Mapping of the X-linked Agammaglobulinemia Locus by Use of Restriction Fragment-length Polymorphism

Sau-Ping Kwan, Louis Kunkel, Gail Bruns, Ralph J. Wedgwood, Samuel Latt, and Fred S. Rosen
Divisions of Immunology and Genetics, Children's Hospital and Departments of Pediatrics and Genetics, Harvard Medical School, Boston, Massachusetts 02115; Departments of Pediatrics, University of Washington School of Medicine, Seattle, Washington 98195

Abstract

A molecular linkage analysis in 11 families with X-linked agammaglobulinemia (XLA) localized the XLA gene to the proximal part of the long arm of the human X chromosome. Significant linkage was detected between XLA and loci defined by two polymorphic DNA probes called 19–2 for the DXS3 locus and S21 for the DXS17 locus. Both localize to the region Xq21.3–Xq22. Most likely recombination distances ($\hat{\theta}$) and associated logarithm of the odds (lod) scores for the XLA-DXS3 and XLA-DXS17 pairs were $\hat{\theta}$ = 0.04 morgans (lod, 3.65) and $\hat{\theta}$ = 0 (lod, 2.17), respectively. Tight linkage between XLA and the locus DXS43 defined by the X short arm probe D2 (localized to Xp22-Xp21) was strongly excluded and we obtained no evidence for significant linkage between XLA and any other X short arm probe. The probe pair 19-2 and S21 should be informative for molecular linkage-based analysis of XLA segregation in the majority of families afflicted with this disorder.

Introduction

In one form of agammaglobulinemia, which is inherited in an X-linked recessive mode (XLA),[1] the serum of males expressing the disease contains markedly diminished or undetectable quantities of all immunoglobulins (1), and B lymphocytes are markedly decreased or absent in peripheral blood, bone marrow, and lymphoid tissues from affected patients. Mature plasma cells cannot be found, although the bone marrow contains normal numbers of pre–B lymphocytes, a fact that might ultimately lead both to basic understanding of the pathogenesis of this disorder and to specific therapy (2). At present, one is limited to empirical therapeutic action, which consists of prophylaxis against recurrent pyogenic infections, achieved by the regular administration of pooled human immune serum globulin.

Address reprint requests to Dr. Kwan, Department of Immunology/Microbiology, Rush Medical School, 1753 West Congress Parkway, Chicago, IL 60613.
Received for publication 5 August 1985.

1. *Abbreviations used in this paper:* lod, logarithm of the odds of linkage; RFLPs, restriction fragment-length polymorphisms; XLA, X-linked agammaglobulinemia.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/86/02/0649/04 $1.00
Volume 77, February 1986, 649–652

Diagnosis of XLA is restricted at present to males who are well past the newborn period. Female heterozygotes for this disease (1) cannot be detected directly, although obligate heterozygotes can be identified by pedigree analysis. Heterozygous females are immunologically normal. It has not yet been possible to perform prenatal diagnosis of XLA in male fetuses. Affected newborn males with XLA are passively protected against infection by maternal IgG for the first year of life (1). Moreover, the clinical and immunologic abnormalities in affected males vary both within and between families, the latter feature promoting the suspicion that "X-linked agammaglobulinemia" might not be a single defect at a unique locus (3). Mapping XLA on the X chromosome, before establishing its linkage relationships with other X chromosome loci, about which published data do not yet exist, would help address this question of XLA heterogeneity and lead to more definitive diagnosis of the disorder.

We undertook this study to ascertain if loci defined by restriction fragment-length polymorphisms (RFLPs) (4) could be found sufficiently close to the XLA locus in available families to provide information on chromosome map location and perhaps to be of use in prenatal diagnosis. Toward this purpose, segregation analysis in 13 families at risk for the transmission of XLA was performed. In view of the rarity of this disorder and the difficulty in finding kindred suitable for this type of analysis, it was fortuitous that we were able to determine that two cloned DNA fragments from the human X chromosome (named S21 [5] and 19–2 [6, 7]) recognize RFLPs mapped as loci DXS17 and DXS3, respectively, to the q21.3–q22 region that are genetically linked to the putative gene for XLA. This provides a first step toward the desired gene mapping and diagnostic capabilities.

Methods

Patients with XLA were identified by the criteria of the Expert Committee on Immunodeficiency of the World Health Organization (8). These patients have been previously described (3, 9, 10). Blood samples were collected into tubes containing EDTA after obtaining informed consent from the propositi and their relatives. Blood samples of 20–40 ml were obtained from 121 individuals in 13 unrelated kindred.

DNA isolation. High molecular weight genomic DNA was isolated from peripheral blood samples according to the protocol of Aldridge et al. (6, 9, 11) except that DNAase-free pancreatic RNAase was added to the mixture at a concentration of 50 μg/ml 1½ h *before* phenol-chloroform extraction and the mixture was dialyzed against 10 mM Tris, 1 mM EDTA, and 20 mM NaCl buffer.

DNA labeling. In vitro labeling of DNA was performed using T4 DNA polymerase according to the method of O'Farrell (12) as modified by Kunkel et al. (13). Briefly, 0.5 μg of DNA was predigested in T4 buffer

(12) with 2–3 U of T4 DNA polymerase for 5 min at 37°C before the addition of 40–80 μCi of [^{32}P]dCTP. The reaction mixture was incubated for 10 min and cold dNTP was added to finish the reaction. The radiolabeled fragments were purified on 0.6% low melt agarose and the appropriate ^{32}P-fragment was recovered from the gel.

Gel analysis and filter hybridization. Genomic DNA was digested for several hours in the appropriate buffer with various restriction enzymes. Electrophoresis was carried out in 0.8% agarose gels in Tris borate buffer (90 mM Tris, 90 mM boric acid, 25 mM EDTA). The gel was denatured in 0.5 M NaOH, 0.5 M NaCl, and neutralized in 0.5 M Tris, pH 7.6, 0.6 M NaCl. DNA from the gel was transferred to nitrocellulose in 20× SSC (3 M NaCl, 0.3 M Na citrate) using the method of Southern (14). After transfer, the filters were rinsed in 4× SSC, and baked for 2 h at 80°C. Prehybridization was performed in a mixture containing 50% formamide, 4× SSC, 8% dextran sulfate, 10× Denhardt's solution (15), 25 μg/ml denatured salmon DNA, 100 μg/ml transfer RNA (tRNA), 1.0 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) for several hours at 42°C. Hybridization was carried out for 18 h in the same solution containing ^{32}P at $\sim 1 \times 10^6$ cpm/ml of ^{32}P-labeled probes. After hybridization the filters were washed in 0.1% SSC, 0.1% SDS at 55°C. Autoradiography was performed at −70°C for 12–36 h, using X-Omat R film (Eastman Kodak Co., Rochester, NY) and an intensifying screen.

Results

DNA from a total of 121 individuals, derived from 13 families, was digested with a panel of restriction enzymes, subjected to Southern blotting, and then probed with one of 18 chromosome-specific DNA fragments.[2] Probes defining loci that map on the short arm of the X chromosome reveal high frequencies of recombination in XLA families (e.g., Table I, DXS43 for probe D2; other data not shown). However, loci defined by two different probes (DXS3 for probe 19–2 and DXS17 for probe S21) (5, 6), that map to Xq21.3-Xq22, show close linkage between the XLA gene and DNA polymorphisms with the enzymes Taq I or MspI.

Fig. 1 shows the segregation of Taq I alleles of probe 19–2 in the pedigree of one family with XLA. The obligate heterozygotes, lane II-2 and II-3, exhibit the heterozygous profile of the 5.2,3.0/2.2 kb Taq I alleles. The lower allele (3.0/2.2 kb) is present in two affected probands (lane III-1, IV-1), while the allele with the 5.2-kb band is present in the unaffected son (lane III-2). This family reveals Mendelian inheritance pattern with no recombinants in three meiotic events. The obligate heterozygote III-3 is homozygous for the lower allele (3.0/2.2 kb) and her affected son has the lower allele.

Linkage analysis was performed on the 10 families that could be scored by analysis with the 19–2 probe, the four families that yielded information that could be scored with probe S21, and four families that were informative with probe D2. Recombinants were detected by comparing the genotypes of heterozygous females with those of their offspring. The phase in the obligate female heterozygote carriers was determined from the genotype of their fathers, whenever possible; otherwise it enters into linkage

Table I. Linkage Analysis of Loci Defined by Three Molecular Probes with the X-linked Agammaglobulinemia Locus

Locus Probe Location	DXS3 19-2 Xq21.3–Xq22	DXS17 S21 Xq21.3–Xq22	DXS43 D2 Xp22.3–Xp21
morgans			
θ			
0	$-\infty$	2.17	$-\infty$
0.05	3.64	1.99	−3.71
0.1	3.41	1.80	−2.31
0.15	3.03	1.59	−1.54
0.2	2.59	1.38	−1.04
0.25	2.12	1.16	−0.69
0.3	1.62	0.93	−0.43
0.35	1.15	0.69	−0.25
0.4	0.70	0.46	−0.12
0.45	0.31	0.34	−0.04
0.5	0	0	0

Summary of the lod scores for possible linkage of XLA with loci DXS3, DXS17, and DXS43 defined by probes 19-2, S21, and D2 at 5 centimorgan recombination (θ) intervals. Shown under the designation for each probe is the approximate probe location on the human X chromosome, based on information provided in the text. Please note that since some of the data for probes 19-2 and S21 were obtained from the same pedigrees and individuals, the sum of the lod scores for their respective loci at each value of θ is not a valid indication of their aggregate evidence for linkage but is an overestimate.

calculations as an uncertainty. The logarithm of the odds of linkage (lod) scores, using all information available, was computed directly, using principles as described by Cavalli-Sforza and Bodmer (23) and Conneally and Rivas (24). The lod score values associated with various values of θ with each probe tested are shown in Table I. The lod score curve for XLA and the locus DXS3 defined by probe 19–2 (Fig. 2) indicates a most probable estimate of the recombination fraction, $\hat{\theta}$, of 0.04 morgans (>95% confidence limits, by direct calculation, between values of $\hat{\theta}$ slightly greater than zero and less than 0.20 morgans) with a lod score of 3.65 at $\theta = 0.04$ morgans. Hence, the odds favoring linkage of XLA with DXS3, compared with the absence of linkage, are more than 4,000 to 1. One obligate recombination in 26 meiotic events between the XLA locus and the DXS3 locus in XLA families has been observed with probe 19-2. This gave rise to the calculated most probable recombination fraction between XLA and probe 19-2 of greater than zero (0.04 morgans). In the case of the locus DXS17, defined by probe S21, however, four families were informative, with no obligate recombinants in 13 meiotic events ($\hat{\theta} = 0$, lod = 2.17). DXS17 did not recombine with XLA in the family in which the single DXS3 crossover event occurred, although DXS17 was not informative in the obligate DXS3 recombinant meiosis.

Discussion

We have analyzed 13 families at risk for XLA, and using RFLPs we were able to detect informative heterozygosity for probes 19–2, S21, or D2 in 12 of the families. In 10 of these families there were 26 informative meiotic events detected with the DNA probe

2. The polymorphic probes used, their approximate locations on the human X (given in parentheses), and their official locus designations [given in brackets] were: Dic 56(pter-p22) [DXS143] (22); RC8(p22.3–p21) [DXS9] (16); D2(p22.3-p21) [DXS43], 99-6(p22-p21) [DXS41], B24(p21) [DXS67], 58-1(p11-cen) [DXS14], 19-2(q21.3-q22) [DXS3], 22-33(q24-qter) [DXS11], 43-15(q24-qter) [DXS42] (6, 7); C7(p21.3-p21.2) [DXS28] (18); p754(p21.2-p21.1) [DXS84] (32); OTC(p21) (20); L.128(p11.3–p11) [DXS7] (17); p8(cen-q12) [DXS1]; pDP34(cen-q13) [DXYS1] (21); PGK(q13) (19); S21(q21.3-q22) [DXS17] (4); and 52A(q27-qter) [DXS51] (7).

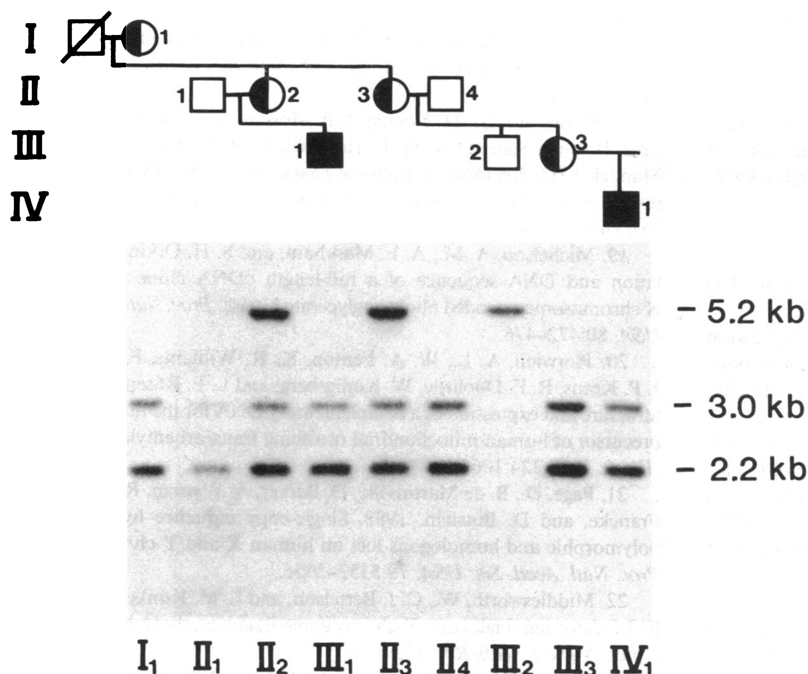

Figure 1. Cosegregation of Taq I alleles of the probe 19-2 and the XLA locus in one of the 10 families studied. The pattern of 5.2-kb and/or 3.0/2.2-kb alleles in Taq I-digested DNA, as revealed by probe 19-2, is shown directly under the symbol for the corresponding member of the pedigree. Affected males are represented by darkened spaces, and obligate female heterozygotes by half-darkened circles. I-1 had a son with XLA who died.

19–2 (6, 7) and in four families, 11 informative meiotic events were detected with another DNA probe S21 (5). Both of these DNA segments mapped to the region Xq21.3-Xq22 (6, 7; and S.-P. Kwan, L. Kunkel, and G. Bruns, unpublished observations). One crossover was observed between the XLA locus and the locus DXS3 polymorphic for the probe 19-2 and none was observed between XLA and the locus (DXS17) polymorphic for the probe S21, consistent with tight linkage of XLA to both of these probes. The combined heterozygosity of probes S21 and 19-2, based on published data (5–7) is greater than 0.75, making this probe combination of significant potential use in the diagnosis of XLA.

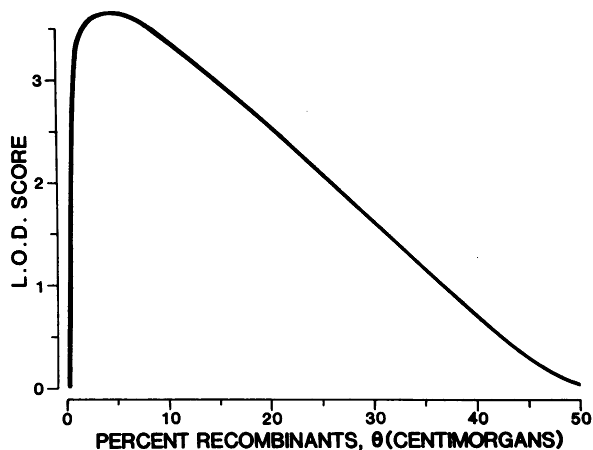

Figure 2. Logarithm of the odds (lod) of the cosegregation observed between the locus DXS3 defined by probe 19-2 and the XLA locus relative to the absence of any genetic linkage as a function of the possible recombination distances (expressed as percentages) between them. The curve derived from data on the 10 informative families described here has a maximum at a value of $\theta = 0.04$ morgans (lod score, 3.65). The nominal confidence range defined by lod scores are one less than the maximum. $0 < \theta \leq 20$ centimorgans includes 96% of the area of a curve of the actual ratio of the odds corresponding to the plot shown.

Tabor et al. (25) have studied a boy with deletion in the X chromosome between bands q13 and q21.3. Although no specific information was reported about the child's immunoglobulin levels, it does not appear from the clinical details given that he had XLA. Nurmi et al. (26) and Eshola et al. (27) have reported three Finnish females with an extensive deletion in the short arm of the X chromosome, localized to Xp22.13-p11.11 or Xp22.11-p11.23 (28, 29). One of these females had decreased T helper lymphocytes but normal numbers of B cells. They concluded that an immunoregulatory defect was present but that the defect did not resemble XLA. Schuurman (30) has studied a number of families with XLA with two short-arm DNA probes, RC8 and L128. He concluded that the XLA gene must lie between Xp21 and Xp11, although the maximum calculated lod scores published for these probes did not reach 1.0. As stated earlier, we have been unable to find any linkage between the X short-arm marker locus DXS43 defined by probe D2 and the XLA gene, and none of the fragmentary data on other X short-arm marker loci and XLA in our families are indicative of an Xp location for XLA. Other abnormalities and deletions of the short arm of X chromosome in males have been described, and none of these patients were reported to have XLA (31, 32).

From our studies it appears that the XLA in the families we examined is a single locus defect, despite the clinical variability among the cases. Now that the XLA gene has been mapped to the proximal region of the long arm of the X chromosome, it appears useful to apply additional RFLPs to refine the map location and ultimately, to use the information obtained for prenatal diagnosis of XLA. For probes 19-2 and S21 described in this study, greater than three-quarters of XLA heterozygotes might be amenable to such an analysis.

Acknowledgments

We thank Elizabeth Gatt, Karen Renaud, and Christopher Zimmerman for excellent technical assistance. We would like to thank Dr. David

Page for the use of the cloned pDP34(DXYS1), Dr. Dennis Drayna and associates for the use of cloned probe S21(DXS17), and Dr. Hans Ochs and Dr. Rebecca Buckley for obtaining blood and Mr. Anthony Monaco for executing some of the ^{32}P labeling experiments.

This work was supported by grants from the March of Dimes Birth Defects Foundation (6-394 and 1-353) and from National Institutes of Health (RR-37, RR-02172, GM 22121/33579, AI 21163, and HD 04807/18658), and from the Muscular Dystrophy Association.

References

1. Rosen, F. S., M. D. Cooper, and R. J. P. Wedgwood. 1984. The primary immunodeficiencies. *N. Engl. J. Med.* 311:235–242.

2. Pearl, E. R., L. B. Vogler, A. J. Okos, W. M. Crist, A. R. Lawton III, and M. D. Cooper. 1978. B lymphocyte precursors in human bone marrow: an analysis of normal individuals and patients with antibody-deficient states. *J. Immunol.* 120:1169–1175.

3. Wedgwood, R. J. 1978. X-linked agammaglobulinemia. *In* CRC Handbook Series in Clinical Laboratory Science. Section F. Vol I., Part 1. D. Seligson, editor. CRC Press, Inc., Boca Raton, FL. 41–50.

4. Botstein, D., R. L. White, M. Skolnick, and R. W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32:314–331.

5. Drayna, D., K. Davies, D. Hartley, J.-L. Mandel, G. Camerino, R. Williamson, and R. White. 1984. Genetic mapping of the human X-chromosome by using restriction fragment length polymorphisms. *Proc. Natl. Acad. Sci. USA.* 81:2836–2839.

6. Aldridge, J., L. Kunkel, G. Bruns, U. Tantravahi, M. Lalande, T. Brewster, E. Moreau, M. Wilson, W. Bromley, T. Roderick, and S. A. Latt. 1984. A strategy to reveal high-frequency RFLPs along the human X chromosome. *Am. J. Hum. Genet.* 36:546–564.

7. Bruns, G. A. P., J. F. Gusella, C. Keys, A. C. Leary, D. Housman, and P. S. Gerald. 1982. Isolation of X-chromosome DNA sequences. *Adv. Exp. Med. Biol.* 154:60–72.

8. Rosen, F. S., R. J. Wedgwood, F. Aiuti, et al. 1983. Primary immunodeficiency diseases. Report prepared for the WHO by a Scientific Group on Immunodeficiency. *Clin. Immunol. Immunopathol.* 28:450–475.

9. Geha, R. S., F. S. Rosen, and E. Merler. 1973. Identification and characterization of subpopulations of lymphocytes in human peripheral blood after fractionation of discontinuous gradients of albumin: the cellular defect in X-linked agammaglobulinemia. *J. Clin. Invest.* 52:1726–1734.

10. Lederman, H. M., and J. A. Winkelstein. 1985. X-linked agammaglobulinemia: an analysis of 96 patients. *Medicine (Baltimore).* 64:145–156.

11. Kunkel, L. M., U. Tantravahi, M. Eisenhard, and S. A. Latt. 1982. Regional localization on the human X of DNA segments cloned from flow sorted chromosomes. *Nucleic Acids Res.* 10:1557–1578.

12. O'Farrell, P. 1981. Replacement synthesis method of labeling DNA fragments. *Focus (Bethesda Res. Lab.).* 3:1–3.

13. Kunkel, L. M., K. D. Smith, S. H. Boyer, D. S. Borgaonkar, S. S. Wachtel, O. J. Miller, W. R. Breg, H. W. Jones, Jr., and J. M. Rary. 1977. Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. *Proc. Natl. Acad. Sci. USA.* 74:1245–1249.

14. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.

15. Denhardt, D. J., D. Cressler, and D. S. Rays, editors. 1978. The Single-Stranded DNA Phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

16. Murray, J. M., K. E. Davies, P. S. Harper, L. Meredith, C. R. Mueller, and R. Williamson. 1982. Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy. *Nature (Lond.).* 300:69–71.

17. Davies, K. E., P. L. Pearson, P. S. Harper, J. M. Murray, T. O'Brien, M. Sarfarazi, and R. Williamson. 1983. Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome. *Nucleic Acids Res.* 11:2303–2312.

18. Camerino, G., M. Koenig, J. P. Moisan, I. Oberle, F. Morlé, M. Jaye, H. de la Salle, D. Weil, B. Hellkuhl, K. H. Grzeschik, and J. L. Mandel. 1984. Regional mapping of coagulation factor IX gene and of several unique DNA sequences on the human X chromosome. *Cytogenet Cell Genet.* 37:431–432. (Abstr.)

19. Michelson, A. M., A. F. Markham, and S. H. Orkin. 1983. Isolation and DNA sequence of a full-length cDNA clone for human X chromosome-encoded phosphoglycerate kinase. *Proc. Natl. Acad. Sci. USA.* 80:472–476.

20. Horwich, A. L., W. A. Fenton, K. R. Williams, F. Kalousek, J. P. Kraus, R. F. Doolittle, W. Konigsberg, and L. E. Rosenberg. 1984. Structure and expression of a complementary DNA for the nuclear coded precursor of human mitochondrial ornithine transcarbamylase. *Science (Wash. DC).* 224:1068–1074.

21. Page, D., B. de Martinville, D. Barker, A. Wyman, R. White, U. Francke, and D. Botstein. 1982. Single-copy sequence hybridizes to polymorphic and homologous loci on human X and Y chromosomes. *Proc. Natl. Acad. Sci. USA.* 79:5352–5356.

22. Middlesworth, W., C. J. Bertelson, and L. M. Kunkel. 1985. An RFLP detecting single copy X-chromosome fragment, dic56, from Xp22-Xpter. *Nucleic Acids Res.* 13:5723.

23. Cavalli-Sforza, L. L., and W. F. Bodmer. 1971. The genetics of human populations. W. H. Freeman and Co. Publishers, New York.

24. Conneally, P. M., and M. L. Rivas. 1984. Linkage analysis in man. *Adv. Hum. Genet.* 10:209–266.

25. Tabor, A., O. Andersen, C. Lundsteen, E. Niebuhr, and H. Sardemann. 1983. Interstitial deletion in the "critical region" of the long arm of the X chromosome in a mentally retarded boy and his normal mother. *Hum. Genet.* 64:196–199.

26. Nurmi, T., M. Uhari, S-L. Linna, R. Herva, K. Tiilikainen, and K. Kouvalainen. 1981. Immunodeficiency associated with a deletion in the short arm of the X-chromosome. *Clin. Exp. Immunol.* 45:107–112.

27. Eskola, J., T. Nurmi, and O. Ruuskanen. 1983. Defective B cell function associated with inherited interstitial deletion of the short arm of the X chromosome. *J. Immunol.* 131:1218–1221.

28. Herva, R., B. Kaluzewski, and A. de la Chapelle. 1979. Inherited interstitial del(Xp) with minimal clinical consequences: with a note on the location of genes controlling phenotypic features. *Am. J. Med. Genet.* 3:43–58.

29. Ingle, C., R. Williamson, A. de la Chappelle, R. R. Herva, K. Haapala, G. Bates, H. F. Williard, P. Pearson, and K. E. Davies. 1985. Mapping DNA sequences in a human X-chromosome deletion which extends across the region of the Duchenne muscular dystrophy mutation. *Am. J. Hum. Genet.* 37:451–462.

30. Schuurman, R. K. B., E. J. B. M. Mensink, J. D. L. Schot, S. A. Thompson, A. Tippett, K. E. Davies, and P. L. Pearson. 1984. X-linked agammaglobulinemia: current state of mapping of the XLA gene for carrier detection and intrauterine diagnosis. *In* Prog. Immunodef. Research and Therapy I. Intl. Congr. Series 645. C. Griscelli and J. Vossen, editors. Elsevier/North Holland, Amsterdam. 77–82.

31. Curry, C. J. R., R. E. Magenis, M. Brown, J. T. Lanman Jr., J. Tsai, P. O'Lague, P. Goodfellow, T. Mohandas, E. A. Bergner, and L. J. Shapiro. 1984. Inherited chondrodhysplasia punctata due to a deletion of the terminal short arm of an X chromosome. *N. Engl. J. Med.* 311:1010–1015.

32. Francke, U., H. D. Ochs, B. de Martinville, J. Giacalone, V. Lindgren, C. Distèche, R. A. Pagon, M. K. Hofker, G.-J. B. Ommen, P. L. Pearson, and R. J. Wedgwood. 1985. Minor Xp21 chromosome deletion in a male associated with expression of Duchenne muscular dystrophy, chronic granulomatous disease, retinitis pigmentosa and McLeod syndrome. *Am. J. Hum. Genet.* 37:250–267.