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A genome-wide screen for asthma-associated quantitative trait loci in a mouse model of allergic asthma

Youming Zhang[1], Jean Lefort[2], Virginia Kearsey[1,3], José Roberto Lapa e Silva[4], William O. C. M. Cookson[1,*,+] and B. Boris Vargaftig[2,+]

[1]Wellcome Trust Centre for Human Genetics, University of Oxford, Windmill Road, Headington, Oxford OX3 7BN, UK, [2]Unité de Pharmacologie Cellulaire, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France, [3]Oxagen Ltd, 91 Milton Park, Abingdon, Oxon OX14 4RY, UK and [4]Servico de Pneumologia, Hospital Universitario Clementino Fraga Filho, Universidade Federal de Rio de Janeiro, Rio de Janiero, Brasil

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Asthma is the most common illness of childhood, affecting one child in seven in the UK. Asthma has a genetic basis, but genetic studies of asthma in humans are confounded by uncontrolled environmental factors, varying penetrance and phenotypic pleiotropy. An animal model of asthma would offer controlled exposure, limited and consistent genetic variation, and unlimited size of sibships. Following immunization and subsequent challenge with ovalbumin, the Biozzi BP2 mouse shows features of asthma, including airway inflammation, eosinophil infiltration and non-specific bronchial responsiveness. In order to identify genetic loci influencing these traits, a cross was made between BP2 and BALB/c mice, and a genome-wide screen carried out in the F_2 progeny of the F_1 intercross. Five potentially linked loci were identified, four of which corresponded to human regions of syntenic homology that previously have shown linkage to asthma-associated traits.

INTRODUCTION

Asthma affects one child in seven in the UK (1). It is due to the interaction of an unknown number of genes and strong environmental factors. Ninety-five per cent of childhood asthma is associated with immunoglobulin E (IgE)-mediated allergy to common inhaled proteins. Genetic studies of asthma in humans are confounded by uncontrolled environmental factors, varying penetrance and phenotypic pleiotropy (2). In contrast to the human state, inbred animal models of asthma offer controlled exposure, limited and consistent genetic variation and unlimited size of sibships. Biozzi high-responder mice were produced originally by selective breeding for antibody responses to multi-determinant immunogens (3). Following immunization and subsequent challenge with ovalbumin (OVA), the Biozzi BP2 mouse shows many features of human allergic asthma. These include airway inflammation, eosinophil infiltration and non-specific bronchial responsiveness to inhaled broncho-constrictors such as histamine and methacholine. These traits are quantitatively and qualitatively different from the same phenotypes in other mice (4). Bronchial responsiveness and the numbers of eosinophils in airway tissues are quantified reliably (4), and consequently were used as the principal phenotypes in this study.

In order to identify genetic loci influencing these traits, a cross was made between BP2 and BALB/c mice, and a genome-wide screen was carried out in the F_2 progeny of the F_1 intercross.

RESULTS

The BP2 and BALB/c parental strains were examined for bronchial responsiveness to methacholine [expressed as the difference between basal and maximal values of enhanced pause (ΔP_{enh})] after antigen challenge (Table 1). The two strains had significantly different, but overlapping, distributions of ΔP_{enh}. The mean of ΔP_{enh} in the F_1 mice was significantly lower than the average of that of the two parents, indicating overall partial dominance for low ΔP_{enh} score. The F_2 mean was not significantly different from the average of the two parents. Eosinophil counts on bronchoalveolar lavage fluid were carried out in the F_0 and F_1 mice, but the trait showed a wide variance and did not differ significantly between the parental strains (data not shown). Direct counting of eosinophil numbers in the bronchial epithelium (between the luminal border and the basal lamina) was therefore performed histologically on the F_2 mice.

A total of 217 mice in the F_2 generation were tested for ΔP_{enh}. Lungs from approximately half (112) of the F_2 mice were submitted for histology. There was no significant correlation between the two traits scored in the F_2 mice. Genetic linkage was sought to a panel of 180 microsatellites. The combined length of the chromosomes totalled 1263.5 cM, according to the Kosambi mapping function.

*To whom correspondence should be addressed. Tel: +44 1865 221335; Fax: +44 1865 221455; Email: wocc@radius.jr2.ox.ac.uk
+These authors contributed equally to this work

Table 1. Statistics for ΔP_{enh} (square root transformed) in non-segregating and segregating generations

$\sqrt{(\Delta P_{enh})}$	BALB/c	BP_2	BALB/c×BP_2	BP_2×BALB/c	F_2
Mean	0.941	1.783	1.186	1.134	1.372
Variance	0.046	0.173	0.074	0.073	0.159
Standard error	0.051	0.098	0.052	0.085	0.027
n	18	18	27	10	217

Potential quantitative trait loci (QTL) effects which controlled ΔP_{enh} were found on chromosomes 9 (lod score 2.5), 10 (lod score 3.8), 11 (lod score 3.65) and 17 (lod score 2.1) (Fig. 1 and Table 2). According to published criteria for interpreting the significance of linkages in genome-wide searches (5), the linkage to chromosomes 9 and 17 would be classified as 'suggestive', and the linkages to chromosomes 10 and 11 as 'significant'. Together, the loci explained 25.4% of the phenotypic variance of ΔP_{enh} in the F_2 mice.

ΔP_{enh} was decreased by the BP2 allele on chromosomes 9 and 10, and increased by that allele on chromosome 11. Interpretation of the QTL effect on chromosome 17 was not straightforward. The additive effect (a) of the QTL on chromosome 17 was close to zero, while the dominance effect (d) was relatively large, leading to a dominance ratio (d/a) of 12. Such a situation may arise from two closely linked QTL, with similar additive effects in repulsion (i.e. on chromosomes derived from different parental strains), but each showing dominance in the same direction. The results therefore suggested that the QTL effect on chromosome 17 comprised more than one QTL. The region contains the major histocompatibilty complex (MHC), which holds many genes which may influence immunologically mediated traits.

One region of linkage, was found between the eosinophil numbers in the bronchial epithelium and chromosome 11, with a lod score of 3.4 (Fig. 1 and Table 2). This locus accounted for 12.9% of the variation in the trait, with the BP2 allele increasing the trait.

DISCUSSION

Several of the potential linkages may be of relevance to human loci linked to asthma-associated traits. The chromosome 10 ΔP_{enh} QTL shows syntenic homology with human chromosome 12q21.1–12q24.22. This region previously has been shown to be linked to human asthma-associated traits in several studies (6–8). It contains the important candidate interferon-γ (IFN-γ). Although the lod score linking mouse chromosome 11 to ΔP_{enh} was only suggestive of linkage, this region contains loci which contributed to survival after ozone-induced pulmonary inflammation in an AJ×C57BL/6J cross (9) and to ozone-induced pulmonary neutrophil infiltration in a C57BL/6J×C3H/HeJ cross (10). The chromosome 11 ΔP_{enh} QTL shows syntenic homology to human chromosome 17, which has been implicated in previous linkage studies of asthma (8). Linkage to the human locus previously has not been replicated convincingly, and localization has been poorly defined. The region nevertheless contains a cluster of chemokine genes which are involved in many inflammatory pathways. One of these, eotaxin, is a chemokine that acts as a potent inducer of eosinophil migration (11). The region also contains the important candidate inducible nitric oxide synthase (iNOS).

The suggestive linkage of ΔP_{enh} to mouse chromosome 17 supports the previous study of De Sanctis *et al*., who showed the region to be linked to spontaneous bronchial responsiveness in an AJ×C57/Bl6 cross (12). These authors also observed epistasis at the locus (12). This region contains the MHC and tumour necrosis factor (TNF) genes, which may have diverse effects on antigen recognition and the promotion of airway inflammation. The MHC and TNF genes have also been implicated in gold salt-induced IgE nephropathy in a BN×LEW rat cross (13). In human families, class II human leukocyte antigen (HLA) genes are known to restrict the ability to react to particular allergens (14,15), and polymorphism with TNF genes has been associated with asthma independently of class II effects (16). The suggestion that two (or more) loci are acting within this QTL in our murine model is, therefore, consistent with the observations in humans.

The mouse QTL influencing eosinophil infiltration into the bronchial epithelium has complex human syntenic homologies, which include the interleukin-4 (IL-4)–IL-5 cytokine cluster on human chromosome 5. This region has been identified by several human linkage studies (17–20), and may contain more than one locus influencing asthma. It has also been implicated by a BN×LEW cross which had been used to investigate IgE nephropathy (13).

Although the BP2 mouse does show many features which typify human asthma, the induction of quite florid changes by intraperitoneal injection and inhalation of OVA does not match the events that produce human disease. It should not be assumed, therefore, that either the patho-physiological or genetic mechanisms producing changes in airway histology or responsiveness are the same in mice and humans. Nevertheless, the presence of loci which potentially are shared between our model and human families segregating asthma suggests that underlying genetic factors may also be shared to some extent. The fine mapping of many regions linked in humans to asthma-associated phenotypes has been slow and problematic, and the subsequent identification of the genes underlying shared human and mouse linkages may be greatly facilitated by concomitant genetic and physical mapping in the two species. The sharing of loci between different mouse models of bronchial hyper-responsiveness and airway inflammation may also aid in the dissection of the complex genetics underlying asthma.

Table 2. Linkage to bronchial responsiveness (P_{enh}) and airway eosinophil count (EP) QTL: descriptive statistics

Chromosome	Trait	QTL position (cM)	± (cM)	m	a	d	d/a	lod	$\%Var_{exp}$	Regions of human syntenic homology: candidate genes
9	P_{enh}	18	10	1.300	–0.105	0.022	–0.210	2.5	5.2	Chromosome 11q23: IL-10R
10	P_{enh}	44	7	1.38	–0.22	0.116	–0.530	3.8	8.3	Chromosome12q22–q24: IFN-γ
11	EP	7	6	3.230	1.359	0.494	0.364	3.4	12.9	Complex: includes Chr 5q31 IL-4 cytokine cluster
	P_{enh}	52	7	1.372	0.146	0.097	0.664	3.65	7.5	Chromosome 17q12–q22: iNOS, eotaxin
17	P_{enh}	10	4	1.37	0	–0.155	11.9	2.1	4.4	Chromosome 6p21: MHC, TNF

m, mean; a, additive effect; d, dominance deviation; d/a, dominance ratio; lod, lod score; $\%Var_{exp}$, percentage variance explained.
Both traits were square root transformed before analysis.

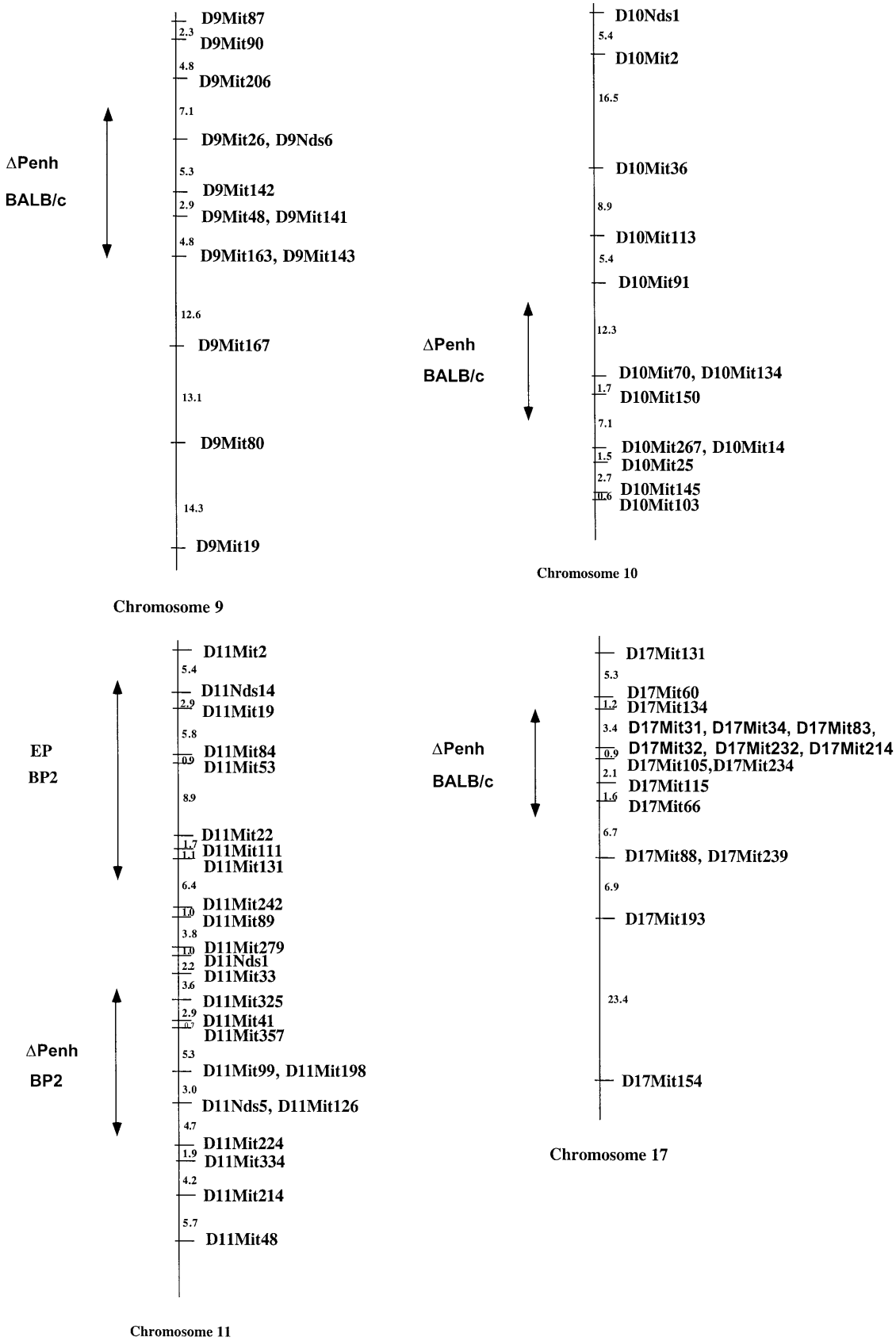

Figure 1. Diagrammatic representation of chromosomes and QTL locations. The bar shows the 95% confidence interval for the QTL location, beside which is indicated the trait and the allele which increases the trait value.

MATERIALS AND METHODS

Phenotypic studies

Mice were provided by the Centre d'Elevage R. Janvier (Le Genest Saint-Isle, France) and were immunized with 100 µg of OVA subcutaneously at weeks 6 and 7 of life, and at week 8 were challenged intranasally under light ether anaesthesia with 50 µl of 10 mg of OVA in 50 ml of 0.9% (w/v) NaCl. Control mice were challenged with saline alone. Each mouse was challenged twice a day for 2 days. Twenty-four hours after the last challenge, unrestrained conscious mice were placed in a whole plethysmograph chamber and airway resistance was measured as P_{enh} (enhanced pause). P_{enh} was calculated as $[T_e/40\% \text{ of } T_r - 1] \times P_{ef}/P_{if} \times 0.67$, where T_e is expiratory time, T_r is relaxation time, P_{ef} is peak expiratory flow and P_{if} is peak inspiratory flow, as previously described (4). After stabilization for at least five measurements, a 20 s aerosol of methacholine was given (1.5×10^{-2} M). The ΔP_{enh} (difference between basal and maximal values) was calculated from the average of five maximal values.

The lungs from 112 mice were examined for histology. Mice were exsanguinated via the abdominal aorta and the contents of the thoracic cavity resected '*en bloc*'. The lungs were inflated via the trachea with 1 ml of Histocon (Polysciences, Warrington, PA), the lobes dissected and mounted over cork disks, covered by optimum cutting temperature compound (OCT; BDH, Poole, UK) and snap frozen in isopentane (Prolabo, Paris, France) cooled by liquid nitrogen. The frozen blocks were kept at –80°C prior to use. Sections were cut in a cryostat kept at –21°C and collected on glass slides previously coated with poly-L-lysine (Sigma, Poole, UK), fixed in chloroform–acetone v/v (Merck, Poole, UK) for 10 min, wrapped in a plastic film and kept at –20°C prior to use. Representative sections of each block were also stained with haematoxylin–eosin (Rhône-Poulenc, Viliers-Saint Paul, France) for conventional histology.

Consecutive sections of each block were stained for cyanide-resistant eosinophil peroxidase (EPO) activity, employing potassium cyanide (Merck), diaminobenzidine and hydrogen peroxide (Merck) to count eosinophils. Sections were stained, coded and read in a 'blind' fashion. Positive cells were enumerated in the bronchial epithelium (between the luminal border and the basal lamina) by means of an eyepiece graticule comprising 100 squares of known area. The area of the compartments and the number of positive cells were determined on each microscope field, and at least 10 fields were analysed. The results of each stained slide were expressed as the number of positive cells per unit area (6.25×10^4 µm^2, the total area of the graticule). Results were calculated for each experimental group.

DNA for genetic studies was extracted by phenol–chloroform methods from mouse tails.

Genotypic studies

A cross was made between female BP2 and male BALB/c mice. An F_1 intercross produced 217 F_2 mice. A polymorphic marker set of 180 microsatellites was created to differentiate the BP2 and BALB/c alleles and the F_2 mice were genotyped. The markers and the primer pairs are available on the WTCHG website (http://www.well.ox.ac.uk).

Forward primers for PCRs were labeled with 6-FAM, HEX, TET or TAMRA fluorescent dyes (Oswel DNA, Edinbugh, UK; Perkin-Elmer, Warrington, UK). PCR of mouse microsatellite loci was performed in 25 µl reactions containing 50 ng of genomic DNA, 67 mM Tris–HCl, pH 8.8, 16.6 mM NH_4SO_2, 0.1% Tween-20 (Bioline, London, UK), 0.2 mM each of dATP, dTTP, dCTP and dGTP, 1.5–3 mM Mg^{2+}, 62.5 ng of each primer and 0.3 U of BIOTAQ polymerase (Bioline). Samples were overlaid with 50 µl of mineral oil. Reactions were performed in Hybaid (Ashford, UK) Omnigene thermocyclers by use of 32 successive cycles, each cycle consisting of 60 s at 94°C, followed by 60 s at 48–60°C, and then 30 s at 72°C.

Products from separate PCRs were pooled to enable simultaneous electrophoretic analysis. Aliquots of 10 µl from each PCR were pooled, and 0.3 µl of the resultant mixture was added to 0.5 µl of Genescan 350 TAMRA or ROX internal lane size standard (Applied Biosystems, Foster City, CA) and 2 µl of formamide containing 3 mg/ml dextran blue dye. Samples were denatured at 95°C for 5 min, put on ice and then loaded onto a gel.

Electrophoresis of samples was carried out on an ABI 373A Sequencer (Applied Biosystems) with 12 cm well-to-read gels composed of 6% (w/v) acrylamide/bisacrylamide at 19:1 ratio, 7 M urea, TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA; Severn Biotech, Kidderminster, UK). Gels were run in TBE buffer for 5 h at a constant 900 V.

PCR products were sized with GeneScan (version 1.2) and Genotyper software (version 1.0; Applied Biosystems).

Statistical analysis

JoinMap version 2.0 (21) was used for all stages of map construction. Initially markers were assigned to the 19 linkage groups corresponding to the autosomal and X chromosomes. Subsequently, each marker in each linkage group was tested for deviation from the expected single factor segregation ratio. Markers which exhibited significant distortion were checked for genotyping errors and, from these revised data, preliminary maps were constructed. The latter served to highlight unexplained double recombination events, thus identifying further possible genotyping errors. The JoinMap module for genotype checking calculates for all loci and for all individuals the probability of obtaining the present genotype, conditional on each genotype at the two flanking loci and on their recombination frequency. We considered unexplained genotypes to be those having a threshold of >3 for the test statistic $\log_{10}(1/p)$. Having corrected genotypes where necessary, maps were constructed anew and tested again for distorted segregation ratios and unexplained double recombination events. This cycle of checking and mapping was repeated until problematical genotypes and markers were either corrected or eliminated from the analysis. The final order of markers and pair-wise recombination frequencies were verified against existing maps. Linear map distances were established using the Kosambi mapping function. X-linked markers were analysed with the males and females treated separately.

The frequency distribution for each trait was tested for skewness and kurtosis using the g_1 and g_2 statistic. Consequently, the square root transformation was applied to P_{enh} and eosinophil counts to normalize the F_2 data.

For each trait, a single marker analysis of variance was performed, followed by three multi-locus QTL mapping procedures: interval mapping (22), regression mapping (23) and marker regression (24). The software, QTL Café (http://www.g.g.seaton@bham.ac.uk), was used for all, except interval mapping, for which Mapmaker/QTL was employed. As

expected, the results obtained by all multi-locus methods were similar, hence we report here the QTL statistics obtained by marker regression and regression mapping. The values taken for significant and suggestive linkages were as previously proposed (5).

The variation among the parental and F_1 individuals provides a measure of the environmental variation, V_E, whilst the variation among the F_2 mice (V_P) is both environmental and genetic, V_G. For each QTL identified, we have estimated the additive effect (a) and dominance deviation (d) (Table 2). Thus, the contribution to the genetic variation of an F_2 by any given QTL will be $\frac{1}{2}a^2 + \frac{1}{4}d^2$ (25), and the variation explained by each QTL was $(\frac{1}{2}a^2 + \frac{1}{4}d^2)/V_P$.

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