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# Genes on the X chromosome affect development of collagen-induced arthritis in mice

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## SUMMARY

Susceptibility to collagen-induced arthritis (CIA) in mice is associated with a class II gene in MHC ($A^q$) but also with unknown genes outside MHC. Investigated here is the influence of genes on the X chromosome as well as the role of the X-linked immunodeficiency (xid) mutation. Reciprocal male $F_1$ hybrids, bred to be heterozygous or homozygous for $A^q$, showed a genetic influence in their susceptibility to develop CIA. Crosses were made between B10.G, B10.Q, DBA/1, SWR/J, C3H.Q and CBA/Ca, and all $F_1$ mice were castrated to avoid sex hormone modulation of the susceptibility. A differential timing of arthritis onset and severity were seen in the reciprocal $F_1$ males. An exception was the reciprocal $F_1$ male offspring from SWR/J and DBA/1 crosses which differed only in disease severity late in the course of the disease. The female $F_1$ crosses did not show the same pattern of differential susceptibility to CIA as the $F_1$ males. To exclude the possible influence of the Y chromosome, $F_1$ males of reciprocal crosses were back-crossed to the parental strains creating offspring with equal X chromosomes but divergent Y chromosomes. No difference in development of arthritis was observed in these. The influence of the xid mutation was investigated next. The xid loci from the CBA/N mouse was bred into DBA/1 strain which is highly susceptible to CIA. The resulting congenic DBA/1-xid strain was resistant to induction of CIA and did not develop an antibody response to type II collagen. We conclude that polymorphic genes on the X chromosome modulate susceptibility to CIA. The results from the experiments with mice carrying xid mutations confirm that such immune modulating genes exist on the sex chromosomes.


**Keywords** type II collagen-induced arthritis X chromosome-linked immune deficiency

## INTRODUCTION

Immunization of mice with type II collagen (CII) induces a chronic, systemic polyarthritis, resembling human rheumatoid arthritis (RA) [1,2]. Peripheral joints are preferentially affected and show a similar type of histopathology to RA. In addition, both RA and CII-induced arthritis (CIA) are clearly associated with MHC genes [2,3]. Mice carrying the $A^q$ class II gene are particularly susceptible to CIA [4]. However, outside MHC, little is known about genetic influence in both RA and CIA, and both diseases are of polygenic susceptibility [5–7]. One possible non-MHC gene influence could be mediated by sex chromosomes. Females have a three-times higher risk of developing RA than males. Theoretically this could be dependent on an influence by the Y chromosome, or polymorphic genes on the X chromosomes; females are mosaics of two X chromosomes whereas males have only one. However, the causes of gender differences are likely to be complex; differences in sex hormones [8] and central nervous system imprinting [9] have also been suggested to be important. In RA, the female preponderance cannot be explained by an oestrogen-mediated phenomenon. Most of the reports concerning the influence on RA of pregnancy or contraceptive pills favour a disease-protective effect by oestrogens [10]. Alternatively, testosterone may exert a relatively stronger suppressive effect on RA. Although this is compatible with a recent open trial with testosterone [11], other reports do not favour a role for androgens in RA [12]. The role of gender is not less complex in the CIA model. As in RA, there is a sex-dependent linkage in susceptibility to arthritis development. In rats there is a female preponderance, while in mice males are more susceptible [13]. As in RA, oestrogen seems to be protective, since treatment with it, even in physiological doses, suppresses CIA development in both sexes and in both mice [14] and rats [15]. The difference in relative susceptibility of male mice and male rats could be explained by a difference in action of testosterone in the two species. In contrast to the male rats of DA and Lewis strains, the presence of testosterone in normal mice causes aggressive behaviour and fighting which seems to promote CIA development. Moreover, testosterone promotes

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the spontaneous development of arthritis in DBA/1 male mice [16].

In addition to the effects mediated by sex hormones there are reasons to believe that genes on the sex chromosomes influence CIA and contribute to gender differences. During our studies of the effect of sex hormones on CIA we have noticed that a sex-linked susceptibility to CIA could be more pronounced if reciprocal $F_1$ crosses were compared [13]. This observation prompted us to address this question more carefully. We have now investigated a number of inbred mouse strains by reciprocal $F_1$ and back-cross experiments. We have also extended these studies by including a known X chromosome-linked mutation known to affect development of autoimmune lupus disease; the X chromosome immunodeficiency gene (xid) [17]. The xid mutation is known to prevent normal maturation of the B cell lineage and to block development of murine lupus in MRL-lpr, BXSB and NZB [18–21]. We have analysed the role of xid in the development of CIA for two reasons; first to follow a gene known to be located on X chromosomes, and second to understand further the role of B cells in CIA. The xid proved to exert dramatic effects on both CIA and the B cell autoreactivity to CII.

## MATERIALS AND METHODS

*Mice*

The parental strains used are listed in Table 1. All mice were kept and bred in a climate-controlled environment with 12 h light/dark cycles, housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum*. Age-matched $F_1$ male mice at the age of 7–12 weeks were castrated 2 weeks before immunization. Testes were removed by a single scrotal incision during barbiturate anaesthesia (Mebumal®). When $F_1$ crosses are mentioned, the maternal strain is the first strain and the paternal strain is the second; i.e. (maternal strain × paternal strain)$F_1$.

**Table 1.** H-2 haplotype and CIA susceptibility of the parental strains used in this study

| Strain | H-2 | CIA | Reference |
|---|---|---|---|
| DBA/1 | q | + | [22] |
| DBA/1xid | q | − | * |
| B10.G | q | + | [22] |
| B10.Q | q | + | [22] |
| C3H.Q | q | + | † |
| SWR/J | q | − | [6] |
| BALB/c | d | − | [13] |
| CBA/Ca | k | − | † |

* The susceptibility to CIA described in this paper. The congenic DBA/1-xid strain was helpfully established by Dr Carl Hansen (Small Animal Section, NIH, Bethesda, MD). The CBA/N strain was used as the donor of xid.

† Unpublished observation. The C3H.Q mice were originally derived from Professor Shreffler (St. Louis, USA) and the CBA/Ca mice from ALAB Laboratories (Stockholm, Sweden).

*Induction of arthritis*

Rat CII from a rat chondrosarcoma and mouse CII from xiphoid cartilage, were prepared by pepsin digestion and purification as described previously [22]. For immunization, native rat CII was dissolved in 0·1 M acetic acid and emulsified in an equal volume of Freund's complete adjuvant (FCA) (Difco, Detroit, MI) at +4°C. Different doses of CII (between 25 and 100 μg) were used because of differences in susceptibility to arthritis development in different strains. In some experiments a booster dose of CII emulsified in Freund's incomplete adjuvant (FIA) was given. The immunization protocol was designed to give a suboptimal effect in order to facilitate detection of differences in susceptibility between reciprocal $F_1$ crosses. The clinical severity of arthritis was quantified according to a graded scale: 1 = detectable swelling in one joint, 2 = swelling in more than one but not in all joints, 3 = severe swelling of the entire paw and/or ankylosis. The severity of arthritis was analysed by the Mann–Whitney *U*-test, by comparing the median scores of each group and the frequency of arthritis by their proportionate group frequencies (Fisher's exact test). The mean clinical onset of arthritis was analysed with Student's *t*-test.

*Enzyme-linked immunosorbent assay*

Quantification of anti-CII reactive antibodies in sera was performed as previously described [22].

## RESULTS

*Reciprocal $F_1$ male mice*

The parental strains used were from different genetic backgrounds [23] of which some are susceptible to CIA (DBA/1, B10.G, B10.Q, and C3H.Q) and some not (BALB/c, CBA/Ca, SWR/J) (Table 1). All $F_1$ hybrids carried H-$2^q$ and were susceptible to CIA, although different strengths of the immunization protocol were required. Before immunization, the mice were castrated to avoid a sex hormone influence. A summary of the arthritis susceptibility of the various $F_1$ males is shown in Table 2 and examples of arthritis development are shown in Fig. 1. In all $F_1$ male hybrids, there was a similar pattern in developing CIA; one of the two reciprocal $F_1$ crosses developed arthritis earlier than their counterparts. Similarly, during the onset period, one of the reciprocal $F_1$ crosses showed significant differences in maximal arthritis and severity. Five weeks after immunization, when approximately 50% of all animals in the groups had acquired CIA, no significant differences in onset rate and/or disease severity were seen. An exception to this pattern was the reciprocal $F_1$ crosses between SWR/J and DBA/1 which appeared to be equal during onset but differed significantly in disease severity later. The SWR/J strain has previously been shown to carry dominant genes determining resistance to CIA which also affect their $F_1$ hybrid offspring and are not directly related to MHC or to T cell receptor genes [6,7]. A booster immunization was required in this strain to overcome the resistance and induce arthritis. Heterozygosity or homozygosity of H-$2^q$ did not affect the susceptibility to CIA. The genetic differences between male offspring of reciprocal $F_1$ crosses are related to the sex chromosomes and the overall results, shown in Table 2 and exemplified in Fig. 1, show that sex chromosomes of different strain origins affect the onset of arthritis or, if SWR genes are involved, only its severity. The autoimmune response to CII was estimated by measuring autoantibodies to CII in

**Table 2.** Development of CIA in reciprocal $F_1$ combinations of castrated male mice

| $F_1$ cross (sex chromosome donator and H-2 indicated) | | | Number of mice | Mean onset of arthritis* | Frequency and severity of arthritis and anti-CII antibody levels at the onset period (5 weeks after immunization)† | | |
|---|---|---|---|---|---|---|---|
| X | Y | H-2 | (*n*) | (days) | % | Mean scores | μg/ml |
| BALB/c | DBA/1 | d/q | 8 | 46·6 | 0 | 0 | 431±263 |
| | | | | NS | $P<0·05$ | $P<0·05$ | NS |
| DBA/1 | BALB/c | q/d | 8 | 39·8 | 50 | 1·0 | 496±359 |
| CBA/Ca | B10.Q | k/q | 21 | 40·4 | 5 | 0·1 | 202±146 |
| | | | | NS | $P<0·05$ | $P<0·05$ | NS |
| B10.Q | CBA/Ca | q/k | 30 | 35·8 | 37 | 0·9 | 221±154 |
| CBA/Ca | DBA/1 | k/q | 19 | 40·7 | 16 | 0.4 | 110±17 |
| | | | | $P<0·05$ | $P<0·05$ | $P<0·05$ | NS |
| DBA/1 | CBA/Ca | q/k | 25 | 31·1 | 52 | 2·3 | 96±13 |
| DBA/1 | B10.G | q/q | 20 | 49·3 | 5 | 0·2 | ND |
| | | | | $P<0·05$ | $P<0·05$ | $P<0·05$ | |
| B10.G | DBA/1 | q/q | 23 | 39·3 | 40 | 1·4 | ND |
| SWR | DBA/1 | q/q | 27 | 53·3 | 19 | 0·2 | 1043±416 |
| | | | | NS | NS | NS | $P<0·05$ |
| DBA/1 | SWR | q/q | 17 | 56·9 | 12 | 0·1 | 1449±828 |

* Mice were inspected for arthritis development and progression for at least 70 days. The total incidence of arthritis was 65–100%. At termination no significant difference in incidence was observed between reciprocal crosses.

† Frequency and severity of arthritis at 5 weeks after immunization when the difference between reciprocal $F_1$ crosses was usually most pronounced. Significance levels are indicated, NS = not significant.

ND = not determined.

serum. The anti-CII response tended to be higher in the reciprocal $F_1$ cross which was more susceptible to arthritis (Table 2). However, there is a large biological variation in the levels of anti-CII antibodies in serum, and in only one of the experiments was the observed difference significant. Nevertheless, these data suggest that the exaggerating effect by sex chromosomes on arthritis development involves activation of autoreactive B cells.

*Reciprocal $F_1$ female mice*

To exclude the possibility that environmental, non-genetic, influences were responsible for the differences in development of arthritis among the reciprocal $F_1$ crosses of male mice shown in Table 2, both female and male offspring of reciprocal $F_1$ hybrids were investigated. If the differences observed among castrated male reciprocal $F_1$ hybrids were due to factors such as a strain-specific mothering or other parental influences, castrated

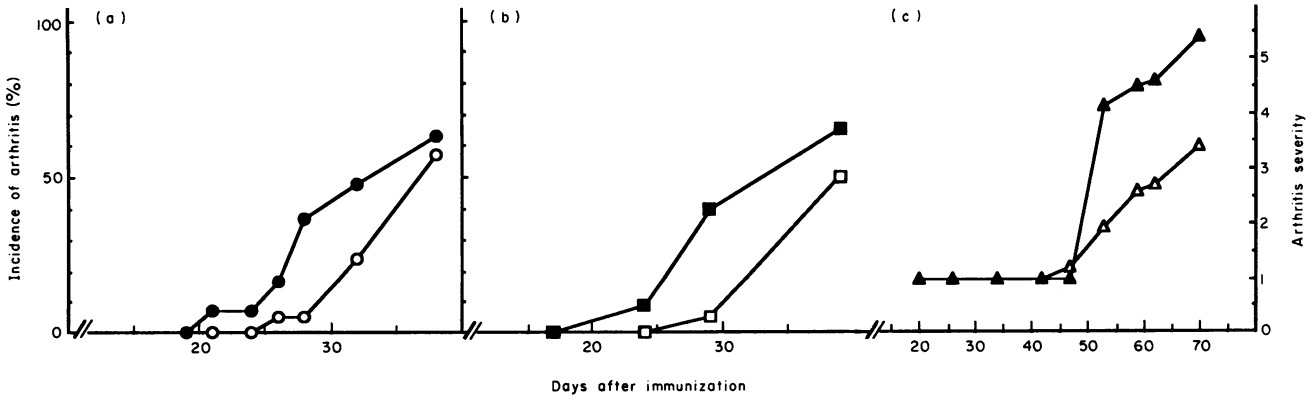


**Fig 1.** Development of arthritis in (a) CBA/Ca × B10.Q $F_1$ crosses, (b) B10.G × DBA/1 $F_1$ crosses and (c) SWR/J × DBA/1 $F_1$ crosses. (a,b) Arthritis frequency; (c) arthritis severity. In the SWR/J × DBA/1 $F_1$ crosses no difference in arthritis development was seen during the onset period but the (DBA/1 × SWR/J)$F_1$ mice later developed a more severe arthritis than the (SWR/J × DBA/1)$F_1$ males (5·4 *versus* 3·4 in mean arthritic scores, $P < 0·05$ at day 70). ●, (B10.Q × CBA/Ca)$F_1$; ○, (CBA/Ca × B10.Q)$F_1$; ■, (B10.G × DBA/1)$F_1$; □, (DBA/1 × B10.G)$F_1$; ▲, (DBA/1 × SWR/J)$F_1$; △, (SWR/J × DBA/1)$F_1$.

**Table 3.** The influence of non-genetic environmental factors; comparison of reciprocal $F_1$ male and $F_1$ female mice

| $F_1$ cross (sex chromosome donator and sex indicated) | | | Number of mice | Mean onset of arthritis* | Frequency and severity of arthritis and anti-CII antibody levels at the onset period (4 weeks after immunization)† | | |
|---|---|---|---|---|---|---|---|
| X | Y | Sex | (*n*) | (days) | % | Mean scores | μg/ml |
| C3H.Q/B10.Q | | Females | 25 | 28·2 | 32 | 0·8 | 793±575 |
| | | | | NS | NS | NS | NS |
| B10.Q/C3H.Q | | Females | 27 | 28·3 | 22 | 0·6 | 1153±889 |
| C3H.Q | B10.Q | Males | 22 | 34·0 | 5 | 0·1 | 555±369 |
| | | | | $P<0·05$ | $P<0·05$ | $P<0·05$ | NS |
| B10.Q | C3H.Q | Males | 25 | 28·4 | 36 | 2·0 | 1050±1160 |

* The mice were inspected for arthritis development and progression for at least 70 days. The total incidence of arthritis was 90–100% with no significant difference between reciprocal crosses.

† Frequency and severity of arthritis at 4 weeks after immunization when the difference between these reciprocal $F_1$ crosses was most pronounced. Serum was collected at day 36 after immunization.

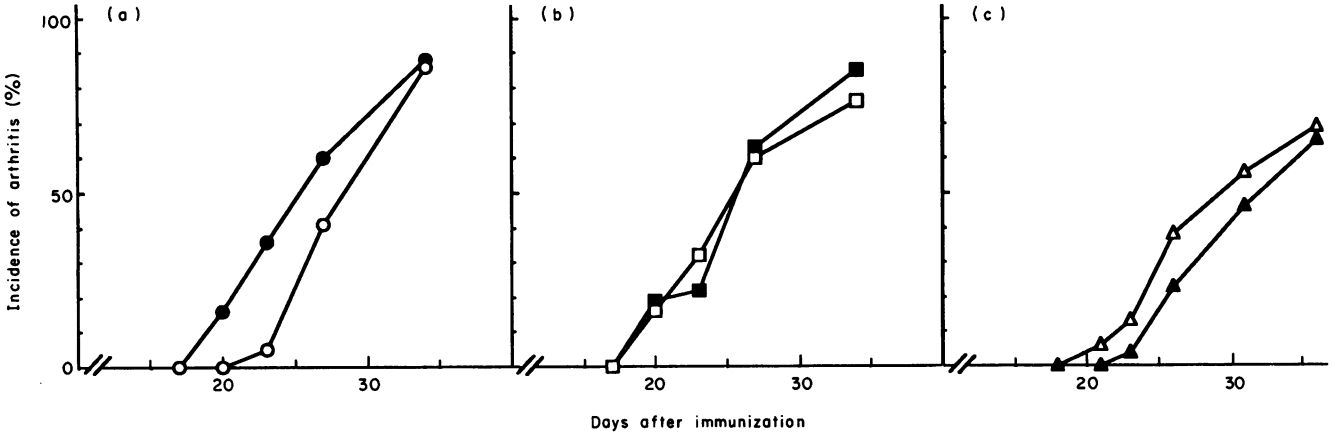


**Fig. 2.** Development of arthritis in (a) male offspring of C3H.Q and B10.Q, (b) female offspring of C3H.Q and B10.Q, and (c) reciprocal back-crosses in which the offspring carry different Y chromosomes. ●, ■, (B10.Q × C3H.Q)$F_1$; ○, □, (C3H.Q × B10.Q)$F_1$; ▲, (C3H.Q × (B10.Q × C3H.Q)$F_1$)bc; △, (C3H.Q × (C3H.Q × B10.Q)$F_1$)bc.

**Table 4.** The X chromosome-linked immunodeficiency gene (xid) blocks development of CIA; comparison of male DBA/1 and the congenic DBA/1-xid strains

| Strain (X chromosome donator indicated) | | Number of mice | Mean onset of arthritis* | Frequency and severity of arthritis and anti-CII antibody levels at the onset period (5 weeks after immunization)† | | |
|---|---|---|---|---|---|---|
| Strain | X | (*n*) | (days) | % | Arthritic index | μg/ml |
| DBA/1-xid | CBA/N(xid) | 14 | — | 0 | 0 | 22±27 |
| | | | | $P<0·05$ | $P<0·05$ | $P<0·01$ |
| DBA/1 | DBA/1 | 25 | 38·8 | 52 | 1·2 | 214±175 |

* The mice were inspected for arthritis development and progression for 100 days. The total incidence of arthritis was 100% in DBA/1 mice and 0% in DBA/1-xid mice.

† Frequency and severity of arthritis at 5 weeks after immunization. Serum was collected at day 38 after immunization.

**Table 5.** The influence of Y chromosomes; comparison of back-crossed mice (reciprocal $F_1$ mice back-crossed to C3H.Q)

| Back-cross (sex chromosome donator and sex indicated) | | | Number of mice | Mean onset of arthritis* | Frequency and severity of arthritis and anti-CII antibody levels at the onset period (4 weeks after immunization)† | | |
|---|---|---|---|---|---|---|---|
| X | Y | Sex | (*n*) | (days) | % | Mean scores | μg/ml |
| C3H.Q | C3H.Q × B10.Q | Males | 16 | 31·0 | 38 | 1·2 | 352±235 |
| | | | | NS | NS | NS | NS |
| C3H.Q | B10.Q × C3H.Q | Males | 26 | 33·5 | 23 | 0·5 | 462±414 |

* The mice were inspected for arthritis development and progression for at least 70 days. The total incidence of arthritis was approximately 80% with no significant difference between the reciprocal crosses.

† Frequency and severity of arthritis at 4 weeks after immunization when the difference between the two groups was most pronounced. Serum was collected at day 36 after immunization.

female siblings should develop arthritis with the same pattern as the males. As shown in Table 3 and Fig. 2, the difference between the reciprocal male $F_1$ hybrids (C3H.Q × B10.Q)$F_1$ and (B10.Q × C3H.Q)$F_1$ was not confirmed among the reciprocal female $F_1$ hybrid siblings, suggesting that the sex chromosomes mediate the differences in susceptibility to CIA.

*Influence by xid*

To analyse the role of xid, a congenic xid-strain in the DBA/1 background was investigated. The presence of xid effectively blocked development of CIA as compared with normal DBA/1 males (Table 4). Only very low levels of CII autoantibodies could be detected in DBA/1-xid, demonstrating that the xid gene dramatically suppressed the B cell response to CII (Table 4).

*X or Y chromosome?*

To exclude a possible influence of Y chromosomes for the observed sex chromosome associated effect, (C3H.Q × B10.Q)$F_1$ males and (B10.Q × C3H.Q)$F_1$ males were back-crossed to C3H.Q. The male back-cross offspring carry identical X chromosomes but different Y chromosomes. The difference in development of arthritis, as was noticed between males in the reciprocal $F_1$ crosses between the same strains, was not retained by the back-cross offspring (Table 5 and Fig. 2c). From these results it follows that the influence of sex chromosomes demonstrated in Table 2 is most likely to evolve from polymorphic genes present on the X chromosome. Assuming that these genes promote development of arthritis, the following order is proposed (if the genes involve resistance the reverse order should be true): B10.G = B10.Q > DBA/1 > SWR/J = BALB/c = C3H.Q = CBA/Ca > DBA/1.xid.

## DISCUSSION

The development of CIA and CII autoimmunity is associated with certain MHC class II genes but is also under strong influence by as yet unknown non-MHC genes. It is shown here that genes located on the X chromosome are also important in CIA and CII autoimmunity. The genetic association to sex chromosomes can be determined using reciprocal $F_1$ mice. Here we used mouse strains with the H-$2^q$ haplotype to neutralize the MHC influence. The mice were also castrated before the experiments to avoid influence by sex hormones. Castrated male mice of reciprocal $F_1$ crosses showed a different pattern of onset early in the disease course. The most obvious explanation is that they carry sex chromosomes inherited from different parental strains whereas they have the same set of autosomal chromosomes. One could argue, however, that another difference between offspring of reciprocal crosses is that they have been brought up by different mothers. It has, for example, been recently shown that some mouse strains produce an infectious mammary tumour virus (Mtv). This virus is transferred to litters by milk and deletes T cells expressing certain TCR Vb genes. $F_1$ hybrids could therefore have a different repertoire of T cells mediating a different response to CIA. Another possible genetic difference between the reciprocal male $F_1$ hybrids is their expression of autosomal genes from maternal or paternal inheritance. It has been proposed that parental imprinting of genes on chromosome 11 is responsible for an increased transmission of insulin-dependent diabetes mellitus to offspring from a diabetic father [24]. These possibilities could be excluded by a comparison with the female siblings of the crosses. To determine whether X or Y chromosomes are involved we carried out a back-cross analysis in which the male offspring differed only in the Y chromosomes but essentially shared the rest of the genome, including the X chromosome. By this comparison we could exclude the influence of the Y chromosome. This result was expected since the Y chromosome is known to carry very limited polymorphism while a number of polymorphic loci are known on the much larger X chromosome. A direct comparison between species is possible because both sex chromosomes are known to be strongly conserved. The X chromosome harbours many gene loci with potential importance for susceptibility to autoimmune arthritis. Among these are genes controlling sex hormone responsiveness, such as the androgen receptor gene which is known to determine androgen responsiveness [25], and the steroid sulphatase gene which regulates the activity of sex steroids in various tissues [26]. Another gene located on the X chromosome which might have an influence on the inflammatory process involving collagen is TIMP, the glycoprotein tissue inhibitor of metalloproteinases that inhibits collagenase, stromelysine and gelatinase, which are all thought to be involved in extracellular matrix turnover [27]. In humans there are several primary immunodeficiencies depending on genetic defects located on the X chromosome and with related roles in determining lymphocyte differentiation; X-linked agammaglo-

bulinaemia (XLA), X-linked severe combined immunodeficiency (XSCID), Wiskott–Aldrich syndrome (WAS) and X-linked lymphoproliferative syndrome (XLP) (reviewed by Conley [28]). They all involve various B cell defects. In the mouse, three related genes (named LyX) have been mapped to the X chromosome. These genes control different lymphocyte membrane molecules and are associated with the immune response to thymus-independent antigens [29]. A gene family most likely to be related to these lymphocyte differentiation determining genes is the X-linked lymphocyte regulation family (XLR), present in both mouse and man [30]. The xid mutation has been suggested to be associated with XLR in mouse [31]. The xid was first discovered in the CBA strain and the mutant was called CBA/N [17] which has since been extensively investigated. The presence of the xid gene primarily affects B lymphocyte differentiation leading to a lack of certain B cell subpopulations such as the B1 cells, and the mature B cells responding to certain T cell independent antigens (TI-2 antigens). The T cell compartment is, however, unaffected and the T cell dependent B cell response largely intact [32,33]. Due to the serious B cell modulation it is not surprising that the xid gene blocks murine lupus disease in MRL-lpr, NZB/W and BXSB [18] in which precipitation of circulating immune complexes in the glomeruli is essential in the pathogenesis of the disease. The present finding that xid blocks development of CIA is perhaps more surprising, since development of CIA is clearly an antigen-specific T cell dependent and MHC-associated disease [2,34]. Autoreactive B cells play a critical role in the development of CIA [35,36] and the autoantibody response after immunization with CII reflects a T cell dependent process [34]. The observed blockage of both arthritis development and autoantibody production to CII by the xid mutation indicates a fundamental role of B cell activation in CIA which is poorly understood. The xid mutation is known to affect T cell independent B cell populations and not expected to affect T cell dependent B cells such as those producing autoantibodies to CII. One possible explanation is that differentiation and selection of CII-reactive B cells is disturbed in xid mice. A comparison can be made with another T cell dependent antigen, phosphoryl choline (PC), which can also be regarded as a self-antigen when taking into account the recognition of symbiotic intestinal microorganisms. In this system the xid blocks selection of PC-specific B cell precursors [21]. However, the X-linked immunodeficiencies in humans and the xid in mice are genetic defects, and natural polymorphisms in X-linked genes affecting lymphocyte differentiation are still to be found. The observed altered B cell response in the presently described reciprocal $F_1$ combinations points towards such a possibility.

To our knowledge this is the first report of an X-linked influence on the susceptibility to arthritis in experimental animals. If a dominant allelic variant on a normal X chromosome influences human autoimmune disease, the risk to females of developing the disease is double the risk for men. Furthermore, the inactivation of one of the X chromosomes is not absolute, and parts of this X chromosome are still transcribed, causing higher levels (about $1{\cdot}5\times$ the level measured in men) of products coded from genes located in this region [37]. If the gene promoting development of autoimmunity has that location it increases the risk for females even further. The isolation of the contributing genes in the animal model and studies on the role of X chromosomes in RA await further investigation.

*Note added in proof*

Since submission of this manuscript, xid mice have been shown to carry a mutation in a B cell-specific tyrosine kinase, *btk*. The same gene is affected in XLA patients [38,39].

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