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Further Studies of Xh, a Serum Protein Antigen in Man[1]

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Abstract. Xh has been identified as a serum protein antigen whose expression is both age and sex dependent, and is shown here to be identical with the antigen previously reported as Pa 1. On immunodiffusion, approximately 75% of randomly selected sera from females of all ages form a precipitin line with specific rabbit antiserum. Frequency of the antigen in females is correlated with age; 94% over 19 years of age are positive while only 55% under 19 are positive. Approximately 47% of male sera are positive; no significant changes in frequency associated with age of males were found. The mean level of the antigen in the sera of females over 19 years of age is approximately twice that in male sera. The heritability of Xh expression on the basis of a study of male twins was 0.66.

A study of 88 families, with 181 offspring selected so that female offspring were at least 20 years old, was inconclusive in two ways: (1) two female offspring exceptional to the hypothesis of sex linkage were found and (2) no negative by negative matings were observed. Thus, although the Xh data are suggestive of a genetic polymorphic system, the influence of physiological factors on Xh expression and the findings, reported here, of similarly high frequencies in adult females of all racial groups, make it more probable that this antigen is an isotypic marker.

Reactivity with anti-Xh was found among sera of some of the great apes and Old and New World monkeys but not among prosimians and lesser mammals. The isotypic specificity is presumed to have arisen during the speciation process leading to the present day anthropoid apes and man.

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Introduction

In 1966, BUNDSCHUH [1] and MACLAREN *et al.* [8] described a genetically determined component of human α_2-globulin detected with heterologous antiserum. BUNDSCHUH [1] called this antigenic component Xh. Immunodiffusion analysis indicated that sera from 21.8 % of males and 77.7 % of females were Xh-positive. In addition, BUNDSCHUH proposed that this antigen was inherited as a sex-linked dominant character though some inconsistencies in the data were apparent. The specificity described by MACLAREN *et al.* [8] was called the Pa 1 antigen. The frequency of Pa 1 in males and young females was reported as 18 %; autosomal inheritance was proposed. More recently, KUEPPERS [7] reported that by filling antigen wells three times on immunodiffusion he could detect Xh antigen in the serum of approximately 92 % of females and 88 % of males.

In this report we show that anti-Xh and anti-Pa 1 detect the same antigenic specificity and we extend the population and genetic studies of this antigen under the premise that a more sensitive typing method might clarify discrepancies in existing frequency and genetic data.

Materials and Methods

Preparation of antigen. Macroglobulins were precipitated from pooled human sera in 50 % ammonium sulfate, resuspended in buffered saline, dialyzed overnight in the same buffer at 4 °C, and reduced under vacuum to a volume yielding a protein concentration of approximately 10 times normal levels. The preparation was further fractionated by gel filtration on Sephadex G-200 equilibrated in 0.1 M tris, 0.02 M NaCl buffer. Proteins excluded from the gel (void volume) were pooled, lyophilized and reconstituted in phosphate-buffered saline at a concentration of 40 mg/ml.

Preparation of antisera. Four New Zealand white rabbits were injected intravenously every 3rd day for 5 weeks with 1 ml of the macroglobulin preparation. Blood was collected from each rabbit 10 days after the last injection and several times thereafter. Specific anti-Xh was prepared by absorbing the rabbit immune sera with an equal volume of whole serum from Xh-negative individuals.

Immunological analyses. Freshly drawn sera for typing were obtained daily from patients seen at the University of Michigan Hospital. Initially, the double diffusion method of OUCHTERLONY [10] was used in screening and typing. These tests were set up on 3.25 × 4 inch glass plates covered with 12.5 ml of 0.8 % buffered agarose and incubated in moist boxes at 36 °C for 24 h. After variability in the quantity of antigen among sera was noted, a control serum which formed a sharp precipitin line with the antiserum was always put in a well adjacent to serum to be typed. The detection of weakly reactive sera was then facilitated by the deflection of the sharp

precipitin line. However, we later chose to type all sera by the more sensitive method of double diffusion in tubes [11]. These tests were set up in 6 x 50 mm culture tubes. The reagents and amounts layered in each tube were 40 μl of antiserum (mixed with an equal volume of buffered agarose solution), 200 μl of 0.4 % buffered agarose, and 40 μl of the serum to be typed. The tubes were sealed with paraffin and incubated at 36 °C for 5–6 days. Each positive serum was expressed as the ratio of the distance from the antigen-agar interface to the middle of the region of greatest density in the precipitin band over the total length of the agar column.

Population and genetic studies. Population frequencies were based on random serum samples collected daily from the Clinical Laboratories of the University Hospital. The major racial components of this group were determined to be approximately 88 % Caucasian and 11 % Negro. Genetic studies were performed with frozen sera from: (1) 215 adult male Caucasian twin pairs, previously diagnosed as to zygosity by a number of blood groups, serum proteins, and morphological characteristics [6], and (2) families in the Tecumseh Michigan Health Study [3].

Other human and non-human primate sera used in these studies were generously supplied by various investigators.

Results

The rabbit antiserum used in these studies was identified as anti-Xh both by its reaction on immunodiffusion with control sera and by comparison with a known anti-Xh sent to us by Dr. KUEPPERS. Subsequently, this anti-Xh was found to have the same antibody activity as anti-Pa 1 supplied by Dr. ALPER (figure 1).

Xh antigen migrated on immunoelectrophoresis with the α_2-globulins and eluted from Sephadex G-200 with the large molecular weight globulins (e.g., α_2-macroglobulins, ceruloplasmins, etc.). However, there were no detectable immunological reactions between this α_2-globulin and specific antisera for α_2-macroglobulin, ceruloplasmin, haptoglobin, group specific component and α_2-lipoproteins.

The frequency of Xh-positive sera among females was approximately twice that of males (table I). Population frequencies of 0.45 for males and 0.75 for females were consistent with the ratio expected for an X-linked marker. The high frequency of positives among females reduced considerably the chances of obtaining matings with negative mothers. Unfortunately, matings involving negative mothers are potentially the most discriminating to the hypothesis of X-linkage, since positive females are of indeterminate zygosity and, therefore, offspring of neither phenotype can be excluded.

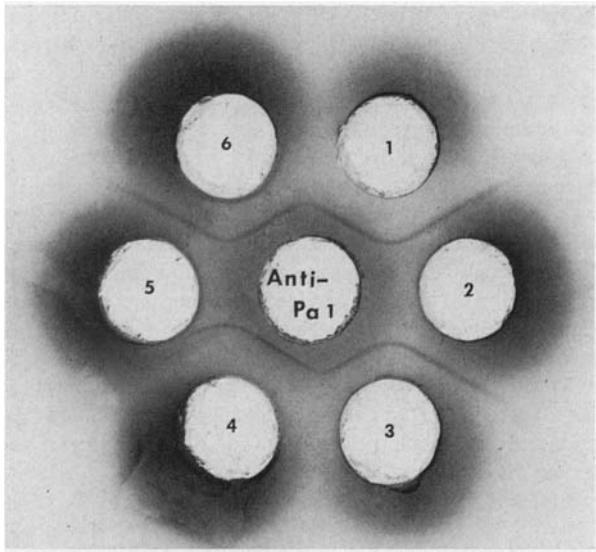

Fig. 1. Immunodiffusion plate illustrating the identity of anti-Pa 1 and anti-Xh. Well 1 contains the Pa 1-positive control serum diluted 1 : 2 in saline; well 2 contains anti-Xh; well 3 contains Xh-positive serum diluted 1 : 2 in saline; well 4 contains undiluted Pa 1-positive serum; well 5 contains anti-Xh; well 6 contains undiluted Xh-positive serum, and the center well contains anti-Pa 1.

Table I. Frequency of Xh as ascertained from fresh serum samples of unselected patients at the University of Michigan Hospital

Sex	Phenotype		Total	Frequency positive
	positive	negative		
Male	98	119	217	0.45
Female	172	58	230	0.75
Total	270	177	447	0.60

The results of the first family study are presented in table II. In the first mating type, (+) female x (+) male, 27 % of the female progeny were negative, and in the third mating type, (+) female x (–) male, 3 out of 4 female progeny were typed as negative. The phenotype of these progeny was inconsistent with the proposed hypothesis of X-linkage. The probable

Table II. Distribution of Xh phenotypes in unselected offspring of 4 mating types from families in Tecumseh, Mich.

Mating type		Number of families	Progeny				Total
♀	♂		female		male		
			–	+	–	+	
+	+	38	(15)[1]	39	32	17	103
+	–	43	22	34	29	24	109
–	+	4	(3)	1	4	(1)	9
–	–	6	7	(3)	6	(1)	17
Total		91	47	77	71	43	238

Frequency of positives among female parents 89 %, male parents 47 %, female progeny 62 %, male progeny 38 %.

[1] Parentheses denote the number of exceptional offspring according to the hypothesis of sex linkage.

Table III. Distribution of Xh phenotype among various age groups

Age group	Sex	Frequency Xh (+)	Sample size	Mean ratio [1]	Variance
1–12	♂	0.44	39	0.25	0.004
	♀	0.51	35	0.27	0.003
13–19	♂	0.51	35	0.28	0.006
	♀	0.58	33	0.38	0.001
20–29	♂	0.42	55	0.23	0.004
	♀	0.94	79	0.46	0.019
30–39	♂	0.35	23	0.28	0.004
	♀	0.97	34	0.44	0.013
40–49	♂	0.54	22	0.31	0.004
	♀	1.00	41	0.41	0.011
50–59	♂	0.54	50	0.34	0.010
	♀	0.95	22	0.46	0.053

Total sample size 468.

[1] Distance from top of agar to middle of precipitin band over total length of agar.

Table IV. Heritability of Xh: a comparison of concordance in male monozygotic and dizygotic twins

Xh type of twin pairs	Monozygotic	Dizygotic	Total
+ +	23	8	31
+ −	11	36	47
− −	65	72	137
Total [1]	99	116	215
Like	88	80	168
Un-like	11	36	47
Total [2]	99	116	215

[1] $\chi^2 = 19.69$, d.f. = 2.
[2] $\chi^2 = 12.4$, d.f. = 1.

explanation for this inconsistency became evident when it was noted that the frequency of Xh-positives was 89 % among female parents and only 62 % among female progeny. Among males, the frequencies were 47 % in parents and 38 % in progeny.

Table III presents data on the relationship between age and Xh expression. An abrupt increase in Xh frequency among females occurs after age group 13–19. This increase was not observed among males in comparable age groups. Failure to control for this sex-associated age variation in Xh expression clearly affects the expected frequencies of the mating types. If one assumes that the population is mating at random with regard to the Xh antigen and that the frequency of the antigen in age-unselected females is 0.75 and 0.45 in males (table I), then significantly fewer matings than expected involving negative females are observed. If the frequencies observed in the parent (i.e. age-selected) population (table II) are used to derive gene frequencies, the observed mating type frequencies do not differ significantly from expected frequencies for a randomly mating population. It is worth noting that the variance among males was less than that of females. The difference between the mean level of antigen in adult males and females is statistically significant ($p < 0.05$).

The heritability of Xh expression was estimated from data on a group of sera from monozygotic and dizygotic twins. The data are presented in table IV. 89 % of the monozygotic twins were alike in Xh phenotype,

Table V. Family study II: distribution of Xh phenotypes in families selected with female progeny 20 years or older; and male progeny, 17 years or older

Mating type		Number of families	Progeny				Total
♀	♂		female		male		
			(–)	(+)	(–)	(+)	
+	+	37	(1)[1]	30	22	20	73
+	–	48	10	37	39	16	102
–	+	3	(1)	2	3	0	6
–	–	0	0	0	0	0	0
Total		88	12	69	64	36	181

Frequencies: female parents 0.96, male parents 0.45, female progeny 0.85, male progeny 0.36.

[1] Parentheses denote the number of exceptional offspring according to the hypothesis of sex linkage.

Table VI. Non-Caucasian distribution of Xh phenotype

Population	Sex	Xh (+)	Sample size	Frequency Xh (+)	Mean age, years	Median age, years
Indian (Apache)	♂	20	32	0.62	40.6	32.0
	♀	39	43	0.90	28.4	23.0
Negro (Ghana)	♂	68	122	0.56	20.6	17.2
	♀	55	67	0.82	20.3	17.8
Oriental (Korea)	♂	42	73	0.57	40.0	39.0
	♀	52	62	0.82	41.5	37.0
Negro (US)	♂	18	41	0.43	–	–
	♀	38	50	0.76	–	–

$\chi^2 = 2.95$ in males and 2.68 in females. d.f. = 3.

Table VII. Distribution of Xh phenotype among non-human mammals

Population	Phenotype	
	(+)	(–)
Non-primates		
Cat	0	1
Sheep	0	2
Mice	0	3
Goat	0	3
Guinea pig	0	1
Rat	0	3
Dog	0	5
Primates		
Prosimians		
Three shrew	0	20
Lemur	0	6
Loris	0	20
New World monkeys		
Capuchin	15	4
Owl monkey	6	3
Squirrel monkey	13	2
Old World monkeys		
Patas	4	0
Stump-tailed	6	0
Cynomologus	3	0
Cynocephalus	19	0
Rhesus	18	3
Pig-tailed	7	0
Great apes		
Gibbon	7	0
Orangutan	4	2
Gorilla	10	0
Chimpanzee	36	27

Total non-primates 18, total non-human primates 235.

whereas only 60 % of dizygotic twins were alike ($p < 0.001$). A heritability estimate of 0.66 was obtained by the method described by NEEL and SCHULL [9].

Because of the quantitative variation in expression of the antigen, another family study was undertaken in which typing was performed by the

semi-quantitative immunodiffusion method of PREER [11]. In addition, age variation was controlled by selecting families with female progeny at least 20 years of age. The results are presented in table V. Two excluded females occurred in families with positive fathers.

Different racial groups were also investigated (table VI), in the hope of finding a population with a higher frequency of negative females. Presumably, family studies in such a population would be more informative with regards to segregation data. Though there was some variation in phenotype frequencies between different populations, the differences were not significant. The χ^2 for the comparison of female populations was 2.68 and that of the males was 2.95 (3 d.f.). The antigen is present in relatively high frequencies in each of the races sampled and the differential sex ratio in Xh frequency was approximately the same.

Table VII shows the distribution of the Xh antigen among various genera and species of mammals. An antigen which cross-reacts with anti-Xh was detected in the sera of both Old and New World monkeys, but not in the prosimians and more distantly related mammals. Among the 55 chimpanzees identified by sex, 4 of 16 males and 19 of 26 females were positive ($p < 0.01$). Among the 21 Rhesus, 4 of 5 males and 14 of 16 females were positive. Thus the sex-associated difference in frequency of the antigen is clearly established among chimpanzees.

Discussion

We have demonstrated here the identity of two independently reported sex-associated antigens, Pa 1 and Xh. The fact that the antiserum specific for this antigen reacts with sera from some individuals but not others is, in itself, not sufficient evidence to assume allotypy, for such an observation could be due simply to the presence or absence of the molecules on which the antigen is located. Therefore, until an isotypic specificity is recognized for this antigen, we submit that an allotypic designation such as Pa 1 is unfounded [2]. In these studies, we have continued to use the term Xh, not to indicate the inheritance pattern of this antigen, but because its expression is influenced by sex and because its nature is unknown.

This report confirms the difference in Xh phenotypic frequencies between the sexes and demonstrates that the frequency of this antigen is elevated in women over 19 years of age. In addition, we report that the level of antigen among women is both higher and more variable than that

of men. GESERICK *et al.* [4] found that Xh is elevated during pregnancy. Unpublished data from this laboratory show the elevation of Xh in normal healthy women following oral administration of ethinyl estradiol. The quantity of Xh in women is clearly subject to considerable variability.

The case for genetic control of a positive versus negative phenotype in an antigen whose expression is influenced by physiological factors (sex, age and pregnancy) is a risky proposition. BUNDSCHUH [1] and MACLAREN *et al.* [8] studied the inheritance of this antigen in families with progeny of unspecified ages and detected progeny whose phenotypes were inconsistent with both X-linkage and autosomal inheritance. BUNDSCHUH [1] proposed a hypothesis of X-linkage in association with sex-limitation to explain his data. MACLAREN *et al.* [8] concluded that the mode of inheritance of the Pa 1 antigen (shown here to be identical to Xh) was autosomal but that recessiveness or dominance could not be determined in the presence of overwhelming hormonal influences.

A number of observations lead to the conclusion that the Xh antigen may in fact be an isotypic specificity, i.e. present in all normal individuals. These include its elevation in older women, its elevation during pregnancy and a rather widespread inability to demonstrate single gene control. Perhaps the most supportive evidence of this proposition is the antigen's near absolute frequency in women of all racial groups. It seems probable that the Xh-negative phenotype reflects more a technical than genetic deficiency [7].

Isotypic antigens are, however, subject to genetic control of quantity and/or structure. The heritability estimate of 0.66 suggests that a genetic component may be responsible for some of the observed variation, at least in males. Electrophoretic variants of Xh have not been detected in a limited study of human sera, although the possibility exists that structural polymorphism, analogous to that in Gc [5], will be found. An electrophoretic variant was, however, observed in the serum of one Rhesus monkey; unfortunately, no family sera could be obtained.

The high frequency of the antigen among chimpanzee females is analogous to the situation found in humans. One might suppose that the same association between sex and antigen frequency will also be found in other species in which the antigen has been found but for which sex data is presently unavailable. The correlation between conditions causing elevations in female sex hormones and those associated with increases in Xh suggest that the protein identified by this specificity functions as a basic component of mammalian sexual physiology. The 'isotypic speci-

ficity' may then have resulted from a structural change in an ancestral protein as part of the speciation process leading to the present day anthropoid apes and man.

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