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Decreased proportion of B cells in lectin-stimulated lymphocyte cultures from patients with Pallister-Killian syndrome

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ABSTRACT

The response to lectins of lymphocytes from Pallister-Killian syndrome patients was studied. Blood lymphocytes were cultured in the presence of phytohemagglutinin or pokeweed mitogen for 96 h. The cells were identified using the MAC (morphology, antibody, chromosomes) technique which enables the immunological identification of both mitotic and non-proliferating cells in unfractionated lymphocyte populations. The proportion of B (CD20/22+) and T (CD3+) cells at the start of culturing was equal in the Pallister-Killian syndrome and control samples. After culturing, the proportion of interphase and mitotic CD20/22+ cells was significantly lower, and that of CD3+ cells higher in Pallister-Killian syndrome cultures compared with control cultures. This result may be explained by the inability of B lymphocytes from Pallister-Killian syndrome patients to respond to stimulation by phytohemagglutinin or pokeweed mitogen, or by a selective loss of B cells during culturing *in vitro*.

INTRODUCTION

Pallister-Killian syndrome (PKS) is a rare sporadic chromosomal mosaicism syndrome caused by tetrasomy of the short arm of chromosome 12 [mos46,XX or XY/47,XXorXY,+i(12p)]. Patients with this condition have a typical syndrome, characterized by severe mental and psychomotor retardation, other defined congenital anomalies and autistic behaviors (Pallister *et al.*, 1977; Gorlin *et al.*, 1990). Diagnosis of PKS is based on a characteristic cytogenetic abnormality, i.e. the presence of an extra metacentric chromosome, i(12p), which is usually confined to skin fibroblasts. Nevertheless, it is sometimes observed in cultured peripheral blood lymphocytes, but at a very low frequency (Gorlin *et al.*, 1990; Larramendy *et al.*, 1993). Thus, a correct diagnosis is sometimes missed, since skin fibroblasts are not routinely used for karyotyping (Fryns *et al.*, 1982; Hunter *et al.*, 1982; Kwee *et al.*, 1984; Hall, 1985; López *et al.*, 1985; Gorlin *et al.*, 1990). Nevertheless, over 45 PKS cases with documented i(12p) have been reported so far (Peltomäki *et al.*, 1987; Steinbach and Rehder, 1987; Chrzanowska and Fryns, 1989; Soukup and Neidich, 1990; Greig *et al.*, 1991; Speleman *et al.*, 1991; Larramendy *et al.*, 1993).

In the present study, we used the MAC (morphology, antibody, chromosomes) technique, which allows simultaneous analysis of the cell morphology, immunologic phenotype, and karyotype of individual mitotic and interphase cells (see reviews by Knuutila and Teerenhovi, 1989; Larramendy *et al.*, 1992; Knuutila *et al.*, 1994) to investigate the proliferative response of B and T lymphocytes from PKS patients of phytohemagglutinin and pokeweed mitogens *in vitro*.

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MATERIAL AND METHODS

Blood samples and PKS patients

Blood samples were obtained from five PKS patients and from four nonsmoking healthy donors selected according to recommendations reported elsewhere (Bianchi *et al.*, 1979). The blood samples (20 ml) were taken by venipuncture. Cytogenetic and molecular cytogenetic characteristics of these PKS patients have been published elsewhere (Larramendy *et al.*, 1993).

Unfractionated mononuclear leukocyte cultures

Mononuclear leukocytes were isolated from whole blood samples by the Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation technique (Böyum, 1968). After centrifugation (400 *g*, 45 min), the mononuclear cell layer was removed, washed three times in Hank's balanced salt solution, and resuspended in complete culture medium [80% RPMI 1640 medium (Gibco, Grand Island, NY, USA), 20% fetal calf serum (Gibco), 0.29 mg/ml L-glutamine (Gibco), 100 units/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco)]. Part of the cell suspension was used to make cytocentrifuge slide preparations (Cytospin, Shandon Elliot, Runcorn, UK) (Knuutila *et al.*, 1994). The remaining cells were used to set up unfractionated mononuclear leukocyte cultures containing 1.5 x 10^6 cells/ml in TC25 Falcon flasks. For *in vitro* lymphocyte stimulation, either phytohemagglutinin (PHA, 0.5 µg/ml, Gibco) or pokeweed mitogen (PWM, 100 µg/ml, Gibco) was used. The cultures were incubated at 37°C in a 5% CO_2 atmosphere for 96 h. Cultures were set up in duplicate for each donor, and cultures from at least one PKS patient and one control were run in parallel.

Chromosome and MAC slide preparations

During the final four hours of culture, the cells were treated with Colcemid (0.1 µg/ml, Difco Laboratories, Detroit, MI, USA). Cells were collected by centrifugation, washed three times with serum-free RPMI 1640 medium, and processed into MAC slide preparations, as described in detail elsewhere (Knuutila and Teerenhovi, 1989). The phenotype of the interphase and mitotic cells was determined by the alkaline phosphatase anti-alkaline phosphatase method (Cordell *et al.*, 1984; Keinänen *et al.*, 1988; Knuutila *et al.*, 1993) using monoclonal antibodies and Giemsa counterstain. The following monoclonal antibodies were used in the study: B1 (CD20, Coulter Immunology, Hialeah, FL, USA), Leu14 (CD22, Becton-Dickinson, Eremdodegem, Belgium), and Leu4 (CD3, Becton-Dickinson). The frequencies of positive and negative interphase and mitotic cells were obtained for each monoclonal antibody by analyzing at least 1000 interphase cells and 100 mitotic cells per antibody. Mitotic indices were estimated for each leukocyte subset in each culture by scoring at least 1000 cells, and expressed as the number of mitoses per 100 cells.

Statistical analysis

The statistical significance of the results was tested by means of the unpaired *t*-test, using the StatWorks software (Heyden and Son, London, UK). P values are given for differences in mean frequencies. Differences yielding a P value under 0.05 were considered statistically significant.

RESULTS

At the start of culturing, the unfractionated lymphocyte population from PKS patients and controls ranged from small to medium size, and the cells possessed a small to moderate amount of cytoplasm. The results of the immunophenotype analysis using various monoclonal antibodies are presented in Table I. The unfractionated mononuclear leukocyte population consisted mainly of CD3+ cells. The mean frequency of CD20/22+ cells was similar in PKS and control donors. No significant differences in the proportion of CD3+ ($P = 0.22$) and CD20/22 ($P = 0.98$) cells were observed between PKS and control donors.

After 96 h of culturing, a significant decrease in the proportion of interphase CD20/22+ cells ($P < 0.02$) and an increase in the frequency of CD3+ cells ($P < 0.003$) was observed in PKS cultures in comparison with control cultures (Table I). In PHA- and PWM-stimulated PKS cultures the mean frequency of CD20/22+ cells among all interphase cells was less than 1%, whereas in PHA- and PWM-stimulated control cultures, the corresponding figure was about 10% (Table I). No differences in the proportion of interphase CD20/22+ and CD3+ cells were observed between cultures stimulated with PHA or PWM in either control ($P > 0.22$) or PKS samples ($P > 0.13$) (Table I).

After 96 h of culturing, significant differences were observed between the PKS and control cultures in

Table I - Proportions (%) of antibody positive B and T lymphocyte subsets among all interphase and mitotic cells in uncultured mononuclear leukocytes and from corresponding cultures from Pallister-Killian syndrome patients and controls stimulated with phytohemagglutinin (PHA) and pokeweed (PWM) mitogens (a).

Cells	Controls				PKS patients				
	1	2	3	4	1	2	3	4	5
Uncultured									
B-interphase	14	6	3	4	7	3	2	9	12
T-interphase	83	90	87	84	90	87	87	90	76
PHA-stimulated									
B-interphase	18	13	4	5	0	1	0	1	2
T-interphase	76	83	90	93	98	98	99	99	97
B-mitotic	28	30	7	8	0	0.5	0	0	2
T-mitotic	77	82	94	92	99	99	99	99	98
PWM-stimulated									
B-interphase	20	10	2	7	1	0.5	0	0	1
T-interphase	78	78	84	80	85	97	93	98	98
B-mitotic	56	25	10	15	1	0.5	0	0	3
T-mitotic	61	67	89	74	95	99	99	99	98

(a) At least 1000 interphase and 100 metaphase cells were analyzed per each monoclonal antibody. The cultures were harvested 96 h after stimulation. The following monoclonal antibodies were used: B1 (CD20) and Leu14 (CD22) for B cells, and Leu4 (CD3) for T cells.

Table II - Mitotic indices in T and B lymphocytes subsets in leukocyte cultures from Pallister-Killian syndrome patients and controls after stimulation with phytohemagglutinin (PHA) or pokeweed (PWM) mitogens (a).

Cells	Mitogen	Controls				PKS patients				
		1	2	3	4	1	2	3	4	5
B-Lymphocytes	PHA	9	20	20	16	< 1	< 1	< 1	< 1	< 1
	PWM	13	23	14	20	< 1	< 1	< 1	< 1	< 1
T-Lymphocytes	PHA	5	4	10	7	13	15	7	10	12
	PWM	3	4	4	5	8	7	5	7	9

(a) At least 1000 cells were analyzed per each monoclonal antibody and mitogen. The cultures were harvested 96 h after stimulation. The following monoclonal antibodies were used: B1 (CD20) and Leu14 (CD22) for B cells, and Leu4 (CD3) for T cells.

the frequencies of mitotic CD20/22+ and CD3+ cells. In PKS cultures nearly all cells were CD3 cells. There were no significant differences in the relative frequencies of CD20/22+ and CD3+ mitoses between PHA- or PWM-stimulation in either control or PKS cultures ($P > 0.11$) (Table I).

No significant differences in mitotic indices of CD3+ cells were observed between PKS and control T cells in the presence of either PHA or PWM (Table II). In the control cultures, the mitotic index of CD20/22+ lymphocytes was significantly higher than that of CD3+ lymphocytes, regardless of the mitogen used ($P < 0.05$) (Table II).

DISCUSSION

In the present study the proportion of B cells among all interphase and mitotic lymphocytes from PKS patients was found to decrease in the 96-h culture, in the presence of PHA or PWM mitogens. There are three possible explanations for these observations: relative greater proliferation of T lymphocytes in the culture, poor response of B lymphocytes to the lectins used, or a selective death of B cells during culture.

Analysis of the mitotic indices speaks against the first hypothesis, as no differences in the mitotic activity of CD3+ cells were observed between PKS and control cultures. It therefore appears unlikely that there would be T lymphocyte overgrowth in response to the lectins used in the samples from PKS patients.

In a previous molecular cytogenetic analysis performed on the same group of PKS patients (Larramendy *et al.*, 1993), we were able to detect no cells with the isochromosome 12p in three out of the five PKS patients, and the frequency of these cells was less than 0.5% in the remaining two patients. As the frequency of B cells in the present study was less than 7% among uncultured cells and close to 0% after culturing, we cannot exclude the possibility of the isochromosome being present in a proportion of B lymphocytes. Whether the presence of the isochromosome in a restricted B cell subset causes the decrease in the frequency of B cells in lectin-stimulated cultures remains to be studied.

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RESUMO

Estudou-se a resposta de linfócitos de pacientes com síndrome de Pallister-Killian a lecitinas. Linfócitos sangüíneos foram cultivados na presença de fitohemaglutinina ou mitógeno de "pokeweed" durante 96 h. As células foram identificadas usando a técnica MAC (morfologia, anticorpo, cromossomo), que permite a identificação tanto de células mitóticas como as não proliferantes em populações não fracionadas de linfócitos. A proporção de células B (CD20/22+) e T (CD3+) no início da cultura foi igual nas amostras controle e da síndrome de Pallister-Killian. Depois da cultura, a proporção de células CD20/22+ mitóticas e em interfase foi significantemente menor, e a de células CD3+ significantemente maior, em culturas da síndrome de Pallister-Killian do que em culturas controle. Este resultado pode ser explicado pela incapacidade dos linfócitos B na síndrome de Pallister-Killian de responder à estimulação pela fitohemaglutinina ou pelo mitógeno de "pokeweed", ou por uma perda seletiva de células B durante a cultura *in vitro*.

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