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MONOCYTES MODULATE THE *IN VITRO* BASAL FREQUENCY OF SISTER CHROMATID EXCHANGES OF PLASMA LEUKOCYTE CULTURES AND MITOTIC ACTIVITY OF SUPPRESSOR-CYTOTOXIC T8 HUMAN LYMPHOCYTES

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The effect of monocytes (MNs) on baseline SCEs and kinetics of human lymphocytes in plasma leukocyte (PLCs) and whole blood cultures (WBCs) was studied. Baseline SCEs in PLCs were nearly two-fold over WBCs. No differences in SCEs were observed between PLCs and MN-depleted PLCs, indicating that SCEs from PLCs are independent of MNs. MNs titration into PLCs decreased proportionally SCEs. Reconstitution of depleted PLCs with concentration of MNs equivalent or higher than those of PLC decreased SCEs. No variations of lymphocyte kinetics in PLCs were observed in the absence/presence of MNs. The proportion of B and T-cell subsets among interphasic lymphocytes were similar in PLC in the absence/presence of MNs, but a significant increase in the proportion of mitotic T8 lymphocytes was observed. Accordingly, MNs modulate both the *in vitro* basal SCEs and the mitotic activity of T8, but not their cell-cycle kinetics.

KEYWORDS: cell-cycle progression; human monocytes; human B lymphocytes; human T lymphocytes; sister chromatid exchanges; MAC (Morphology, Antibody, Chromosomes) technique.

INTRODUCTION

Since 1974, methods for demonstrating differentially stained sister chromatids have been routinely employed in studies of peripheral lymphocytes from humans and other mammals conducted to estimate the increase of sister chromatid exchanges (SCEs) induced by environmental clastogens and to monitor cell-cycle progression *in vitro*. The existence of spontaneous SCE induction and the existence of a wide variability in their baseline frequencies as well as in their cell-cycle kinetics are still open questions (Carrano *et al.*, 1980; Latt *et al.*, 1980; Gebhart, 1981; Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990, 1993, 1996; Reigosa *et al.*, 1997; Soloneski *et al.*, 1999). Traditionally whole-blood (WBCs), plasma leukocyte (PLCs) and mononuclear leukocyte cultures (MLCs) are routinely used for the SCE- and cell-cycle progression assays. Discrepancies in the baseline SCE frequency and cell-cycle kinetics of the same lymphocyte among these three types of cultures have been observed to occur in several species, including humans (Wilmer *et al.*, 1984; Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990, 1993, 1996), pigs (Larramendy and Reigosa, 1986; Larramendy *et al.*, 1990, 1993, 1995; Reigosa *et al.*, 1997; Soloneski *et al.*, 1999) and rats (Kligerman *et al.*, 1982; Wilmer *et al.*, 1983).

We have found that human, but not pig PLCs and MLCs exhibited nearly a two-fold increase in the baseline frequency of SCEs when compared with WBCs, and that the incorporation of either human or swine erythrocytes in human PLCs and MLCs produced a red blood cell dose-dependent decrease in the frequency of SCEs (Larramendy and Reigosa, 1986). Besides, we have observed that in both human and pig erythrocyte-free cultures (PLCs and MLCs), the proliferation of

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lymphocytes is slower than in WBCs (Larramendy *et al.*, 1990). We have reported that pig and human red blood cells modulated the *in vitro* cell-cycle progression of both human and pig lymphocytes in a time- and dose-dependent manner, whereas the baseline frequency of SCEs is affected only in human cells (Larramendy *et al.*, 1990, 1993, 1996; Reigosa *et al.*, 1997). An estimation of the length of the S period revealed that PLCs have a markedly lengthened S period compared to WBCs (Larramendy *et al.*, 1996).

We analysed the effect of co-culturing varying concentrations of monocytes (MNs) on the baseline frequency of SCEs and cell-cycle progression of pig lymphocytes from PLCs and WBCs (Soloneski *et al.*, 1999). We observed that pig MNs modulate the *in vitro* cell-cycle progression of swine lymphocytes in a dose-dependent manner and that the low baseline SCE frequency is independent of the presence or absence of MNs in the culture (Soloneski *et al.*, 1999).

Different lymphocyte subsets may be distinguished by the so-called MAC (Morphology, Antibody, Chromosomes) methodology. This technique, as well as producing acceptable chromosome spreading, preserves the cell cytoplasm, thus allowing simultaneous analysis of morphology, immunological phenotype, and karyotype characteristics in each mitotic cell (Knuutila *et al.*, 1994). Furthermore, this methodology has been adapted for determination of the differential sensitivities of human lymphocyte subpopulations to different genotoxic agents (Slavutsky and Knuutila, 1989; Larramendy and Knuutila, 1991; González-Cid *et al.*, 1997) and to the study of cell proliferation in unfractionated hematopoietic cell cultures (Larramendy and Knuutila, 1990; Larramendy *et al.*, 1992).

In the present report we investigated whether delay of the cell-cycle kinetics and increase in the SCE baseline frequency of human lymphocytes in erythrocyte-free cultures are influenced by the presence or absence of adherent cells such as MNs in the culture system. The results show that monocytes modulate both the *in vitro* basal frequency of SCEs and the mitotic activity more readily of suppressor/cytotoxic T8 than in T human lymphocytes as a whole, but not their cell-cycle kinetics.

MATERIAL AND METHODS

Blood samples

Human blood samples were obtained by venipuncture from healthy male donors (20–40 years old) (Blood Bank of Buenos Aires Province, La Plata, Argentina) selected as recommended elsewhere (Bianchi *et al.*, 1979).

Whole blood cultures (WBCs)

Human WBCs were set up as described previously (Larramendy and Reigosa, 1986). Briefly, 1.0 ml of whole blood was seeded in 9.0 ml of complete culture medium (80% Ham's F10 (Gibco, Grand Island, NY, U.S.A.), 20% fetal calf serum (Gibco), 0.3 ml of phytohemagglutinin M (Gibco), 100 units penicillin/ml (Gibco), 100 µg/ml streptomycin/ml (Gibco) and 10 µg/ml bromodeoxyuridine (Sigma Chemical Co., St Louis, MO, U.S.A.)). According to the hemotological data from patients, the final RBC concentration was 4.55×10^8, 4.90×10^8, and 4.50×10^8 erythrocytes/ml (donors A, B, and C, respectively) while the concentration of leukocytes was 6.50×10^5, 6.70×10^5 and 6.25×10^5 cells/ml (donors A, B, and C, respectively). Final concentrations of MNs in culture are summarized in Table 1. During the last 3 h of culture, cells were treated with 0.1 µg/ml colchicine/ml (Sigma). Seventy-two hours after seeding, cells were harvested, exposed to a hypotonic solution (0.075 M KCl, 37°C, 15 min), and fixed in methanol-acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique. Cultures were established in duplicate for each donor, and at least two different donors were run simultaneously. The same batches of culture media, sera and reagents were used throughout the study.

Plasma leukocyte cultures (PLCs)

Human PLCs were set up as described by Larramendy and Reigosa (1986). Briefly, after gravity sedimentation of whole blood for 1–2 h at room temperature, a volume of plasma leukocyte suspension (1.0–1.5 ml) containing 1.2×10^7 cells was added to a volume of complete culture medium to reach to final volume of 10 ml. The final MN concentrations are depicted in Tables 1–3. Cell treatment, culture and harvesting conditions were as described for WBCs.

Effect of addition and depletion of monocytes (MNs) to plasma leukocyte cultures (PLCs)

The same volume of gravity separated plasma leukocyte suspension used for setting up PLCs from each donor was resuspended in complete culture medium (4.0 ml) and pipetted onto plastic tissue culture plate (60 mm). A 90 min incubation

Table 1.
Effect of human monocytes (MNs) in plasma leukocyte cultures (PLCs) on sister chromatid exchange (SCE) frequency and cell-cycle progression of human lymphocytes[a]

Culture type	*MN concentration (× 10⁴ MNs/ml)*			*SCE frequencies[b]*			*Cell-cycle progression (% cells in metaphase)*								
							Donor A			*Donor B*			*Donor C*		
	Donor A	*Donor B*	*Donor C*	*Donor A*	*Donor B*	*Donor C*	*1st*	*2nd*	*≥3rd*	*1st*	*2nd*	*≥3rd*	*1st*	*2nd*	*≥3rd*
WBC	1.95	1.34	1.87	4.30 ± 0.56	3.90 ± 0.36	3.39 ± 0.21	18	56	26	11	48	41	17	52	30
PLC	3.60	2.40	3.60	8.15 ± 0.44*	8.95 ± 0.25*	7.09 ± 0.34*	54*	40	6*	44*	56	0*	38*	61	2*
MNs-depleted PLC	0.00	0.00	0.00	8.60 ± 0.59	9.20 ± 0.80	9.20 ± 0.91	50	48	2	39	61	0	39	58	3
MNs-depleted PLC+MNs	1.50	1.50	1.50	6.85 ± 0.40	7.23 ± 0.39	6.90 ± 0.40	39	60	1	40	60	0	41	59	0
MNs-depleted PLC+MNs	3.00	3.00	3.00	6.75 ± 0.42	7.10 ± 0.61	6.50 ± 0.49	36	60	4	42	55	3	33	65	2
MNs-depleted PLC+MNs	7.50	7.50	7.50	5.45 ± 0.66*	Failed	4.85 ± 0.28*	57	43	0		Failed		44	56	0
MNs-depleted PLC+MNs	9.00	9.00	9.00	5.60 ± 0.31*	5.80 ± 0.40*	4.70 ± 0.39*	45	51	4	44	50	6	35	65	0
MNs-depleted PLC+MNs	12.00	12.00	12.00	5.50 ± 0.31*	4.40 ± 0.34*	5.00 ± 0.41*	42	56	2	40	56	4	36	64	0

[a]Human MNs were introduced into human PLCs after culture initiation, stimulated with PHA and harvested 72 h later.
[b]Results are expressed as mean SCEs/cell ± standard error of the mean.
*$P<0.05$ compared to WBC, and to PLC, respectively.
WBC, whole blood culture.

Table 2.
Effect of human monocytes (MNs) in MN-depleted plasma leukocyte cultures (MN-d PLCs) on the relative proportion (%) of different lymphocyte subsets among all interphase cells[a]

Lymphocyte subset[c]	*Culture type*									
	Donor D[b]					*Donor E*[b]				
	PLC 3.60×10^4 *MNs/ml*	*MN-d PLC* + 0×10^4 *MNs/ml*	*MN-d PLC* + 3.0×10^4 *MNs/ml*	*MN-d PLC* + 7.5×10^4 *MNs/ml*	*MN-d PLC* + 12.0×10^4 *MNs/ml*	*PLC* 3.50×10^4 *MNs/ml*	*MN-d PLC* + 0×10^4 *MNs/ml*	*MN-d PLC* + 3.0×10^4 *MNs/ml*	*MN-d PLC* + 7.5×10^4 *MNs/ml*	*MN-d PLC* + 12.0×10^4 *MNs/ml*
B cells										
CD20 (pan B)	7	9	10	12	11	11	10	12	11	10
T cells										
CD3 (pan T)	72	77	81	75	78	70	74	71	76	80
CD4 (T-helper/inducer)	52	57	54	55	56	47	55	55	61	57
CD8 (T-suppressor/cytotoxic)	25	18	22	27	29	21	22	24	17	28

[a]Human MNs were introduced into human MN-depleted PLCs (MN-d PLC) after culture initiation. Cells were stimulated with PHA and harvested 72 h later.
[b]Results are presented as mean percentage of immunoperoxidase positive interphase cells.
[c]The following monoclonal antibodies were used: L26 for CD20; antiCD3 for CD3; antiCD4 for CD4, and antiCD8 for CD8.

Table 3.
Effect of human monocytes (MNs) in MN-depleted plasma leukocyte cultures (MN-d PLCs) on the relative proportion (%) and mitotic indices (between parentheses) of different lymphocyte subsets among all mitotic cells[a]

Lymphocyte subset[c]	*Culture type*									
	Donor D[b]					*Donor E*[b]				
	PLC 3.60×10^4 *MNs/ml*	*MN-d PLC* + 0×10^4 *MNs/ml*	*MN-d PLC* + 3.0×10^4 *MNs/ml*	*MN-d PLC* + 7.5×10^4 *MNs/ml*	*MN-d PLC* + 12.0×10^4 *MNs/ml*	*PLC* 3.50×10^4 *MNs/ml*	*MN-d PLC* + 0×10^4 *MNs/ml*	*MN-d PLC* + 3.0×10^4 *MNs/ml*	*MN-d PLC* + 7.5×10^4 *MNs/ml*	*MN-d PLC* + 12.0×10^4 *MNs/ml*
All cells	(7.2)	(6.8)	(8.8)	(12.7)*	(14.7)*	(4.7)	(5.6)	(4.5)	(6.4)*	(6.6)*
B cells										
CD20 (pan B)	15 (6.0)	12 (4.4)	21 (4.6)	22 (5.8)	17 (6.6)	12 (5.6)	11 (6.0)	9 (5.6)	9 (4.6)	13 (4.2)
T cells										
CD3 (pan T)	75 (10.3)	68 (7.3)	74 (9.7)	80 (12.3)	72 (10.2)	76 (9.7)	71 (7.8)	73 (7.3)	75 (8.2)	72 (10.2)
CD4 (T-helper/inducer)	58 (10.0)	48 (8.4)	52 (9.5)	58 (10.2)	65 (9.7)	56 (8.4)	49 (6.9)	55 (8.0)	54 (8.9)	60 (7.9)
CD8 (T-suppressor/cytotoxic)	25 (8.2)	24 (7.3)	20 (6.5)	42* (20.8)*	49* (17.5)*	18 (10.6)	23 (9.8)	24 (10.2)	40* (19.6)*	42* (18.3)*

[a]Human MNs were introduced into human MN-depleted PLCs (MN-d PLC) after culture initiation. Cells were stimulated with PHA and harvested 72 h later.
[b]Relative proportions of different lymphocye subset are presented as mean percentage of immunoperoxidase positive mitotic cells while mitotix indices as percentage of immunoperoxidase positive mitoses among all immunoperoxidase positive cells.
[c]The following monoclonal antibodies were used: L26 for CD20; antiCD3 for CD3; antiCD4 for CD4, and antiCD8 for CD8.
*$P<0.05$.

in 5% CO_2 incubator at 37°C was done to allow MNs to adhere as suggested elsewhere (Yasaka *et al.*, 1981). Non-adherent cells were then pipetted into culture tubes and used to start MN-depleted PLCs according to standard procedure. Adherent cells were removed from the tissue culture plates with a rubber policeman, and re-added to the depleted PLCs at concentrations ranging from 0 to 1.2×10^5 MNs/ml as specified in the Results section. Cell treatment, culture and harvesting conditions were as described for WBCs.

Fluorescence-plus-Giemsa (FPG) method for sister chromatid differentiation

Chromosome spreads were stained using the FPG technique for sister-chromatid differentiation described in detail by Larramendy and Knuutila (1990).

Sister chromatid exchange, cell-cycle progression and mitotic index analyses

All slides were coded and a single observer scored the SCE frequencies in 50 diploid metaphases per sample. A minimum of 200 metaphase cells per sample were scored to determine the percentages of cells that had undergone one, two, three or a greater number of mitoses. All metaphases showing differential staining of sister chromatids in less than one-fourth of the chromosomal complement were considered to be in at least the fourth cell-cycle. The mitotic index was determined by scoring 1000 cells from each culture and expressed as percentage of mitoses.

MAC (Morphology, Antibody, Chromosomes) study

The MAC method has been described in detail elsewhere (Knuutila *et al.*, 1994; Larramendy *et al.*, 1992). Briefly, after culturing, the cells were bathed (5 min, room temperature) with solution composed of one part of hypotonic solution (50.0 mM glycerol, 10.0 mM KCl, 0.8 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 10.0 mM sucrose in water) and one part of complete culture medium. At the end of the hypotonic treatment, the cells were placed in cyto-centrifuge chambers (Cytospin, Shandon Elliot, Runcorn, U.K.) and centrifuged at $400 \times \boldsymbol{g}$ for 5–10 min. The slides were air-dried for 18–24 h and then fixed for 1 min in a solution consisting of 50 ml of acetone and 50 µl of 30% formaldehyde. For the immunological phenotyping of the cells, the slides were stained following the immunoperoxidase method using several monoclonal antibodies and Giemsa counterstain (Knuutila and Teerenhovi, 1989). In brief, the cells were incubated with primary antibody (1:5–1:100) for 1 h. They were then washed in phosphate-buffered saline containing fetal calf serum, and treated with secondary biotinylated antibody (1:250) and avidin/DH biotinylated horseradish peroxidase (1:160) (Vectastain ABC Kit, Vector, Burlingame, CA, U.S.A.) for 30 min. Afterwards, the slides were bathed for 20 min in the presence of H_2O_2 and 3-amino-9-ethylcarbazole (0.2 mg/ml). The following monoclonal anti-human antibodies were used in the study: L26 (CD20) (Dako A/S, Glostrup, Denmark), anti-CD3 (CD3) (Sigma), anti-CD4 (CD4) (Sigma), and anti-CD8 (CD8) (Sigma). CD 8 is a marker for suppressor/cytotoxic T cells and CD4 for helper/induced T cells. CD3 and CD20 are a pan-T and a pan-B cells, respectively. For visualization, the slides were counterstained with 5% Giemsa in Sörensen's buffer (pH 6.8).

The frequency among of interphasic and mitotic lymphocytes of cells from each of the lymphocyte subsets we studied, was obtained by analysing at least 1000 and 100 positive for each monoclonal antibody, respectively. The mitotic index was determined by scoring 1000 positive cells from each culture for each monoclonal antibody and expressed as percentage of mitoses.

Statistical analysis

Student's *t*-test for comparisons was used to determine the significance of differences between the mean SCE frequencies. The χ^2 test was used for cell-cycle progression data, mitotic index and proportions of different lymphocyte subsets among mitotic and interphasic cells in culture. The chosen level of significance was 0.05.

RESULTS AND DISCUSSION

Table 1 summarizes experimental results from control WBC and PLC in which human lymphocytes were cultured in the presence of different adherent cell concentrations after *in vitro* stimulation. Baseline SCE frequencies in control PLCs were nearly twice as high as those in control WBCs ($P<0.05$). These observations confirm the differential response of human lymphocytes depending on whether they are cultured together with red blood cells or not, as previously reported by us (Larramendy and Reigosa, 1986; Larramendy

et al., 1990, 1993, 1996) and Mehnert *et al.* (1984). However, none of these previous studies addressed the effect of MN concentration on the baseline SCE frequency. No significant differences in the baseline SCE frequency were observed between control PLCs and MN-depleted PLCs ($P>0.05$). Accordingly, these results may demonstrate that, regardless of the donor, the high SCE frequency of human lymphocytes found in erythrocytes-free cultures is independent of the presence or absence of MNs during the culture period, and then, most probably committed to the absence of red blood cells. Years ago, we demonstrated that human and pig erythrocytes have a direct effect on human lymphocytes, preventing the induction of a heightened basal frequency of SCEs by releasing a 'corrective' factor present after hemolysis in the erythrocyte ghosts (Larramendy and Reigosa, 1986). In addition, it has been observed that this corrective factor is not dialysable, and it is not specific to species, since pig erythrocytes are nearly as efficient as human red blood cells in decreasing the high SCE frequency of human erythrocyte-free cultures (Larramendy and Reigosa, 1986). Despite efforts, the nature of such factor remains unknown. The information we possess is not enough to elucidate whether this corrective factor present in erythrocytes is the same found by others in other normal mammalian cells different than erythrocytes (vanBull *et al.*, 1978; Bartram *et al.*, 1979, 1981; Rüdiger *et al.*, 1980; Shiraishi *et al.*, 1981; Leroux *et al.*, 1984).

The addition of different concentration of MNs to MN-depleted PLCs gave some interesting results. Increasing the concentration of MNs caused a proportional decrease in the frequency of SCEs as well as an increase in the mitotic activity of PLC (Tables 1, 3). Reconstitution of depleted PLCs with concentration of MNs lower than that present in PLC (0–3.0×10^4 MNs/ml) did not modulate SCE frequencies ($P>0.05$). Only the decrease of SCEs reached significance at concentrations of MNs equivalent or higher than those present in PLC (7.5×10^4–1.2×10^5 MNs/ml) ($P<0.05$). Moreover, although significant, SCE baseline frequencies were intermediate between PLC and WBC (Table 1). When concentrations of MNs ranging from 0–3.0×10^4 cells/ml were titrated into depleted PLCs, no variations in the mitotic activity was observed among cultures ($P>0.05$). On the other hand, concentrations higher than 7.5×10^4 MNs/ml titrated into cultures induced a significant increase in the mitotic indexes over non-depleted PLCs ($P<0.05$) (Table 3).

To date, the function of the MNs within the leukocyte culture system and their possible modulative function exerted on the baseline SCE frequency of mammalian lymphocytes remains unknown. So far, only our recent study of pig lymphocytes (Soloneski *et al.*, 1999) and the results by Wilmer *et al.* (1983) using rat lymphocytes have been reported. In pig cells, we have demonstrated that the low baseline SCE frequency of WBCs and PLCs is independent of the presence or absence of MNs in the culture (Soloneski *et al.*, 1999). In rat cells, it has been observed that the SCE frequency in MN-depleted mononuclear leukocyte cultures is, though not significant, lower than in non-depleted cultures (Wilmer *et al.*, 1983). Moreover, reconstitution of depleted cultures with adherent cells rendered SCE baseline frequencies intermediate between MN-depleted and mononuclear leukocyte cultures, indicating that MNs are able to increase the SCE frequency of rat lymphocytes (Wilmer *et al.*, 1983). Accordingly, Wilmer *et al.* (1983) suggested that a mechanical dislodging induced during isolation of adherent cells may reduce their functional capabilities responsible for increasing SCEs. On the other hand, the results we present here demonstrate that the basal frequency of SCEs from PLCs are independent of MNs, while MNs titration into adherent cells-depleted PLCs did not reduce the SCE frequencies to that of WBCs, a significant decline occurred. Generally, the former (PLCs containing MNs) can be considered as culture condition representing a physiological situation whereas the latter (reconstitution of depleted PLCs with MNs) should be considered as a non-physiological phenomenon. To date, there has been no additional information to elucidate this peculiar human MNs behaviour and whether this function is exhibited by the MNs from other species. We know, at least, that swine plastic adherent cells do not exhibit this phenomenon (Soloneski *et al.*, 1999) while rat MNs would exhibit an opposite effect than that of human ones (Wilmer *et al.*, 1983). Furthermore, our results would indicate that under physiological condition/s, MNs would be unable to modulate the SCEs of human lymphocytes *in vitro*, and that only under an artificial situation of culturing, e.g. the reconstruction of depleted PLCs, adherent cells could decline SCEs. This assumption could be supported by the fact that even a doubled number of MNs titrated into PLCs were unable to render the baseline SCE frequency at values found in WBC.

To determine whether the titration of MNs into adherent-depleted PLC would modify the proliferation rate of human lymphocytes *in vitro*, the

frequencies of cells in their first, second, and third or subsequent mitoses were calculated (Table 1). The results demonstrate that in all PLC, when compared to the lymphocyte proliferation kinetics in the corresponding WBC, the proportion of cells in their first mitosis increases, while a decrease in cell frequency in the third mitosis is observed ($P<0.05$). However, the proportion of cells in their second mitosis remains unchanged ($P>0.05$). These observations have been previously reported by us (Larramendy *et al.*, 1990, 1993, 1996) and by Mehnert *et al.* (1984), confirming that human lymphocytes *in vitro* when cultured in erythrocyte-free cultures proliferate slower than parallel WBC. Similar observations have been also previously found for pig (Larramendy *et al.*, 1990, 1993, 1995; Reigosa *et al.*, 1997; Soloneski *et al.*, 1999) and for rat lymphocytes as well (Wilmer *et al.*, 1983, 1984). They all agree that mononuclear leukocytes isolated by centrifugation in Lymphodex (Mehnert *et al.*, 1984) and Ficoll-Hypaque (Wilmer *et al.*, 1983, 1984) or cultured in PLCs (Larramendy *et al.*, 1990, 1993, 1995; Reigosa *et al.*, 1997; Soloneski *et al.*, 1999) exhibit a slower cell-cycle progression than WBCs, suggesting that red blood cells release lymphocytes from inhibition to growth and proliferation.

Interestingly, depletion of adherent cells from PLCs does not affect the cell-cycle kinetics of human lymphocytes, although variations among donors exist (Table 1). No differences in the proportion of cells in their first, second, and third or subsequent divisions were observed either between PLC and MNs-depleted PLCs ($P>0.05$) or among MN-free PLCs titrated with different concentrations of adherent cells ($P>0.05$). These observations demonstrate for the first time that the proliferation rate of human lymphocytes *in vitro* is not modulated by the presence or absence of adherent cells during the culture period. Unfortunately, information available on the relationship between *in vitro* lymphocyte proliferative characteristics and MNs is scarce. So far, variations in cell-cycle progression of lymphocytes from PLC both in the absence and presence of adherent cells in the culture have been only reported for pig cells (Soloneski *et al.*, 1999). It has been observed that in MN-free cultures, lymphocytes proliferate faster than in parallel PLC. However, when MNs were seeded into the cultures, cell-cycle progression gradually slowed as a function of the concentration of adherent cells present in the cultures (Soloneski *et al.*, 1999). To date, we did not have enough information to elucidate whether the influence of MNs on the proliferation rate of pig lymphocytes was restricted to this species or, on the other hand, was this a common phenomenon extended to other species. Our present results clearly demonstrate that at least human MNs do not possess any modulative effect on the proliferation rate of lymphocytes *in vitro*.

Cellular immunophenotyping was used to study the modulatory effect of MNs on the phenotype of interphasic and mitotic B cells and different T lymphocyte subsets from cultures grown with different concentrations of adherent cells (Tables 2, 3). Although the phenotypes of both interphasic B cells and different T lymphocyte subsets were similar among cultures either between PLC and MNs-depleted PLCs ($P>0.05$) as well as among MN-free PLCs titrated with different concentrations of adherent cells ($P>0.05$) (Table 2), the mitotic subpopulations showed discrepancies (Table 3). By using the MAC technique it was possible to demonstrate for the first time that the increased mitotic activity found in those PLCs in which 7.5–12.0×10^4 MNs/ml were seeded was almost exclusively due an increased frequency of mitotic suppressor/cytotoxic T8 lymphocytes at the time of harvesting ($P<0.05$) (Table 3). Two plausible explanations for this observation can be suggested: (1), a selective overgrowth and/or a relative greater proliferation of T8 cells being more prone to enter mitosis in general, or (2) these cells are specifically sensitive to the presence of adherent cells in the culture system. Years ago, Kovanen and Knuutila (1989) found that fetal calf serum, the type of serum employed in the culture media we used in our experiments, promotes the proliferation of mainly suppressor/cytotoxic T8 cells among human lymphocytes in culture. We, however, were unable to demonstrate a selective overgrowth of B cells and any of the different T-cell subpopulations in unfractionated human mononuclear cultures growing in fetal bovine supplemented culture media upon stimulation with either phytohaemagglutinin or pokeweed mitogen as reported elsewhere (Larramendy *et al.*, 1992) as well as in the present study (Table 2). Furthermore, the fact that the mitotic frequency of this T-cell subset differs from control values only in those cultures treated with the highest concentrations of MNs (Table 3) argues as well against the first hypothesis. It therefore appears unlikely that there is a selective overgrowth and/or a relative greater proliferation of suppressor/cytotoxic T8 lymphocytes Hence, it appears probable that suppressor-cytotoxic T8 lymphocytes are in general more sensitive than B and others T-cell subpopulations to divide more frequently by the presence of adherent cells in the

culture medium, at least in the concentration range we employed. Moreover, it should be mentioned that the increase we observed in the mitotic activity of those PLCs in which $7.5–12.0 \times 10^4$ MNs/ml were seeded, was exclusively due an increased frequency of mitotic suppressor/cytotoxic T8 lymphocytes ($CD8^+$), and not accompanied by an increase in the frequency of mitotic $CD3^+$ and/or decrease in the relative proportion of mitotic $CD4^+$ cells. Generally, $CD8^+$ cells are also $CD3^+$. Accordingly, in the absence of any alteration in the mitotic activity of $CD4^+$ cells, whether the mitotic indexes of $CD8^+$ increased, the mitotic indexes of $CD3^+$ should also increased. Moreover, the increase in mitotic $CD8^+$, but not in $CD3^+$ and $CD4^+$, we observed after MNs incorporation, could indicate the existence of a $CD3^-/CD4^-/CD8^+$ subpopulation with a differential sensitivity to MNs. Unfortunately, at present we possess no monoclonal antibody specific for such a lymphocyte subset.

Some studies with purified human T and B-cell populations suggest that phytohaemagglutinin induces selective proliferation of T cells, whereas pokeweed mitogen stimulates both T and B cells (Geha and Merler, 1974; Greaves *et al.*, 1974; Mellstedt, 1975; Keightley *et al.*, 1976; Kuritami and Cooper, 1982; Mills *et al.*, 1985). However, using the MAC methodology we previously demonstrated that, when heterogeneous lymphocyte populations are used for the establishment of unfractionated mononuclear cultures as the culture system employed in the study, the effects of these mitogens are not as selective as when purified populations are used, i.e., phytohaemagglutinin is also capable of inducing B lymphocyte proliferation. In fact, no significant differences were observed in the proportion of interphasic and mitotic B cells in phytohaemagglutinin-stimulated and pokeweed-stimulated cultures (for review see Larramendy *et al.*, 1992, and references therein). Previous reports employing immunophenotyping of peripheral blood lymphocytes cultured *in vitro* have revealed that human lymphocyte subpopulations have different sensitivities to several clastogenic agents (Slavutsky and Knuutila, 1989; Larramendy and Knuutila, 1991; Slavutsky *et al.*, 1995; González-Cid *et al.*, 1997; Soloneski *et al.*, 2002). B and suppressor-cytotoxic T8 lymphocytes had higher levels of micronuclei formation than other lymphocyte subpopulations in tobacco smokers (Larramendy and Knuutila, 1991), and in cells exposed *in vitro* to the antineoplastic drugs peplomycin (Slavutsky and Knuutila, 1989) and carboplatin (Slavutsky *et al.*, 1995) or to the dithiocarbamate zineb and its commercial formulation azzurro (Soloneski *et al.*, 2002) suggesting that B and suppressor-cytotoxic T8 cells are generally more sensitive to clastogenic agents than other lymphocyte subsets. Our present results agree well with this assumption.

In addition to the biological importance of the differential sensitivity of the suppressor-cytotoxic T8 lymphocyte subset (even though we do not yet understand the basic mechanism for this difference), our observations have a direct practical implication. When a cell-cycle proliferation study is performed, the MAC methodology should be applied simultaneously with any classical cytogenetic procedure since it permits the study of the proliferation kinetics of different cell subpopulations. In our study T8 lymphocytes were the only affected by the presence of MNs during culturing. However, this subset is the least frequent of all T lymphocytes, constituting about 30% of the lymphocytes (Knuutila and Kovanen, 1987, Larramendy and Knuutila, 1991; Larramendy *et al.*, 1992). Therefore it should be possible to demonstrate statistical significance even with a small number of donors by concentrating the mitotic index analysis to this sensitive cell population.

Primarily our observations underline the lack of knowledge in the mechanism(s) that regulate and cause variations in the baseline SCE frequencies and cell-cycle kinetics of mammalian lymphocytes. It appears that the SCEs and cell-cycle progression rates observed in human as well as in other mammalian lymphocyte cultures are overestimation's, as they have been produced by several modulating factors introduced into the culture system. Standardized culture protocols and the MAC technique should be used together to obtain a valid measure of SCE and cell-cycle progression from lymphocytes *in vitro* when any short-term lymphocyte cultures are employed as monitoring assays for clastogenesis.

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