural TI-2 antigens could become arrested in the cell cycle, trigger the apoptotic machinery and be eliminated as described and suggested by several studies (6,7,9,10).

An alternative way to explain how the xid mutation modifies the development of B cells is that all of the B cells produced in a xid mouse that reach the periphery are short lived and rapidly eliminated. In a recent re-evaluation of the effects of xid on B cell development, Klaus et al. (35) have provided evidence that all B cell compartments may be affected. This suggests the possibility that B cells in xid mice could produce only short-lived B cells and little long-term memory.

Specifically, then, does the xid mutation interfere with the clonal longevity in B-2 lymphocytes that are activated by T-dependent antigens? This question cannot yet be definitively answered. There is evidence that T-dependent responses in conventional B-2 cells are reduced in CBA/N mice (36–40). When thymus-dependent immune responses were induced in (CBA/N×C57BL/6)F<sub>1</sub> hybrids, Ridderdstad et al. (39) found a 10-fold reduction in the primary responses in the male (X<sup>xid</sup>) progeny, but qualitatively and quantitatively wild-type (normal) secondary responses. In other studies the btk<sup>xid</sup> mutation interfered with the formation of long-lived cells; however, the deficit in older mice was much reduced, indicating there was a recovery or a compensating B cell replacement mechanism in old xid mice (38).

One of the cardinal defects in xid mice is a deficiency of a subset of B cells that react to TI-2 antigens (i.e. B-1 cells).
TI-2 antigens are of autogenous origin as well as being common antigens in ubiquitous microbial species. Thus, B-1 cells, which are self-replenishing (20,41) and activated naturally by these antigens, are clones that may have undergone repeated cycles of antigen activation and quiescence. Examples of clonal expansions of CD5+ clones have been described in NZB mice (42). Other direct evidence that clonal longevity exists is found in autoimmune mice where clonal history can be traced through Igomatic hypermutation (43,44). This accumulation of successive Igomatic mutations is assumed to result from repeated interactions with auto-antigens and each mutation may occur in daughter cells of an original clone, separated in space and time. When the xid mutation was introduced into (NZW×NZB)F1 hybrid mice, it was found that immune complex glomerulonephritis and anti-DNA antibodies were dramatically reduced (45), suggesting that clonal evolution did not occur. If a similar process of repeated antigenic exposure occurs in BALB/cAn mice, then it becomes possible that a single clone could accumulate mutations affecting proliferative behavior (oncogenic diversification).

B-1 cells have been reported to be prone to develop clonal expansions both in autoimmune NZB (42) and C57BL/6 mice (46). This phenomenon may be linked to the self-renewing capabilities of B-1 cells in the mouse. The findings in the present study strongly suggest that the reduction in B cells that can respond to TI-2 antigens eliminates the lymphocytic precursors of the PCT cells. These appear to be B-1 cells.

Acknowledgements

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>PCT</td>
<td>plasmacytoma</td>
</tr>
<tr>
<td>XLA</td>
<td>X-linked agammaglobulinemia</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>TI-2</td>
<td>type 2 thymus-independent antigens</td>
</tr>
<tr>
<td>xid</td>
<td>X-linked immunodeficient</td>
</tr>
<tr>
<td>PerC</td>
<td>peritoneal cavity</td>
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</table>

References


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