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Sex-limited protein: *in vitro* and *in vivo* functions

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SUMMARY

Mouse complement component C4 exists in two isoforms, C4 and a protein with expression restricted to male animals called sex-limited protein (Slp). Although Slp is about 95% homologous to C4, it is generally believed to be non-functional, at least in conventional haemolytic complement assays. In a previous study, however, we showed that Slp is haemolytically active in a C1-inhibitor (C1INH)-regulated, EDTA-resistant mouse complement activation pathway. To study other possible implications of this finding, we generated constitutively expressing Slp-transgenic mice. The transgene was crossed into otherwise Slp-deficient C57Bl/6J and NZB mice. Members of the third backcross generation of C57Bl/6J mice were tested for functional Slp and classical and alternative complement pathway activities (CH_{50} and AP_{50} levels, respectively). Slp-transgenic C57Bl/6J mice showed enhanced CH_{50}, but normal AP_{50} levels when compared with non-transgenic littermates. To discover a possible protective role for Slp in spontaneous systemic lupus erythematosus (SLE) in NZB×NZW (NZB×W) mice, the third backcross generation of Slp-transgenic NZB mice was mated with NZW mice and the development of SLE in the female offspring was followed. In these introductory experiments, Slp-transgenic NZB×W animals presented with a significantly extended life span. Our results imply that Slp is a mouse complement component with functions which partially resemble some of those of human C4A.

Keywords complement lupus mouse Slp transgenic

INTRODUCTION

The class III region of the MHC of the mouse, also known as the S region, encodes serum proteins including C2, factor B, C4 (Ss), and sex-limited protein (Slp) [1–4]. Although Slp has long been considered non-functional, data from our group suggest that it is involved in an EDTA-resistant complement activation pathway, which is—at least partially—different from both classical and alternative activation pathways [5]. The Slp gene is considered to be a duplication of the mouse C4 gene with about 95% homology to C4 at both the DNA and protein levels [6]. However, the protein is incapable of rendering C4-deficient guinea pig serum haemolytic [7]. Furthermore, Slp is resistant to cleavage by activated human and mouse C1s and the mouse C1s-like protease Ra-reactive factor, whereas mouse C4 is not [8–10]. These are probably the main reasons why Slp has been considered non-functional. These findings seem to rule out that C1 or the Ra-reactive factor play an important role in Slp-mediated complement activation. The following questions then remain: at what level in the complement cascade does Slp act and what are the *in vivo* implications?

As already stated, the Slp gene is a member of the MHC class III family, a family that includes not only other complement components, but also complement-unrelated proteins [11]. Therefore, a role for Slp-linked genes in Slp-mediated complement activation cannot be entirely ruled out. In order to assign definitely functional activity to Slp and generate mouse models for functional Slp activity, an Slp-expressing allele was introduced into mice lacking a functional Slp gene product. The insert of a cosmid clone encompassing a full-length Slp gene was used for the microinjection of oocytes. The gene chosen originated from the B10.WR7 mouse strain, which expresses Slp without the characteristic dependence on androgen [12]. The origin of this gene is probably the presence of additional Slp genes in the strain that have undergone recombination with a neighbouring C4 gene (and subsequent multiplication). This did result in hybrid genes that have C4-regulatory sequences preceding Slp-coding regions and thus constitutive (C4-like) expression rather than limitation to mature

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males [2,13,14]. The insert of cosmid clone 38.3 was used because the intact Slp gene contained within this clone accounts for the majority of Slp in B10.WR7 mice [2,13].

The objective of the present study was to obtain more insight into Slp function(s) by comparing the activities of sera from Slp-transgenic and Slp-deficient littermates in different assays of mouse complement activation, including the Slp-dependent EDTA-resistant pathway [5]. In an introductory experiment with systemic lupus erythematosus (SLE)-prone, Slp-transgenic NZB×W mice, we aimed at studying the role of Slp in murine lupus erythematosus.

MATERIALS AND METHODS

Animals

Transgenic mice were generated by the Transgenic Animal Model Core of the University of Michigan's Biomedical Research Core facilities. The purified DNA fragment was microinjected into fertilized eggs from F_1 hybrid zygotes from C57Bl/6J and C57Bl/6J×SJL/J parents. The eggs were then transferred to pseudo-pregnant CD-1 mice, as described by Hogan *et al*. [1]. C57Bl/6J×SJL/J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and CD-1 mice from Charles River (Wilmington, MA). Transgenic founders, identified by Southern blotting of tail DNAs, were mated to C3H mice (Jackson) and transgenic progeny to C57Bl/6J and subsequently also to NZB mice (Fig. 1). Slp-transgenic mice from these established, independent lines (A, B and C) were then selected on the basis of functional Slp in serum. After the first backcross procedure in C57Bl/6J mice, offspring were tested for the presence of transgene and the absence of SJL/J Slp gene using Southern blotting. Incidentally, genotypes were confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). All mouse procedures were approved by the University of Michigan Committee on the Use and Care of Animals and were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

The second Slp-transgenic C57Bl/6J generation was mated to NZB/Ola//Hsd mice (Harlan Olac Ltd, Bicester, UK) and, after three backcrosses with NZB, to NZW mice in order to obtain female SLE-prone NZB×W mice (NZW/Ola/Hsd; Fig. 1). The mice used in these experiments and haemolytic complement tests were matched for sex and age (by making comparisons within the same litters). In the Netherlands, all mice were bred and maintained at the Central Animal Facilities of Utrecht University.

DNAs

An androgen-independent Slp gene was obtained from cosmid 38.3, which contains the full-length 17-kb SlpB gene from the B10.WR7 strain [2]. The 26-kb EcoRI fragment encompassing the complete gene and including approx. 5 kb of 5′ flanking sequences was purified from agarose following electrophoresis to remove vector DNA and then microinjected into oocytes. Genomic DNAs used in Southern blotting were prepared either from tail biopsies of 2-week-old animals using a salting-out procedure [3] or from livers of adult mice using standard procedures. The probe used in Southern blotting was the nick-translated insert of a plasmid containing a full-length (5·4 kb) C4d cDNA constructed from overlapping λGT10 phage clones [4]; both Slp and C4 sequences hybridized due to their 97% sequence identity.

Southern blotting

To distinguish unequivocally between the integrated SlpB transgene and endogenous C4 and Slp genes, Southern blots were performed to detect the SlpB-specific 19-kb HindIII fragment [6]. Hybridization intensities also allowed the estimation of gene-copy numbers more readily than simple PCR analysis. Genomic DNA (15 μg) was digested overnight with at least 5 U/mg HindIII

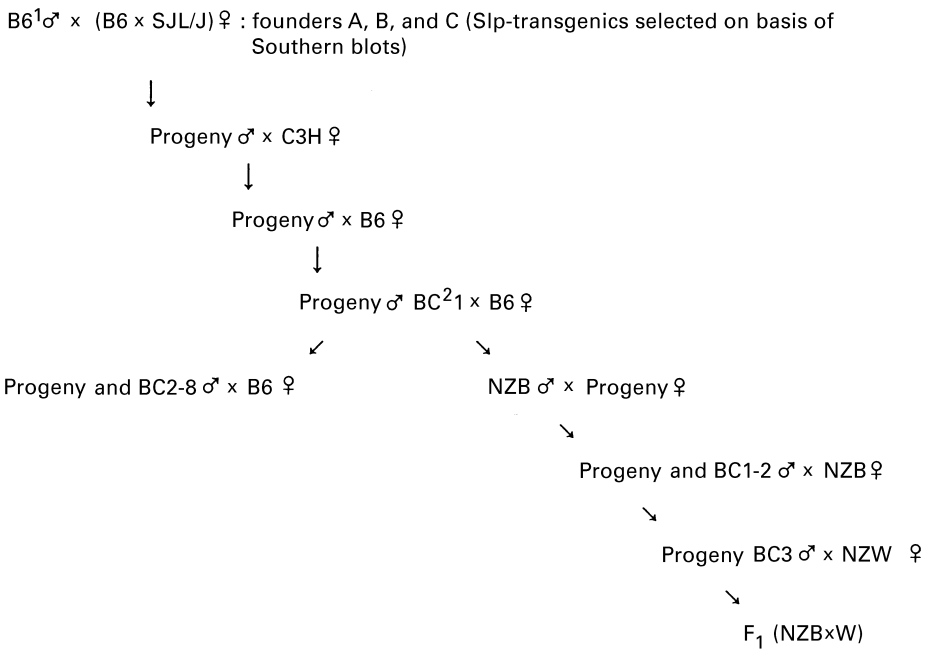

Fig. 1. Flow scheme of sex-limited protein (Slp)-transgenic C57Bl/6J and NZB×W breeding. [1]C57Bl/6J mice; [2]backcross generation numbers.

(Boehringer Mannheim, Mannheim, Germany) and electrophoresed on 0·4% agarose gels. The gels were then DNA-transferred to Zeta-Probe GT membranes according to the manufacturer's instructions (BioRad Labs, Hercules, CA). Hybridization of the nick-translated probe and washing conditions were standard.

Sera

Male C3H/FeJ (H-2^k; Slp-negative) mice originally obtained from Jackson Laboratory and male F_1 (BALB/c × Swiss inbred; Slp-positive) mice, bred and maintained at the Central Animal Facilities at Utrecht University, were used at an age of 10–15 weeks as donors of Slp reagent and Slp-containing reference serum, respectively. Mice were anaesthetized with ether and bled by retro-orbital puncture; after pooled blood was clotted for 1·5 h at 20°C, serum was separated by centrifugation and stored at −70°C until use.

Rabbit erythrocytes

Rabbit blood diluted 1:2 with Alsever's old solution (114 mM citrate/27 mM glucose/72 mM NaCl pH 6·1; bioTrading, Wilnis, The Netherlands) was used as the source of rabbit erythrocytes (RaE). Before use, RaE were washed three times with isotonic NaI to elute adsorbed serum proteins and then resuspended in the appropriate buffer.

Buffers

Buffers included veronal (25 mM)-buffered saline (750 mM, pH 7·35 ± 0·05; VBS ×5) which served as a five-times concentrated stock solution for the preparation of VBS^{2+} (containing 0·15 mM $CaCl_2$ and 0·5 mM $MgCl_2$; buffer for classical complement pathway activation), EGTA-VB (containing 2·5 mM $MgCl_2$ and 8 mM EGTA; buffer for alternative complement pathway activation), and EDTA-VB (containing 10 mM EDTA; buffer for haemolytic Slp assay).

Haemolytic assays

Functional Slp activity was estimated using the microtitre assay described by Van den Berg *et al*. [5]. Briefly, C3H/FeJ serum and Slp-containing test samples were diluted 1:10 in EDTA-VB and precipitated with 11% (w/v) polyethylene glycol 6000 (PEG; Fluka, Buchs, Switzerland) followed by centrifugation (4000 ***g***, 4°C, 1 h). The precipitates were dissolved to their original volumes in EDTA-VB and subsequently incubated at 41°C for 45 min. Precipitated C3H/FeJ serum (diluted 1:2·5) was used as reagent for Slp. After 50 μl of serially diluted Slp samples were mixed with equal volumes of 11% PEG precipitates of C3H/FeJ serum, 50-μl volumes of a 2% RaE suspension in EDTA-VB were added to each well. Absorbance values (405 nm) of the supernates were determined after incubation at 39°C for 1 h. Activities of Slp-containing sera were expressed in units (corresponding to the amount of Slp giving rise to 50% haemolysis of RaE in the presence of 11% PEG precipitate) per ml.

Classical and alternative complement pathway activities (CH_{50} and AP_{50} levels, respectively) were determined by our colourimetric microtitre assays [15]. In addition, AP_{50} values were determined in the presence of 5 mg/ml final concentration of Zymosan A (Sigma, St Louis, MO) [16].

Statistical analysis

Statistical significance of the results obtained for the different groups in haemolytic assays were determined using Student's *t*-test and correlations were analysed with Pearson's correlation test. The Kaplan–Meier method was used to evaluate the survival of mice. The log rank test was used to test differences between groups of mice.

RESULTS

Generation of mice expressing Slp unlinked to other MHC class III components

Data from our group confirm that Slp, rather than being a non-functional pseudogene of C4, plays an essential role in an EDTA-resistant complement activation pathway in mice [5]. Since Slp is closely linked to the mouse H-2 locus [11], the role of other MHC class III genes in this pathway cannot be entirely ruled out. To assign activity definitively to Slp and to generate mouse models for the functional assay of this activity, we introduced an Slp-expressing allele into mice otherwise 'deficient' in the EDTA-resistant complement activation pathway. The insert of a cosmid clone encompassing a full-length Slp gene was used for micro-injection into oocytes. The gene chosen originated from the B10.WR7 strain, which expresses Slp without the characteristic dependence on androgen [12]. This constitutive expression must have resulted from a recombination event between the Slp gene and neighbouring C4 genes and subsequent multiplication [2,13,14]. The insert of cosmid clone 38.3 was used because the intact Slp gene contained within this clone accounts for most of the Slp expression in B10.WR7 mice [2,13].

The cosmid insert was purified of vector DNA by excising a

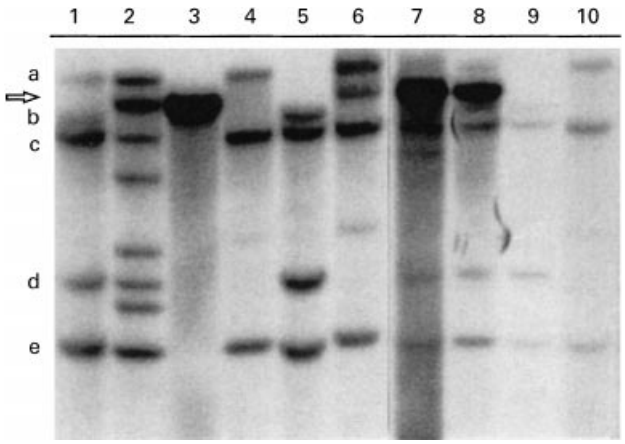

Fig. 2. Identification of transgenic mice by hybridization to a sex-limited protein (Slp)B-specific 19-kb HindIII fragment. Southern blotting was used to identify transgenic founder mice (and subsequently their transgenic offspring) and as to estimate insertion copy numbers and MHC background. Representative blots of genomic DNAs digested with HindIII (see Materials and Methods) indicate the polymorphic (haplotype-dependent) C4 and Slp fragment sizes. The lanes contain the following DNAs: lane 1, F_1(C57Bl/6J × SJL/J); lane 2, B10.WR7; lane 3, SlpB, 5 ng cosmid 38.3 DNA plus 15 mg salmon DNA; lane 4, SJL/J; lane 5, C57Bl/6J; lanes 6–8, progeny C57Bl/6J × (C57Bl/6J × SJL/J) positive for the transgene (founders for lines A, B, and C, respectively); lanes 9 and 10, as lines 6–8 but devoid of carrying transgene. The arrow on the left indicates the 19-kb HindIII fragment that is present in the B10.WR7 (transgene donor) genome, in cosmid 38.3, but not in SJL/J and C57Bl/6J mice. Lower case letters on the left indicate HindIII fragments in C57Bl/6J × SJL/J F_1 mice that are of endogenous C4 and Slp origin; fragment a is specific to SJL/J chromosomes, fragments b and d indicate C57Bl/6J chromosomes.

Table 1. Relationship between estimated gene copy number in founder mice and sex-limited protein (Slp) activity in first generation F_1 (founder × C3H) mice*

Founder	Approx. gene copy	Slp activity
A	15–20	+++
B	10–15	++
C	15–20	+++
–†	0	–

* Progeny from three independent transgenic lines were tested for Slp activity in a functional complement assay. SlpB gene copy numbers were estimated from relative band intensities on blots similar to those in Fig. 2. Relative Slp activities are indicated by pluses and minuses, based on activity of first generation progeny tested for each founder.

† These mice correspond to lanes 9 and 10 of Fig. 2.

26-kb EcoRI fragment within which resides the 17-kb Slp structural gene and approx. 5 kb of 5′ flanking and 4 kb of 3′ DNA sequences [2]. This DNA was microinjected into fertilized mouse oocytes resulting from crossing C57Bl/6J (H-2^b; high C4 and absent Slp expression) with C57Bl/6J × SJL/J (H-$2^{s;}$ high C4 and low Slp expression) heterozygotes. Progeny was screened by Southern blotting to assess not only the presence of the transgene, but also insertion copy numbers and the haplotype of founders at the endogenous C4 and Slp loci. A 19-kb HindIII fragment unique to the introduced gene clearly revealed the presence of the transgene (Fig. 2). Restriction fragment length polymorphism of other bands hybridizing with the C4/Slp cDNA probe allowed discernment of haplotypes b, s, and k. Three (a–c) of the original positive mice, with approximate gene copy numbers ranging from a few to over 20, are represented in Table 1. These three mice were used to found independent transgenic lines by mating them to C3H mice (H-2^k; C4 low, Slp Q0) and subsequently to C57Bl/6J mice. Slp transgene-negative mice were not included in the breeding programme.

Haemolytic complement assays

Crossing the Slp transgene into C57Bl/6J mice and repeated backcrossings yielded the third backcross generation of Slp-transgenic mouse lines A, B, and C. These lines were tested for EDTA-resistant, classical (CH_{50}), and alternative (AP_{50}) complement pathway activities. The results are listed in Table 2. The lines were subgrouped into Slp-negative (< 200 U/ml) and Slp-positive (> 200 U/ml). For all lines and both sexes, we found two-to-three-fold enhanced CH_{50} levels in the Slp-transgenic mice compared with Slp-negative mice. However, the AP_{50} levels of sera from Slp-negative and Slp-positive members were not different. To show that CH_{50} titres correlated with Slp titres, we calculated Pearson's correlation coefficient for the three lines and sexes (Table 3). We found a high correlation in lines A and C, a weak correlation for the male members of line B, and no correlation at all for the female littermates of line B. Because of the unexpected behaviour of line B, breeding of this line was discontinued.

Protective role of Slp in SLE-prone mice

In order to study the role of Slp in SLE-prone mice, an introductory study with NZB×W mice was undertaken. The Slp-transgenic NZB×W mice were obtained in the following manner: mice of the first backcross of Slp-transgenic with C57Bl/6J mice of line C were crossed with NZB mice (H-2^d; high C4 and sex-limited Slp expression) and backcrossed three times. The thus acquired third-generation Slp-transgenic NZB mice were then crossed with NZW mice (H-2^z; high C4 and absent Slp expression). Twenty-four female F_1 Slp-transgenic NZB×W mice and an equal number of non-transgenic littermates (age matched) born from these crossings were used in the pilot study and followed for 1 year. The results are given in Fig. 3. The median survival time of non-transgenic mice was 203 ± 11·43 days (mean ± s.d.), whereas Slp-transgenic animals lived 31% longer (i.e. 266 ± 8·57 days). Statistical analysis

Table 2. Functional sex-limited protein (Slp), CH_{50}, and AP_{50} levels in third generation backcross Slp-transgenic and non-transgenic mice†

		Transgene-negative C57Bl/6J mice (≤ 200 U/ml)			Transgene-positive C57Bl/6J mice (> 200 U/ml)		
		Slp (U/ml)	CH_{50} (U/ml)	AP50 (U/ml)	Slp (U/ml)	CH_{50} (U/ml)	AP_{50} (U/ml)
Line A	M	23 ± 1	317 ± 31	613 ± 27	5125 ± 934	1136 ± 114***	655 ± 25
		(n = 8)	(n = 8)	(n = 8)	(n = 12)	(n = 12)	(n = 12)
	F	61 ± 15	133 ± 11	243 ± 12	4073 ± 900	212 ± 22**	259 ± 10
		(n = 7)	(n = 7)	(n = 7)	(n = 13)	(n = 13)	(n = 13)
Line B	M	152 ± 23	441 ± 85	332 ± 39	9076 ± 1801	789 ± 130*	323 ± 35
		(n = 9)	(n = 9)	(n = 9)	(n = 21)	(n = 21)	(n = 19)
	F	72 ± 12	43 ± 13	182 ± 30	5649 ± 20	79 ± 20	165 ± 10
		(n = 8)	(n = 8)	(n = 8)	(n = 18)	(n = 18)	(n = 18)
Line C	M	6 ± 4	222 ± 15	323 ± 21	760 ± 9	465 ± 59***	280 ± 38
		(n = 9)	(n = 9)	(n = 9)	(n = 9)	(n = 9)	(n = 9)
	F	2 ± 1	96 ± 11	108 ± 4	4652 ± 787	177 ± 16***	116 ± 11
		(n = 9)	(n = 9)	(n = 9)	(n = 6)	(n = 6)	(n = 6)

† Functional Slp activities as well as CH_{50} and AP_{50} levels (mean ± s.e.m.) in male (M) and female (F) Slp-transgenic and non-transgenic mice of three different backcrossings with C57Bl/6J mice. The mice of different lines were matched for age (animals of the same litters were used). Difference between the mean of Slp-transgene-negative and that of Slp-transgene-positive mice is significant: *P < 0·025; **P < 0·005; ***P < 0·0005.

Table 3. Pearson's correlation coefficient between sex-limited protein (Slp) and CH_{50} titres in three different Slp-transgenic C57Bl/6J lines

		Correlation coefficient	*P* value (two-tailed)
Line A	M	0·7503	< 0·0001
	F	0·7614	< 0·0001
Line B	M	0·3917	< 0·025
	F	0·1856	NS
Line C	M	0·7511	< 0·0001
	F	0·7466	< 0·0001

M, Male; F, female; NS, not significant.

based on Kaplan–Meier survival scoring showed that the life spans of Slp-transgenic and Slp-non-transgenic mice were significantly different ($P = 0·009$).

DISCUSSION

The present studies were performed in order to assign functional activity to mouse Slp. Earlier studies indicated that Slp expresses functional activity in a rather artificial, EDTA-resistant mouse complement activation pathway [5]. Here, we report the generation of Slp-transgenic mice and, after mating these animals to C3H mice, the crossing and repeated backcrossing of the Slp transgene with C57Bl/6J and, after two generations, also with NZB mice.

The transgene elevated CH_{50} levels in both male and female, third-backcross generation C57Bl/6J mice, which strongly suggests that constitutively expressed Slp is a functional component involved in mouse classical complement pathway activation. The significant correlation between functional Slp and CH_{50} titres in transgenic mouse lines A and C corroborates the direct involvement of Slp in mouse complement (Table 2). A less likely, but not entirely impossible, alternative interpretation of our results could be that Slp competes with C4 for binding sites on complement regulator C4-binding protein (C4BP), which could render C4 relatively resistant to regulation by C4BP. However, data from Ferreira *et al*. [7] do not support C4BP binding to Slp. Very recent results from our group, in agreement with literature data [17], indicate that Slp is deposited onto the membranes of sensitized sheep erythrocytes during complement activation. This, in turn, favours a direct, functional role for Slp.

Overall, Slp-transgenic animals showed 60–2400-fold enhanced Slp levels compared with non-transgenic littermates.

Experiments are underway that might reveal the possible mechanism of Slp-mediated complement activation. Preliminary results with regard to C4 and Slp deposition on sensitized sheep erythrocytes confirm earlier data which show that Slp can bypass C3 and mediates direct activation of C5 [5]. Similar C3-bypass activation pathways have been identified in humans in which C4 dimers and the activated protease unit of C2a have the ability to activate C5 without the typically participation of C3 [18].

The extended life span of female, Slp-transgenic NZB×W mice suggests that Slp has a protective function with regard to disease development in SLE-prone mice. This function is similar to that of C4A in humans, one of the major protective factors in familial SLE [19,20]. The protective effect of Slp may be based on the prevention of immune complex precipitation (PIP) or the solubilization (SOL) of already formed immune complexes *in vivo*. We are now working on an *in vitro* model system to study PIP and SOL in mice. Preliminary data obtained so far with this system indicate that Slp indeed mediates PIP and SOL in serum of female NZB×W mice.

In conclusion, our data are in favour of a functional role for Slp in mouse classical complement pathway activation and in the prevention of disease development in SLE-prone NZB×W mice. Recently obtained preliminary results suggest that the disease-preventing effect is mediated by a better handling of immune complexes rather than a regulation of anti-dsDNA autoantibody formation in NZW×B mice. Whatever the exact mechanism might be behind the life-extending effect of Slp in Slp-prone mice, our results are in favour of functional roles for Slp.

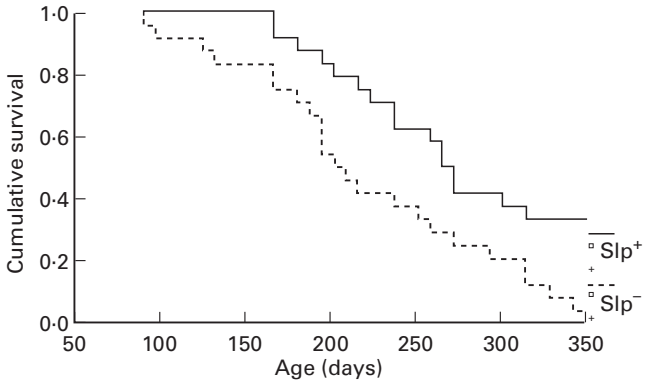

Fig. 3. Sex-limited protein (Slp)-mediated, enhanced survival of female NZB×W mice (line C). Kaplan–Meier survival plots are given which describe the fate of female Slp-positive ($n = 24$) and corresponding Slp-negative NZB×W mice ($n = 24$; from the same litters). Log rank analysis revealed a significant ($P = 0·009$) difference in life span.

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