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Chromosomal Aberrations in Cultures of Skin Fibroblasts of Leprosy Patients[1]

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Over the past 15 years, investigations on cancer and leprosy in several countries have dealt with cancer mortality in patients affected with both of these diseases ([4, 9, 13, 15, 22, 23]). These studies have shown that leprosy does not modify cancer risk and that no significant difference exists between lepromatous and tuberculoid patients concerning the death rate from malignant tumors.

Skin cancer, which has a favorable prognosis, may be an exception to this conclusion, since the risk of this type of cancer was found to be increased among leprosy individuals and to be more prevalent among lepromatous patients ([11]). Thus, in a series of 60,000 histopathologic examinations of skin biopsies, Michalany ([11]) found 539 cases of malignant tumor (nearly 1%); the patients with a recorded type of leprosy (37.5%) were all lepromatous. This prevalence may be even greater, mostly because many cases escape clinical diagnosis, since it is difficult to distinguish a tumor in the skin of lepromatous patients, particularly a carcinoma among lepromas ([6]).

Taking into account these findings and considering that malignant tumors are associated with chromosomal aberrations, we decided to investigate the frequency of these abnormalities in fibroblast cultures initiated from skin biopsies of leprosy patients. This decision was prompted by the knowledge that the frequency of cells with chromatid or chromosome lesions is significantly increased in the leukocyte metaphases of leprosy individuals ([2]).

MATERIALS AND METHODS

Chromosome analyses were made on metaphases obtained by culturing *in vitro* the fibroblasts of skin biopsies taken after informed consent from 16 Brazilian leprosy patients and two healthy women used as controls. The leprosy cases included: 10 lepromatous (L), 2 borderline (B), and 4 tuberculoid (T) patients (13 males and 3 females) whose ages varied from 17 to 72 years. All of them were outpatients of the Divisão de Dermatologia Sanitária at Campinas, São Paulo, Brazil. The two healthy women, aged 27 and 29, were not ingesting drugs at the time of this investigation.

Four of the leprosy patients (2L, 1B, and 1 T) had been on dapsone therapy (100 mg/day) for periods ranging from 1 to 20 years, while 8 of them (4 L, 1 B, and 3 T) had received dapsone and/or other drugs (300 mg/day of rifampin, 0.5 to 1.5 mg/day of dexamethazone, and/or 100 mg/day of thalidomide) for periods varying from 1 week to 16 years. The remainder (4 L cases) had not received any antileprosy therapy at the time of their skin biopsy.

Skin biopsies were taken from lepromatous, borderline, and tuberculoid lesions. In the three lepromatous and one tuberculoid patients (nos. 8, 9, and 10 of Table 1) who did not exhibit skin lesions at the time of the investigation, as well as the individuals used for controls, a biopsy was taken from the inner surface of a forearm. The biopsies were obtained with a 2-mm punch, with or without local anesthesia with xylocaine (2%), and immediately placed in tissue culture medium (TCM) composed of Ham F-10 (Difco Laboratories, Detroit, Michigan, U.S.A.), supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 20% fetal bovine serum (Cultilab, Campinas, São Paulo, Brazil). This medium was used for both transportation of the biopsies to the laboratory and for cell culturing.

Each skin biopsy was transferred to a Petri dish containing TCM and cut into about 20 pieces. With the help of a Pasteur pipette, these fragments were introduced in groups of four or five into Leighton tubes, where

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they were allowed to adhere for three days at 30°C. After this period, 1 ml of TCM was added to each tube which was incubated for seven days at 37°C. After this time, the TCM was changed every second day. The period of cell culturing varied from 8 to 15 weeks, during which time several subcultures were prepared (six at most) by detaching the cells from the flat wall of the Leighton tubes with the help of a 0.25% w/v trypsin (Difco) in Puck's salt solution (TS) at 37°C.

The cell divisions were arrested in metaphase by adding 0.02 ml of colchicine (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) 4×10^{-5} M to each tube 2–6 hr before harvesting the cultures. After transfer of the TCM from each Leighton tube to a conical tube, the culture was washed rapidly with 1.5 ml of TS at 37°C, this volume also being transferred to the same conical tube. The tubes were thereafter allowed to incubate for 5–10 min at 37°C with another 1.5 ml of TS, and shaken after the first 5 min of incubation. If shaking did not detach the cells, a glass spatula was used to accelerate the process. After a 10-min incubation, the contents of the Leighton tube were transferred to the conical tube and centrifuged for 5 min at 200 × *g*. All of the supernatant, except for 0.5 ml, was discarded.

The hypotonic treatment of the sedimented cells was performed as recommended by Professor Walter Pinto, Jr. (personal communication, 1981). Thus, 1 ml of 0.075 N KCl solution at 37°C was added to the concentrated cell suspension and, after 3 min, another 1 ml of this hypotonic solution was added. The cells were again resuspended gently, and the process repeated until a final volume of 4 ml was reached after 12 min of treatment.

The hypotonic treatment was terminated by adding 0.5 ml of freshly prepared fixative (methanol–acetic acid 3:1 v/v) to the conical tube, which was centrifuged immediately for 5 min at 200 × *g*. The supernatant was discarded, and the same procedure repeated three times.

The fixed cells were distributed on slides which were air dried before staining for about 12 min with Giemsa's reagent (5 ml Giemsa Gurr stock solution; 20 ml Sörensen's phosphate buffer, pH 6.8; and 75 ml deionized water). The slides were rinsed in tap water, air dried, and mounted in Permount.

The metaphases were selected under low-power magnification on the basis of the quality of chromosome spreading, and examined under 1000× magnification for chromosomal aberrations. The structural chromosomal aberrations included chromatid and chromosomal lesions as well as chromosomal rearrangements. A structural chromosomal aberration was classified as a chromatid lesion if only one of the two chromatids of a chromosome showed one or more discontinuities, or if both chromatids were injured but not in corresponding sites. If both chromatids were injured in corresponding sites, the structural aberration was considered a chromosomal lesion. A centromeric lesion was defined as a discontinuity seen in the centromeric region. All of these lesions were subclassified as breaks or gaps according to the size of the discontinuity seen in the chromatids and/or the position of the distal fragment. Thus, the discontinuities that were smaller than the width of one chromatid and that maintained the distal and the proximal ends in the same direction were considered as gaps. The discontinuities that were larger than the width of one chromatid and/or showed a change in the direction of the distal fragment were recorded as breaks. The chromosomal rearrangements seen without banding techniques included translocations, pericentric inversions, and deletions.

The numerical chromosomal aberrations included aneuploid, pseudodiploid, and polyploid cells. Among the aneuploid metaphases, those exhibiting less than 46 chromosomes were classified hypodiploid; those exhibiting more than 46, hyperdiploid. Pseudodiploidy was attributed to the metaphases showing a total number of 46 chromosomes which resulted from a monosomy of one chromosome compensated by a trisomy of another. Nuclei showing one or more excedent haploid sets of chromosomes or exhibiting diplochromosomes (endoreduplication) were classified as polyploid.

The number of metaphases selected for investigating the proportion of structural chromosomal aberrations, aneuploidies, and

TABLE 1. *Data recorded on the examined leprosy patients and the controls.*

Patient no.	Sex	Age (yr)	Race	Leprosy form	Bacterial index	Treatment		Days of culture	Selected metaphases				Dividing nuclei	
						Time	Drugs[a]		Total	With structural aberrations (%)	Aneuploid (%)	Pseudodiploid (%)	Total	Polyploid
1	M	48	C	L	+	7 days	R	30	80	10.0	15.0	—	728	5.1
2	M	29	C	L	++	1 mo.	DDS, R	75	44	15.9	47.7	—	358	15.6
3	F	40	C	L	++	No	None	48	42	4.8	45.2	2.4	332	13.9
4	M	23	N	L	+++	No	None	27	121	5.8	20.7	0.8	1207	3.7
5	M	21	N	L	+++	No	None	34	50	8.0	16.0	—	434	6.7
6	M	48	N	L	++	14 days	DDS, Dx, T	31	58	17.2	27.6	5.2	514	15.0
7	M	58	C	L	+	No	None	30	53	9.4	26.4	—	376	1.1
8	M	50	C	L	—	9 yrs	DDS	31	50	6.0	26.0	—	429	2.6
9	M	39	C	L	—	20 yrs	DDS	29	100	15.0	17.0	—	981	5.0
10	F	41	C	L	—	16 yrs	DDS, T	43	40	30.0	27.5	—	259	35.1
11	M	72	C	B	—	1 mo.	DDS	41	40	15.0	40.0	—	650	40.9
12	M	25	C	B	—	2 mos.	DDS, Dx	28	60	13.3	25.0	—	577	2.8
13	M	60	N	T	—	6 mos.	DDS, Dx	36	60	18.3	28.3	—	373	4.3
14	M	35	C	T	—	6 mos.	DDS, Dx, R	26	71	12.7	28.2	1.4	697	1.2
15	M	40	N	T	—	6 mos.	DDS, R	30	76	13.2	15.8	—	660	1.5
16	F	17	C	T	—	7 yrs.	DDS	31	100	8.0	20.0	—	910	2.1
Mean		40.4				3.4 yrs		35.6		12.7	26.6	0.61		9.8
S.D.		15.33				6.35 yrs		12.16		6.27	10.05	1.40		12.07
Control 1	F	27	C					35	60	3.3	40.0	—	863	3.4
Control 2	F	29	C					54	60	1.7	20.0	—	519	1.5

[a] DDS = dapsone; Dx = dexamethazone; R = rifampin; T = thalidomide.

TABLE 2. *Distribution of chromatid, chromosomal and centromeric breaks and gaps according to chromosome group in fibroblast cultures of the leprosy patients.*

Chromosome group	Breaks (N = 46)	Gaps (N = 103)	Total (N = 149)
A	34.8%	41.7%	39.6%
B	17.4%	13.6%	14.8%
C	26.1%	27.2%	26.8%
D	8.7%	9.7%	9.4%
E	13.0%	4.9%	7.4%
F	—	1.9%	1.3%
G	—	1.0%	0.7%

TABLE 3. *Frequency of chromosomal losses in the hypodiploid cells according to chromosome groups.*

Chromosome group	Leprosy patients (N = 285)	Controls (N = 46)
A	7.0%	—
B	8.8%	6.5%
C	25.6%	41.3%
D	12.3%	10.9%
E	19.6%	19.6%
F	11.6%	6.5%
G	15.1%	15.2%

pseudodiploidies varied from 40 to 121 per individual, while the number of nuclei examined for evaluating the frequency of polyploid cells varied from 259 to 1207.

RESULTS

In Table 1, the 16 leprosy patients are distributed according to sex, age, race, bacterial index, drugs used for leprosy treatment, age of fibroblast cultures (mean number of days), and the frequency of structural chromosomal aberrations, aneuploidies, pseudodiploidies, and polyploid nuclei. The data concerning the fibroblast cultures of the two healthy controls are also included in this table.

Almost all of the structural chromosomal aberrations observed in the 1045 fibroblast metaphases of the leprosy patients were chromatid (5.3%), chromosomal (3.0%), or centromeric (2.8%) lesions, the chromosomal rearrangements being seen in only eight cells of three patients (0.8%): Patient no. 6 had one cell with a pericentric inversion in a chromosome number 3, and one cell with a reciprocal translocation affecting two C chromosomes. Patient no. 9 had six cells with 46 chromosomes and a reciprocal translocation affecting chromosomes D and G. Patient no. 11 had one cell with a reciprocal translocation affecting a chromosome number 2 and D chromosome.

Among the 120 analyzed metaphases of the controls, no chromosomal rearrangements were found, the structural chromosomal aberrations being restricted to two (1.7%) chromatid gaps (one in a chromosome of group A and the other in a chromosome of group B) and one (0.8%) chromosome gap in a chromosome of group C.

Table 2 shows the frequency of chromatid, chromosomal, and centromeric breaks and gaps observed in each group of chromosomes of the fibroblast cultures of the leprosy patients studied.

The aneuploidies were usually represented by hypodiploid cells. Thus, among 256 aneuploid metaphases of the leprosy patients only six were hyperdiploid (three 47,XY,+G; one 47,XY,+B; one 47,XY,+D; and one 47,XY,+F), and they were found in the fibroblast cultures of Patients no. 1 (1 cell), no. 2 (2 cells), no. 11 (2 cells), and no. 13 (1 cell). An identical situation was found in the controls; among 36 aneuploid metaphases only one hyperdiploid cell (47,XY,+F) was observed in the fibroblast cultures of Control no. 1.

Table 3 shows the frequency of chromosomal losses in the hypodiploid cells of the fibroblast cultures of leprosy patients and controls according to the chromosome groups.

Pseudodiploidies were found as infrequently as hyperdiploid cells and were only seen in the fibroblasts of four patients (nos. 3 and 14 had one cell with an extra E and a missing C chromosome; no. 4 had one cell with an extra C and a missing A chromosome; and no. 6 had three cells with an extra D and a missing G chromosome). Thus, there was a very low frequency of pseudodiploid cells found in the cell cultures.

Concerning the polyploid nuclei seen in the fibroblast cultures of leprosy patients and controls, it should be emphasized that the frequency of endoreduplicated meta-

phases, that is to say, with diplochromosomes, was low (below 0.5%) in all of the cultures analyzed. However, the proportion of the remaining types of polyploid nuclei was extraordinarily high in the cell cultures of some leprosy patients. On the average, such cultures showed 9.8% polyploid fibroblasts (Table 1).

DISCUSSION

Analyses of the data presented in Table 1 have shown that the frequency of structural aberrations seen in the fibroblast cultures of the leprosy patients is not correlated with the age of these individuals or with the age of the cultures. Moreover, the proportion of such abnormalities is independent of sex, race, bacterial index, and form of leprosy. However, it depends significantly on leprosy treatment [$\chi^2 = 12.327$, 2 degrees of freedom (DF), $p < 0.01$], since the percentage of structural chromosomal aberrations was 6.8% in the cultures of the patients who did not ingest drugs for leprosy therapy, 11% in the cultures of patients who were ingesting dapsone alone, and 15.3% in the cultures of patients treated with dapsone and/or other drugs.

The proportion of structural chromosomal aberrations observed in the fibroblast cultures of the patients who did not ingest drugs for leprosy treatment did not differ significantly from that seen in the cultures of the controls (corrected $\chi^2 = 2.156$, 1 DF, $0.10 < p < 0.20$). These aberrations were below the limits accepted as being due to normal variations in human diploid cell cultures, i.e., 3.5% for breaks and 1.3% for other nonspecified structural abnormalities ([14]). The same was not true for the comparisons between controls and patients treated with dapsone alone (corrected $\chi^2 = 6.683$, 1 DF, $p < 0.01$) or between controls and patients receiving combined therapy (corrected $\chi^2 = 13.093$, 1 DF, $p < 0.001$). Indeed, these data were expected, since it is known that dapsone ([3]), rifampin ([16, 17]), and thalidomide ([18]) are able to show a clastogenic effect on the chromosomes of human lymphocytes.

A comparison of the distribution of breaks and gaps according to the chromosome group seen in Table 2 showed that the chromosomes of leprosy patients were similarly affected by both types of lesions ($\chi^2 = 5.133$, 6 DF, $0.50 < p < 0.70$). Moreover, the distributions shown in Table 2 do not differ significantly from the distributions of breaks and gaps in chromosomes of the lymphocytes of leprosy patients under dapsone therapy. Such results, therefore, reinforce the hypotheses that: a) Gaps are not merely staining discontinuities but injuries as important, and probably of the same nature, as breaks seen in chromosomes, chromatids, and centromeres ([2, 3]); and b) The frequency of structural chromosomal aberrations observed in fibroblast cultures of leprosy patients estimates the frequency of these abnormalities *in vivo*.

The rareness of the chromosomal rearrangements found in almost all fibroblast cultures of the leprosy patients are compatible with the hypothesis that they occurred mostly as a consequence of artifacts of cell culture. Nevertheless, the reciprocal translocation between chromosomes D and G seen in a group of six cells in the cultures of Patient no. 9 is an exception and deserves comment. While the possibility of *in vivo* mosaicism cannot be excluded, it is obvious that the alternative hypothesis, which considers such a group of cells as a consequence of chromosomal instability, should also be considered. In this case, the fibroblast exhibiting the same type of chromosomal rearrangement would be a clone ([10, 14]), comparable to that observed in some rare inherited disorders associated with cancer, such as xeroderma pigmentosum ([6]) or Werner's syndrome ([8, 19, 20]).

The analyses of the frequency of aneuploid metaphases seen in the fibroblast cultures showed that it was not correlated with the age of the leprosy patients. On the other hand, the proportion of aneuploid cells was independent of sex, race, bacterial index, leprosy form, and drugs used for leprosy treatment. Table 1 shows that only five fibroblast cultures of the leprosy patients (nos. 1, 5, 9, 15, and 16) revealed 20% or less aneuploid metaphases. As a consequence, one might conclude that the frequency of aneuploid cells in the cultures of the leprosy patients (26.6% on the average) was significantly increased since, according to Priest ([14]), the proportion of aneuploid metaphases seen in long-term cultures is, at most, 20% (2% of hyperdiploid and 18% of hy-

podiploid nuclei). Nevertheless, we doubt this conclusion, because the data in Table 1 demonstrate a significant and high correlation coefficient between the frequency of aneuploid cells and the age of the fibroblast cultures ($r = 0.77$, $t = 4.449$, 14 DF, $p < 0.001$). Moreover, Table 1 shows that the frequency of aneuploid cells in the fibroblast cultures of the controls was also high.

The analysis of the data concerning the aneuploidies (Table 3) shows that chromosomal losses in the hypodiploid fibroblasts are not at random ($\chi^2 = 50.355$, 6 DF, $p < 0.001$ for leprosy patients and $\chi^2 = 35.113$, 6 DF, $p < 0.001$ for controls), and are not independent of the origin of the cell cultures. As a matter of fact, a significant excess of fibroblasts missing a group A chromosome ($p = 0.046$ by Fisher's exact test) and a less-frequent occurrence of cells missing a group C chromosome ($\chi^2 = 5.676$, 1 DF, $p < 0.05$) are seen among the hypodiploid fibroblasts of leprosy patients as compared to controls.

The frequency of polyploid nuclei seen in the fibroblast cultures of the controls (Table 1) was within the normal range (0–4%) for long-term cell cultures of normal tissues ([1, 6, 12, 14]). In contrast, the proportion of polyploidy observed in the fibroblast cultures of leprosy patients was significantly high and, in five cases, very high (Patients nos. 2, 3, 6, 10, and 11).

The frequency of polyploidy exhibited by the cultures of leprosy patients was not correlated with the age of the patients or with the age of the cultures. The latter result contradicts those of other authors ([21]) who observed an increased frequency of polyploid nuclei in older tissue cultures. In spite of this, the proportion of polyploid cells seen in the fibroblast cultures of the leprosy patients was difficult to interpret, since it was significantly associated with sex ($\chi^2 = 11.121$, 1 DF, $p < 0.001$); race ($\chi^2 = 45.407$, 1 DF, $p < 0.001$); bacterial index (corrected $\chi^2 = 5.259$, 1 DF, $p < 0.05$); leprosy form ($\chi^2 = 490.006$, 2 DF, $p < 0.001$); and leprosy treatment ($\chi^2 = 75.518$, 2 DF, $p < 0.001$). Moreover, the frequency of polyploid cells in the fibroblast cultures of leprosy patients was significantly correlated with the frequency of structural chromosomal aberrations ($r = 0.57$, $t = 2.586$, 14 DF, $p < 0.05$) and with aneuploidies ($r = 0.52$, $t = 2.278$, 14 DF, $p < 0.05$).

In conclusion, structural chromosomal aberrations are the only type of chromosomal abnormality that may be accepted as significantly increased in skin fibroblasts of leprosy patients who are under treatment with dapsone alone or combined therapy. Therefore, it seems of great importance to investigate whether the risk of skin cancer among leprosy patients is also associated with leprosy therapy. Another important line of investigation seems to be the study of the chromosomal pattern exhibited by the cells of the malignant tumors of the skin.

SUMMARY

A search for structural and numerical chromosomal aberrations was made on metaphases obtained by culturing *in vitro* skin fibroblasts of 16 leprosy patients (10 lepromatous, 2 borderline, and 4 tuberculoid cases) and 2 healthy individuals used as controls. The data were analyzed taking into account sex, age, race, form of leprosy, bacterial index, type of therapy, and age of the fibroblast cultures. Structural chromosomal aberrations are the only type of chromosomal abnormality that may be accepted as significantly increased in the skin fibroblasts of leprosy patients who are under treatment with dapsone alone or with combined therapy.

RESUMEN

Se buscaron aberraciones cromosómicas estructurales y numéricas en metafases obtenidas cultivando fibroblastos de piel de 16 pacientes con lepra (10 lepromatosos, 2 intermedios, y 4 tuberculoides) y de 2 individuos sanos usados como control. Los datos se analizaron tomando en cuenta edad, sexo, raza, forma de la lepra, índice baciloscópico, tipo de terapia, y edad de los cultivos. Se acepta que las aberraciones cromosómicas estructurales son el único tipo de anormalidad cromosómica que puede encontrarse significativamente aumentado en los fibroblastos de piel de los pacientes con lepra que están bajo tratamiento con dapsona sola o con terapia combinada.

RÉSUMÉ

On a recherché les abérrations de structure et de nombre des chromosomes chez 16 malades de la lèpre (10 lépromateux, 2 dimorphes, et 4 tuberculoides) et chez 2 témoins en bonne santé. Cette étude a été menée

sur des chromosomes en métaphase obtenus par culture *in vitro* de fibroblastes de la peau. Les données ont été analysées en ce qui concerne le sexe, l'âge, le groupe ethnique, le type de lèpre, l'indice bacilloscopique, le mode de traitement, et l'âge des cultures de fibroblastes. Dans les fibroblastes cutanés de ces malades de la lèpre qui étaient traités par la monothérapie à la dapsone ou par la polychimiothérapie, la seule anomalie chromosomique présentant une fréquence significativement élevée consistait en des abérrations de la structure des chromosomes.

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