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Family studies in scleroderma (systemic sclerosis) demonstrating an HLA-linked increased chromosomal breakage rate in cultured lymphocytes*

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Summary. An increased chromosomal breakage rate (ICBR) was found in 27 of 28 patients with scleroderma (systemic sclerosis, SS) – 5 with the syndrome including calcinosis cutis, Raynaud phenomenon, esophagus hypomotility, sclerodactyly and telangiectasia (CREST), 4 incomplete CREST, 1 overlapping syndrome, 18 progressive systemic sclerosis (PSS). Not only the patients, but also about half of their first-degree relatives showed an increased chromosomal breakage rate (more than 5 breaks per 100 metaphases). This character segregated as a dominant marker in nine families of scleroderma patients. In the six informative of the nine families, the ICBR trait showed close linkage with the HLA region on chromosome 6 (total lod score 5.5 at $\theta = 0$). In these families, ICBR was predominantly observed in linkage with HLA haplotype **A1**, **Cw7**, **B8**, **C4AQ0B1**, **DR3** which is frequently observed in autoimmune diseases. The nature of the agent inducing chromosomal breakage in cultured lymphocytes of some, but not all family members of scleroderma patients remains to be clarified.

Introduction

Scleroderma (systemic sclerosis, SS) is a disease characterized by vascular involvement and progressive sclerosis of the skin, lung, esophagus, heart and other organs. Its aetiology is still unknown. However, several immune phenomena like hypergammaglobulinemia, circulating immune complexes, antinuclear antibodies, mononuclear cell infiltrates and graft-versus-host disease-like reactions may support the view of SS as an autoimmune disease of the connective tissue (see LeRoy 1981 for a review).

The involvement of the immune system gave rise to several reports of a possible association with antigens of the HLA system; however, none of these reports disclosed a clear-cut relationship. HLA B8 was thought to be a marker for a more severe, rapidly progressing disease (Tiwari and Teresaki 1985 for a review). According to Black et al. (1984), the syndrome including calcinosis cutis, Raynaud phenomenon, esophagus hypomotility, sclerodactyly, and telangiectasia (CREST) may be characterized by the occurrence of HLA DR5. In 1984 it was also suggested that a genetic deficiency of the fourth component of complement (C4) could predispose to the disease (Mollenhauer et al. 1984; Rittner et al. 1984a). Briggs et al. (1987a) noted a significant increase of silent or "null" alleles in scleroderma patients at the C4A locus and traced it back to the strong association with the HLA haplotype **A1**, **Cw7**, **B8**, **DR3**, which includes a deletion at the C4A locus (Carroll et al. 1985; Schneider et al. 1986).

In a number of publications, Emerit et al. (1976) have recently described an increased chromosomal breakage in autoimmune diseases like SS, systemic lupus erythematosus (SLE) and others assuming a clastogenic, transferable factor of possible viral origin as inducing agent (see Emerit 1980 for a review). In family studies (Emerit et al. 1976), an increased chromosomal breakage rate was not only found in cultured lymphocytes of the patients, but also in a majority of the relatives. A mode of inheritance, however, could not be deduced, also because unrelated spouses had increased rates. Interestingly, in NZB mice, the classical model for autoimmune diseases, elevated rates of secondary aberrations were in accordance with the assumption of a dominant mode of inheritance (Levy et al. 1982). Since in some families, clustering of different autoimmune diseases like SS, SLE, rheumatoid arthritis (RA), primary biliary cirrhosis (PBC), multiple sclerosis (MS) and Raynaud's disease is known, a genetic predisposition and a common denominator in the pathogenesis leading to increased chromosomal breakage, HLA association and C4 deficiency, have been assumed (Rittner 1983).

In the present paper, we present evidence for increased chromosomal breakage rates (ICBR) occurring not only in SS patients, but also in about half of their first-degree relatives. ICBR segregates as an autosomal dominant marker in close linkage with HLA haplotypes, predominantly HLA **A1**, **Cw7**, **B8**, **C4AQ0B1**, **DR3**, suggesting that an MHC-related clastogenic factor may be responsible for the increased numbers of secondary aberrations observed in cultured lymphocytes of SS patients and about half of their family members.

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Materials and methods

A total of 28 patients with scleroderma (SS) – 5 with CREST syndrome, 4 with incomplete CREST, 1 overlapping syndrome and 18 with progressive SS – 38 first-degree relatives in nine families and 16 unrelated controls from Germany and the United Kingdom were investigated, some of them at different times. Lymphocytes from whole blood of all individuals were cultured under phytohemagglutinin (PHA) stimulation for 72 h in Seromed chromosome medium or in Gibco medium A. In addition, isolated fresh peripheral blood lymphocytes (PBL) from eight patients and thawed PBL from five patients were cultured under the same conditions. Cultures were stopped by adding colcemid and subsequently treated according to Moorhead et al. (1960) to prepare metaphase chromosomes. Homogeneous chromosome staining was done with Giemsa or Orcein. Differential staining was performed by Q-banding with quinacrine mustard (QFQ). At least 50, and if possible 100 metaphases, were screened for secondary structural aberrations by light microscopy. All secondary chromosomal aberrations were classified according to the international system for human cytogenetic nomenclature (ISCN 1985).

Allotyping of chromosome 6 markers included HLA-A, B, C, BF, C4A, C4B, and DR, and was performed as described (Rittner et al. 1984b; Briggs et al. 1987a).

Lod scores were calculated according to Morton (1955) by Max P. Baur, using the linkage program LIPED of Ott (1974).

Results

Definition of increased chromosomal breakage rate (ICBR)

All patients and family members were karyotyped and found to be normal, except one patient who has a primary chromosomal aberration (46,XX/47,XXX mosaic) without phenotypical changes (see patient 22 in Table 1).

The frequency of the spontaneously occurring secondary aberrations in the patients is shown in Table 1. In the table all patients with their individual disease, the percentage of aberrant mitoses, the frequency of aberrations and breaks per mitosis in cultures from whole blood and from isolated lymphocytes of some patients are listed, including mean and standard deviation. As a rule, one break was detected per aber-

Table 1. Spontaneously occurring chromosomal aberrations in lymphocyte cultures of patients with scleroderma. A, % Aberrant mitoses; B/M, breaks/mitosis; A/M, aberrations/mitosis; PSS; progressive systemic sclerosis; CREST, calcinosis cutis, Raynaud phenomenon, oesophagus hypomotility, sclerodactyly, telangiectasia; inc. CREST, incomplete CREST; DM, dermatomyositis; SS, Sjögren syndrome; overl. s., overlapping syndrome

Patient	Whole blood			Lymphocytes			Diagnosis
	A	A/M	B/M	A	A/M	B/M	
1	18	0.20	0.16				PSS
2	12	0.12	0.10				inc. CREST
3	32	0.40	0.20				PSS
4	22	0.26	0.12	26	0.30	0.24	CREST
5	22	0.28	0.20	12	0.12	0.10	CREST
6	0	0.0	0.0	0	0.0	0.0	PSS
7	10	0.10	0.10	28	0.32	0.26	PSS
8	12	0.12	0.12	16	0.16	0.10	PSS
9				12	0.12	0.10	PSS + DM
10	14	0.14	0.08				PSS
11	16	0.16	0.14	16	0.16	0.12	PSS + SS
12	8	0.10	0.06	14	0.14	0.12	PSS
13	12	0.12	0.10	12	0.12	0.08	PSS
14	14	0.14	0.10	12	0.14	0.10	PSS
15	14	0.14	0.06	14	0.16	0.12	PSS
16	18	0.24	0.20	18	0.34	0.32	PSS
17	16	0.26	0.26	20	0.36	0.38	overl. s.
18	14	0.18	0.12				CREST
19	8	0.10	0.08				inc. CREST
20	14	0.16	0.10				CREST
21	8	0.08	0.08				PSS
22	10	0.10	0.10				PSS
23	13	0.13	0.08				CREST
24	12	0.12	0.10				inc. CREST
25	10	0.10	0.08				inc. CREST
26	14	0.14	0.08				PSS
27	18	0.18	0.18				PSS
28	12	0.14	0.14				PSS
Mean ± SD	13.8 ± 5.8	0.16 ± 0.08	0.12 ± 0.06	15.4 ± 7.0	0.19 ± 0.11	0.15 ± 0.11	

Table 2. Chromosomal aberrations and breaks per mitosis in controls

Proband	A	A/M	B/M
1	2	0.02	0.02
2	0	0.0	0.0
3	6	0.06	0.02
4	2	0.02	0.0
5	0	0.0	0.0
6	5	0.05	0.03
7	4	0.04	0.03
8	5	0.06	0.02
9	6	0.06	0.02
10	3	0.03	0.01
11	5	0.05	0.04
12	5	0.05	0.01
13	5	0.05	0.04
14	4	0.04	0.02
15	3	0.03	0.03
16	3	0.03	0.01
Mean ± SD	3.6 ± 1.9	0.04 ± 0.02	0.02 ± 0.01

Table 3. Frequencies of aberrations and breaks per mitosis in relatives with normal breakage rates

Proband	A	A/M	B/M
1	5	0.06	0.03
2	6	0.06	0.05
3	5	0.05	0.02
4	5	0.05	0.04
5	2	0.02	0.02
6	5	0.06	0.04
7	5	0.06	0.03
8	5	0.05	0.04
9	2	0.02	0.02
10	6	0.06	0.02
11	4	0.04	0.04
12	4	0.04	0.02
13	4	0.04	0.02
Mean ± SD	4.5 ± 1.3	0.05 ± 0.01	0.03 ± 0.01

rant mitosis. In some, but not all patients, where the results from whole blood and isolated lymphocytes can be compared, the breakage rate in cultures from isolated PBL was higher. As can also be seen, there is no correlation between the classification of the patients' disease and the percentage of aberrations. Patient 6 served as a negative control. He was the only male patient and may have suffered from an occupational pseudo-scleroderma. At the time of investigation he was in remission. In Tables 2–4, the results of aberrations and breaks per mitosis of the healthy controls and the relatives with normal and increased breakage rates, respectively, are listed. It is noteworthy that the magnitude of aberrations is similar in patients and their relatives with ICBR. This fact strengthens the view that ICBR is not a disease-related phenomenon.

In Fig. 1, all aberrations are represented graphically in the four subgroups studied: patients, normal healthy controls, relatives with normal breakage rates, relatives with increased breakage rates. As a main result of this study, chromatid breaks, gaps and fragments form the major portion of secondary aberrations in scleroderma patients whereas translocations are almost missing. This finding contrasts with the pattern of

Table 4. Frequencies of aberrations and breaks per mitosis in relatives with increased breakage rates

Proband	A	A/M	B/M
1	16	0.18	0.12
2	12	0.12	0.10
3	12	0.12	0.10
4	14	0.16	0.10
5	12	0.12	0.08
6	20	0.20	0.14
7	18	0.18	0.12
8	7	0.07	0.06
9	8	0.08	0.07
10	14	0.16	0.08
11	18	0.18	0.09
12	20	0.22	0.16
13	8	0.08	0.08
14	10	0.10	0.08
15	6	0.14	0.14
16	12	0.12	0.12
17	10	0.10	0.10
18	12	0.14	0.12
19	8	0.08	0.06
20	30	0.32	0.20
21	22	0.28	0.20
22	10	0.12	0.08
23	12	0.12	0.09
24	10	0.10	0.08
25	20	0.22	0.10
Mean ± SD	13.6 ± 5.7	0.15 ± 0.06	0.11 ± 0.04

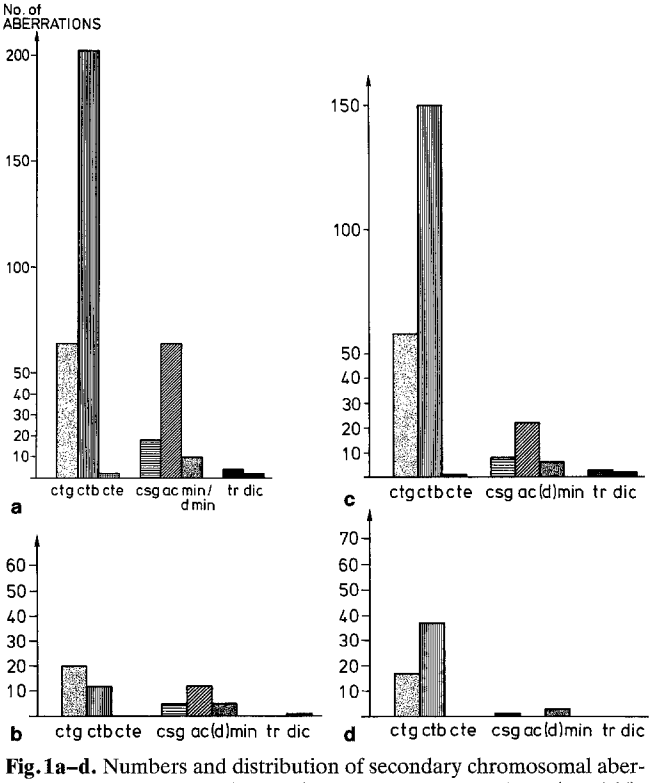

Fig. 1a–d. Numbers and distribution of secondary chromosomal aberrations in lymphocyte cultures of: **a** 28 scleroderma patients ($n = 366$); **b** 16 controls ($n = 55$); **c** 25 relatives with increased breakage rates ($n = 250$); **d** 13 relatives with normal breakage rates ($n = 58$). *ctg* Chromatid gaps, *ctb* chromatids breaks, *cte* chromatid exchanges, *csg* chromosome gaps, *ac* acentric fragments, *(d)min* (Di)min, *tr* translocations, *dic* dicentric chromosomes

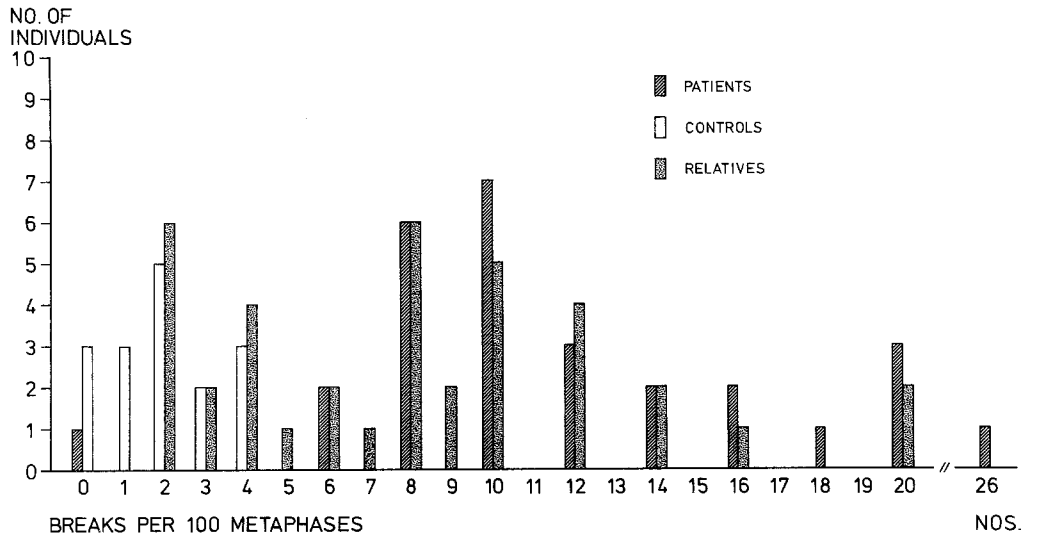

Fig. 2. Histogram of numbers of breaks in 100 metaphases of 28 scleroderma patients, 38 relatives and 16 controls

secondary aberrations in chromosomes of classical breakage syndromes (see Discussion). It can be seen that the pattern of aberrations is similar in all groups studied, depending only on the different numbers observed. It is obvious that chromatid breaks are the most frequently observed aberrations, as already shown by Emerit and Marteau (1971). In the following, we concentrate therefore on the breakage rate.

In Fig. 2, the breakage rates of all groups studied are represented in a histogram. In 16 normal controls 0–4 breaks were counted (open columns). All 28 patients except one showed increased breakage rates (ICBR) (shaded columns). The relatives, however, show a bimodal distribution and can therefore be subdivided into a group with normal breakage rates and another group with ICBR (dotted columns). It could be concluded from this observation that ICBR also occurs in normal individuals and must not lead to the disease. Since there is no significant overlap between the two modi, normal breakage rate is defined as $ICBR^-$, compared to $ICBR^+$. To demonstrate the constancy of the observation in a given individual, some members of family BK were investigated a second time after a period of two years. The results are given in Table 5. On both occasions tested, the family members belong either to the group with normal or increased breakage rate. Therefore, $ICBR^-$ and $ICBR^+$ can be regarded as constant characters of a given individual, which can be genetically determined.

Segregation of ICBR in scleroderma families

This conclusion was supported by the mode of inheritance in nine families of scleroderma patients. In all families the trait increased chromosomal breakage rate (ICBR) segregated like an autosomal dominant marker. At least one parent possesses ICBR if one child exhibits it. ICBR is found in about 50% of the offspring, without sex preference.

Possible linkage of ICBR and HLA

In addition to the ICBR fraction, the MHC haplotypes are indicated in Fig. 3a–e, representing six of the nine families that were informative for linkage analysis of ICBR with the HLA region. The most informative family for linkage analysis, family BK, is shown in Fig. 4: three sibs, II.1, II.3 and II.4,

Table 5. Cytogenetic parameters of family BK. bs_1, First blood sample; bs_2, second blood sample; nt, not tested

Proband	Age at bs_1	A		B/M	
		bs_1	bs_2	bs_1	bs_2
II.1	63	12	16	0.1	0.14
II.2	54	6	4	0.02	0.0
II.3	59	14	12	0.08	0.18
II.4	57	16	10	0.12	0.1
II.5	55	0	0	0.0	0.0
II.6	56	5	nt	0.03	nt
III.1	17	14	20	0.1	0.16
III.2	16	12	8	0.08	0.08
III.3	27	6	6	0.05	0.04
III.4	25	20	nt	0.14	nt
III.5	30	5	6	0.02	0.04
III.6	15	12	8	0.1	0.1

are HLA-identical, one of them suffers from scleroderma. Their deceased mother is said to have had the disease. All three (II.1, 3, 4) exhibit ICBR. Two of them are married and have given the marker ICBR together with the rare HLA haplotype b to four of six offspring. This rare haplotype **A3**, **Cw6**, **Bw47**, **BFS**, **C4A1BQ0**, **DR7** includes a deletion of C4B and the 21-hydroxylase B genes (Schneider et al. 1985, 1986). The other two children inherited haplotype a from their mother and have a normal breakage rate. Therefore, the odds are 6:0 in favour of linkage of ICBR and HLA.

In three of the remaining five families the so-called extended haplotype **A1**, **Cw7**, **B8**, **BFS**, **C4AQ0B1**, **DR3** is involved and is segregating together with ICBR. In family K with three generations studied, ICBR is found in the grandfather, daughter and grandson whereas only the daughter is affected (see Fig. 3a). In family LJ (Fig. 3b) the mother is affected with SS and has given haplotype c to two children together with ICBR. Two sibs received haplotype d and show normal chromosomal breakage rates. In family SI (Fig. 3c) three children share haplotype b, one is affected. All also show ICBR; the sib II.2 carrying haplotype a has a normal breakage rate.

Family H (Fig. 3d) can only partly be analysed since the mother is deceased. The parents must have shared the ex-

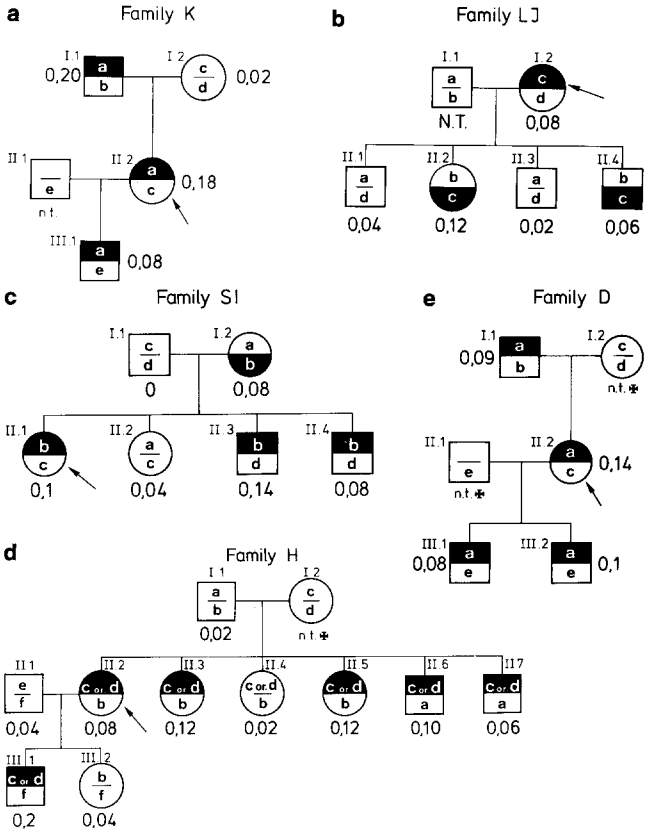

Fig. 3a–e. Pedigree of further families informative for linkage of ICBR with the MHC complex. *Arrows* affected members; *haplotype marked black* HLA haplotype carrying ICBR marker; *numbers under the symbols* breaks per 100 metaphases. The haplotypes are listed below, informative haplotypes are printed in *bold face.* **a** Family K with HLA haplotypes as follows: *a* **A1, Cw7, B8, BfS, C4AQ0, C4B1, DR3, GLO1**; *b* A24, Cw−, B18, BfS, C4A4, C4B2, DR2, GLO2; *c* A2, Cw6, B17, BfS, C4A6, C4B1, DR7, GLO2; *d* A1, Cw7, B8, BfS, C4A3, C4B1, DR2, GLO2; *e* A3, Cw7, B7, BfS, C4A3, C4B1, DR4, GLO2. **b** Family LJ with HLA haplotypes as follows: *a* A2, B27, BfS, C4A3, C4BQ0, DRw6; *b* A3, Bx, BfS, C4AQ0, C4B1, DR3; *c* **A1, B8, BfS, C4AQ0, C4B1, DR3**; *d* A3, B35, BfF, C4A3,2, C4BQ0, DR1. **c** Family SI with HLA haplotypes as follows: *a* A2, B17, DR7; *b* **A30, B8, DR3**; *c* A3, B15, DR3; *d* A1, B7, DR2. **d** Family H with HLA haplotypes as follows: *a* A1, Cw7, B8, BfS, C4AQ0, C4B1, DR3; *b* A2, Cw7, B39, BfF, C4A3,2, C4B1, DR1; *c, d* **A1, Cw7, B8, BfS, C4AQ0, C4B1, DR3**; *e* A3, Cw5, B44, C4A3, C4BQ0, DR4; *f* A1, Cw−, B5, BfS, C4A3, C4B1, DR2. **e** Family D with HLA haplotypes as follows: *a* **A28, Cw5, Bw55, BfS, C4A4, C4B4, DR1**; *b* A3, Cw7, B7, BfS, C4AQ0, C4B1, DR2; *c* A29, Cw−, B44, BfF, C4AQ0, C4B1, DR4; *d* A1, Cw3, Bw62, BfF, C4A3, C4B1, DRw6; *e* A32, Cw4, B35, BfS, C4A3, C4B1, DRw6

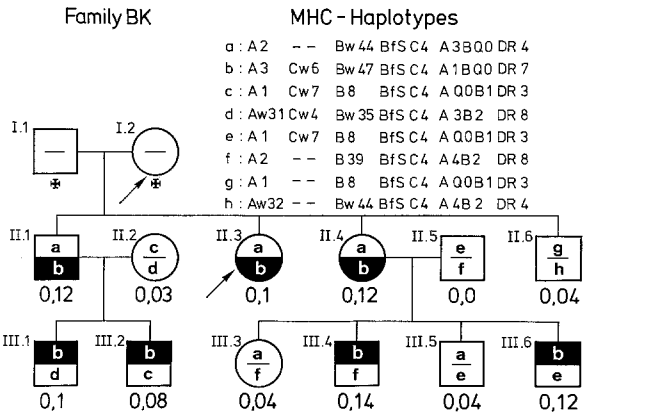

Fig. 4. Pedigree of family BK. *Arrows* affected members; *haplotype marked black* HLA haplotype carrying ICBR marker; *numbers under the symbols* breaks per 100 metaphases

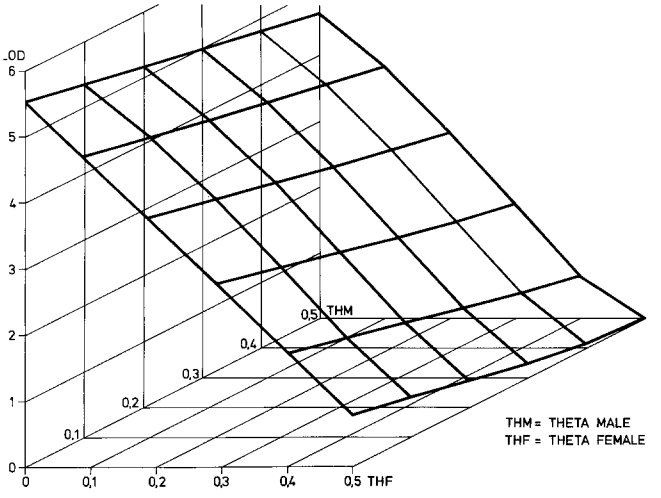

Fig. 5. Three-dimensional diagram of total male and female lod scores in six families informative for linkage between ICBR and HLA. Note that the highest lod score is obtained from analysis of female meioses

tended haplotype **A1, Cw7, B8, BFS, C4AQ0B1, DR3**, since two children are HLA homozygous. As a reasonable assumption, the mother may also have been HLA homozygous, since she has given apparently HLA-identical haplotypes (c or d) to her six children. Another indication for homozygosity of the mother for HLA, but not ICBR, may be that three sibs identical by HLA type can be distinguished by their breakage rate: two of them have ICBR, one has a normal breakage rate. The proposita (arrow) has two children, one, with haplotype c or d, has ICBR, the other, with haplotype b (from the otherwise normal grandfather), has a normal breakage rate.

In family D (Fig. 3e) the marker ICBR segregates with HLA haplotype a which includes the rare C4 haplotype A4B5 with aberrant Ch/Rg determinants (Roos et al. 1984). Therefore, it can be concluded that a hypothetical marker ICBR is in all cases studied associated with particular HLA haplotypes including C4 deletion or aberrations.

The data obtained have been analysed with the LIPED program for linkage analysis of Ott (UCLA version 01/23/80) for both theta male and female assuming for ICBR a two-locus model, $ICBR^+$ and $ICBR^-$, at one genetic locus. The result is depicted in the three-dimensional graph in Fig. 5. The total lod score is 5.5 at $\theta = 0$. No recombinant was found in our family material. By convention, a lod score above 3 can be taken as evidence for linkage between the two loci under study.

Discussion

As a characteristic feature of an HLA-associated disease, Dausset (1976) mentions familial occurrence even though Mendelian inheritance cannot be demonstrated. As noted by Kelley et al. (1981) in families with autoimmune diseases, manifestations such as SS, SLE, RA, and MS occur in the

same family. In many autoimmune diseases the extended haplotype A1, Cw7, B8, DR3 is involved (see Tiwary and Terasaki 1985). Primary biliary cirrhosis (PBC), often found together with SS, also shares unusual C4 allotypes with SS (Briggs et al. 1987b; Manns et al. 1987). Interestingly, PBC patients have also an increased chromosomal breakage rate (5.3 aberrations compared to 2.3 per 100 mitoses in controls, in this case mainly gaps, $P = 0.02$; A. Notghi, unpublished observations). Since all these diseases share common immune phenomena like circulating immune complexes, and, more specifically, anti-nuclear antibodies as also found in SS (Maddison et al. 1986), it could be assumed that these diseases could be traced back to a common aetiological factor. Increased chromosomal breakage rates (ICBR) as demonstrated for scleroderma patients and their first-degree relatives in the present paper may be a reasonable candidate for a factor involved in an increased susceptibility to autoimmune diseases.

The screening for aberrations in metaphase chromosomes after homogeneous staining undoubtedly is as much cumbersome as also influenced by subjective criteria. A common nomenclature for secondary structural aberrations was only agreed upon recently (ISCN 1985) and was also followed in this study. Mainly, the error of small numbers should be avoided. Therefore, as a rule, 100 metaphases were investigated. Only well-differentiable mitoses were evaluated. In famly BK, the individual breakage rates were confirmed after two years in fresh lymphocyte cultures. All persons were apparently healthy. We conclude that the expression of $ICBR^+$ and $ICBR^-$ is only slightly, if at all, influenced by other than genetic factors. The manifestation of scleroderma, however, is known to depend on factors such as age, sex, pregnancy or infection.

According to the data obtained from control individuals as presented in Fig. 1, the marker ICBR is apparently rare in normal individuals. Therefore, the segregation can only be studied in families with SS and possibly other diseases associated with **HLA-A1**, **Cw7**, **B8**, **C4AQ0B1**, **DR3**. ICBR segregates as a dominant autosomal trait. The classical breakage syndromes, however, have a recessive mode of inheritance (see German 1972 for a review). In addition, also the pattern of secondary aberrations in metaphase chromosomes in patients with Bloom's syndrome, hereditary ataxia teleangiectasia, Fanconi's anemia and xeroderma pigmentosum, including translocations, is different from that of scleroderma patients and their relatives.

ICBR appears to be linked to the HLA region on chromosome 6. By convention, a total lod score of 5.5 at $\theta = 0$ can be considered as evidence for close linkage. The marker segregated with particular HLA haplotypes in the families of scleroderma patients. These haplotypes included rare or deleted genes of the fourth component of complement. Of these patients, 88% possessed at least one, and 25% had two C4 null alleles (data not shown). It remains to be seen if there is any relationship between the appearence of C4-deleted genes and ICBR.

ICBR is a quantitative character detected by increased chromosomal breakage in lymphocytes after 72 h of culture. It appears in individual rates regardless of whether PBL were derived from whole blood or had been isolated before. All lymphocytes were cultured under identical conditions, all members of a family in parallel. Therefore, it is unlikely that technical variation of cultural media or additives could be invoked as a cause of different breakage rates. In addition, as could be demonstrated in family BK, the character ICBR remains stable over a considerable period of time. The nature of the agent inducing increased chromosomal breakage in some, but not all members of scleroderma families, however, remains to be shown.

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