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Synergism between phorbol myristate acetate and calcium ionophore in inducing proliferation of *in vitro* γ-irradiated murine lymphocytes

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Abstract. The objective of this study was to analyze the *in vitro* effects of γ-irradiation (0–5 Gy) on lymphocyte proliferation in animals sensitive to radiation as BALB/c mice. Lymphocytes were irradiated and underwent different treatments: quiescent cells were cultured with calcium ionophore A23187 (5 min or 48 h) with or without phorbol myristate acetate (PMA); lymphocytes (control cells or incubated with A23187 and PMA) were also cultured with four mitogens that are specific to the different subpopulations to determine the degree of inhibition of the response to radiation. Results obtained indicated that in quiescent cells, A23187 and PMA treatment had a mitogenic effect, which peaked with long A23187 treatment (48 h); synergism was further demonstrated between both drugs and was enhanced with higher ionizing radiation doses. However, in both irradiated and non-irradiated mitogen-stimulated cells, A23187 (48 h) and PMA had a strong inhibitory effect on cell proliferation. In conclusion these results indicate that irradiated BALB/c mice lymphocytes respond to treatment with A23187 and PMA more actively than controls. Inhibition of the post-exposure mitogen-induced proliferative response and the synergic effect between A23187 and PMA also suggest altered PKC activation mechanisms in cell membranes. Comparing with previous studies with *in viv*o irradiated mice, the effects of IR *in vitro* were less intense.

Key words: Ionizing radiation — Lymphocyte proliferation — Phorbol myristate acetate — Calcium ionophore — Mitogens

Introduction

The cellular effects of ionizing radiation (IR) are mediated by many factors, including radiation doses, exposure time and cell type. Lymphocytes from the BALB/c mouse strain are especially sensitive to IR and allow us to study activation mechanisms and proliferation after an IR dose in both *in vivo* and *in vitro* systems (Hernández-Godoy et al. 2008, 2010).

Lymphocytes are a heterogeneous mixture of different cell subpopulations: CD3 + T-cells, subdivided into CD4 + helper T (Th)-cells and CD8 + cytotoxic (Tc) cells, B-cells and natural killer (NK) cells. By using specific T- and B-cell mitogens it has been shown that the sensitivity response of these cell types to IR is variable. Thus, B-cells are more radiosensitive than Tc, Tc are more radiosensitive than Th, and finally, Th are more radiosensitive than NK cells (Mori and Desaintes 2004; Hernández-Godoy et al. 2008, 2010; Cao et al. 2011).

For lymphocyte activation, the antigen (or the mitogen) has to be recognized by cell membrane receptors, which differ according to the lymphocyte subpopulation. Next phospholipase C (PLC) enzyme is activated and induces the generation of two second messengers, diacylglycerol (DAG), which activates the protein kinase C (PKC) pathway, and inositol 1,4,5-trisphosphate (IP3) which, in turn, increases the intracellular calcium concentration [Ca^{2+}]. Then, activation, proliferation and cell differentiation may occur (Smith-Garvin et al. 2009; Hogan et al. 2010). Two pharmacological agents can be used to mimic the effects of DAG and IP3: phorbol myristate acetate (PMA) activates the PKC enzyme and calcium ionophore (A23187) initiates calcium flow (Planelles et al. 1992; Meng et al. 2002).

By studying the effects of IR on cellular activation mechanisms, highly variable results have been described depending on the cell type studied and its origin. Thus after exposure, certain genes of Jurkat cells were activated more quickly

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(Syljuåsen et al. 1996), others were overexpressed (Guo and Xu 2001), and some others did not respond at all (Banda et al. 2008). Inhibition of apoptosis has also been described (Meng et al. 2002) and similar results have been obtained in other cell types (Tomei et al. 1990; Gong et al. 2008). Moreover, the presence of the ionophore A23187 in Swiss mice potentiated the effects of Ca^{2+} modulators (Agrawal et al. 2003), and when cells were irradiated at low doses *in vitro,* different radiosensitivity regulatory and effector T-cells have been seen (Cao et al. 2011).

Given the sensitivity to IR of BALB/c mice lymphocytes, they are of much interest to study the effects of radiation on the immune response and the cellular mechanisms mediating it, although the information obtained in these cell types is still scarce. Our group has studied the effect of IR in this cell type, and has shown that after exposure in both *in vivo* and *in vitro* (Hernández-Godoy et al. 2008, 2010, 2013), inhibition of the lymphoproliferative response occurs, which is recovered in time, and that B-cells are more sensitive than T-cells, although B populations recover before T ones. In the lymphocytes of animals irradiated *in vivo* with a single dose of radiation, it has been demonstrated that inhibition of the proliferative response can be reversed if cells were incubated with PKC activators, such as PMA and A23187 (Hernández-Godoy et al. 2013). This observation is of great importance because it could imply IR damage mechanisms of PKC activation and cell proliferation. However, the fact that each animal had only been exposed to a single dose of radiation limited the findings of that *in vivo* study and indicated the need to design appropriate *in vitro* experiments.

The *in vitro* experiments described in this work permitted the exposure of lymphocytes from the same animal to escalating doses of IR (0–5 Gy) and to evaluate the effect of PMA and A23187 in cells stimulated or not with mitogens in each dose of irradiation.

Materials and Methods

Animals

Male and female BALB/c mice were used as spleen cell donors, which were free of pathogens, weighed 22–25 g and were 8 to 12 weeks old. For the experiments, all the animals were rapidly and humanely euthanized with 100% CO_2 in accordance with standard international animal care protocols.

Preparation of cell suspensions and in vitro irradiation

Mice spleens were removed in sterile conditions and lymphocyte suspensions prepared through sterile pressure sieves as described previously (Hernández-Godoy et al. 2008). Briefly, the cells were washed twice in saline solution (Grifols, Parets del Vallés, Barcelona, Spain) and then resuspended in complete medium RPMI 1640 (Gibco, Paisley, United Kingdom) containing 5% of fetal calf serum (Gibco), glutamine 2 mM (Flow Laboratories, Irvine, United Kingdom) and cefotaxime 100 µg/ml (Hoechst, Barcelona, Spain) at a density of 4×10^6 cells/ml. Cell suspensions were kept in ice until irradiation.

The irradiation *in vitro* took place in a Sagitarius (25 MV) lineal accelerator from the Oncology Service of La Fe Hospital, Valencia. Dosage administered varied between 1 and 5 Gy at a rate of 2.4 Gy/min, with a dosage error of less than 2%. After irradiation a temperature of 0ºC (in ice) was maintained until seeding. Seeding took place 30 minutes after irradiation. The control cells (0 Gy) followed the same process as irradiated cells and were seeded in microcultures at the same time.

Study of cell viability

A study of cellular viability was conducted with the irradiated and non-irradiated cells using the cytometric acridine orange method (Pantazis and Kniker 1979).

Preparation of irradiated and non-irradiated cell cultures

In order to evaluate the effects of IR *in vitro* on the lymphocyte proliferation aliquots of irradiated and non-irradited cells were treated (or not) with A23187 calcium ionophore (Sigma-Aldrich, Tres Cantos, Madrid, Spain), to induce calcium flow, and/or with phorbol myristate acetate (PMA; Sigma-Aldrich), to activate the PKC enzyme, and cultured with (or without) specific T-cells mitogens, as concanavalin A (ConA; Sigma-Aldrich) and phytohaemaglutinin A (PHA; Sigma-Aldrich), or specific B-cells mitogens as lipopolysaccharide (LPS) from *Escherichia coli* (Difco, San Agustín del Guadalix, Madrid, Spain) or mixed mitogens as pokeweed (PWM, Gibco, Paisley, United Kingdom). Briefly, cell suspensions were preincubated at 37ºC for 5 min with 200 nM A23187, then washed twice in saline solution and readjusted to the original cell concentration. Both the untreated and A23187 preincubated cells were distributed at a ratio 4×10^5 viable cells/well in 96-well flat-bottom microtiter plates and were cultured without mitogen or with different doses of ConA, PHA, LPS and PWM. A23187 was added directly to some of the wells containing untreated cells when culture was begun. In all experiments, parallel cultures were performed in which 10 ng/ml PMA was added.

After seeding, duplicate cultures were incubated at 37°C in a humid atmosphere with 5% CO_2 for 48 hours. Next, 18.5 kBq of tritiaded thymidine (specific activity 185 GBq, Amersham) were added to each well. Cells were collected

Table 1. Effects of ionizing radiation exposure (0, 1, 3 and 5 Gy) on spontaneous cell proliferation with A23187 (200 nM) and/or PMA (10 ng/ml)

PMA		–	–	–	+	+	+
A23187		–	5 min	48 h	–	5 min	48 h
IR (Gy)	0	2134 ± 179	1637 ± 172	2338 ± 512	5417 ± 830	10378 ± 3746*	118250 ± 7045***
	1	825 ± 100	411 ± 41	1142 ± 145	4632 ± 760***	8562 ± 2680*	96082 ± 10855***
	3	256 ± 52	174 ± 11	425 ± 75	2023 ± 369***	4492 ± 1747*	94106 ± 71220***
	5	127 ± 15	87 ± 7	342 ± 67	798 ± 124**	3314 ± 1482*	43475 ± 7758***

Arithmetic mean ± SEM of counts *per* minute are shown. The number of mice was 10 in all experiments. Results obtained with cells treated with A23187 and/or PMA were compared with those from untreated cells using paired Stutent *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; IR, ionizing radiation; PMA, phorbol myristate acetate.

18 hours later by suction through fibreglass filters and the radioactivity in the cells was measured with a liquid scintillation counter. Cell proliferation was measured by quantifying the tritiated thymidine incorporated into cellular DNA during the generated proliferation, expressed as counts *per* minute (cpm).

Interaction index and dose reduction factor

The effect of A23187 and PMA was studied individually and in combination, as was the interaction between these agents and mitogens. To this, the method of Papadogiannakis and Johnsen (1987) was followed. Given two chemicals, A and B, the interaction was calculated according to the following expression: Interaction index = $[(E_{A\cdot B}) - (E_A + E_B)] / (E_A + E_B)$ where E is the number of counts *per* minute obtained in each case. Thus, positive values indicate a synergistic effect, values close to zero indicate an additive effect, and negative values indicate an inhibitory effect of the combination.

In order to compare the inhibition effect of ionizing radiation between different doses of the same mitogen, the dose of IR which caused 50% inhibition in the lymphoproliferative response (ID_{50}) was estimated (Laska and Meisner 1987; Leatherbarrow 1990) and to compare the effect of drugs on irradiated cultures the dose reduction factor (DRF) was used. This was calculated as set out in Manori et al. (1985) by dividing the dose of radiation required to reduce cell proliferation by 50% (ID_{50}) in the presence of the drug in question by the dose required to obtain a similar effect in the cell culture in the absence of the drug. That is: DRF = (ID_{50} presence drug) / (ID_{50} absence drug).

Statistical analysis

Statistical test employed was the Student's *t*-test (for pairwise comparisons).

Results

Effects of A23187 and/or PMA on quiescent cells

From Table 1 it can be seen that IR significantly inhibited cell proliferation in a dose-dependent manner both in untreated and in PMA-treated quiescent cells. Addition of PMA to non-irradiated quiescent cells had no statistically significant effect on cell proliferation. However, in irradiated cell cultures PMA had a protective effect of cell proliferation, being this higher in the presence of PMA than in its absence. If in addition to PMA, A23187 is present in the culture, very high proliferation takes place with a maximum 48-hour preincubation in non irradiated cells, whereas proliferation is lower in irradiated cells, but always remains very high with all the IR doses.

The interaction rates between A23187 (5 min or 48 h) and PMA (Table 2) show a synergism between both drugs which becomes more intense with increasing IR doses. That is, the higher the IR dose, the stronger the synergistic effect of both drugs.

Table 3 shows that PMA increased the ID_{50} values. This effect was enhanced by treatment with A23187, regardless of it being short or long, although a longer treatment (48 h) seemed more effective.

Table 2. Interaction index between A23187 and PMA for differents radiation doses.

	A23187	
IR (Gy)	5 min	48 h
0	0.5	14.2
1	0.7	15.8
3	1.0	36.2
5	2.8	37.2

The number of mice was 10 in all experiments. IR, ionizing radiation; PMA, phorbol myristate acetate.

Table 3. ID_{50} and DRF values obtained for irradiated cells (0–5 Gy) treated with A23187 (200 nM) and/or PMA (10 ng/ml)

A23187	ID_{50}		DRF
	− PMA	+ PMA	
–	0.9	2.5	2.8
5 min	0.7	2.7	3.9
48 h	1.0	4.5	4.5

The number of mice was 10 in all experiments. ID_{50}, the dose of IR which caused 50% inhibition in the lymphoproliferative response; DRF, dose reduction factor (the ratio ID_{50} presence drug/ID_{50} absence drug); PMA, phorbol myristate acetate.

Effects of PMA on mitogen-stimulated cells

PMA significantly increased the cell proliferation induced by the four mitogens (ConA, PHA, PWM and LPS) in both the irradiated cells and controls (Table 4).

Moreover, PMA effects for the non-irradiated cells depended on the mitogen that induced the proliferation. PMA was synergistic for the cells stimulated with PHA, LPS or PWM. In contrast for those stimulated with ConA the effect was additive. For the irradiated cells, the effects of PMA on mitogen-induced proliferation depended on the mitogens, their dose and the IR dose. Thus the cells stimulated with PHA displayed a higher degree of synergy with PMA, while those treated with ConA exhibited the lowest degree of synergy (Table 5).

Effects of A23187 on mitogen-stimulated cells

As Table 6 shows, IR significantly inhibited mitogen-induced cell proliferation in both the absence and presence of A23187. Thus, in the irradiated cells, A23187 (5 min) had different effects depending on the mitogen: hence the ConA-stimulated cells were inhibited or slightly improved basal response. Conversely, the response of the PHA-stimulated cells significantly increased (at all the IR doses) when pre-treated with the calcium ionophore for 5 min.

Furthermore, the 48-hour treatment with A23187 significantly inhibited the mitogen-induced proliferative responses of T-cells. That is, a short pretreatment with A23187 had a synergistic effect, while a long treatment had an antagonistic effect, and was the same with non-irradiated cells (Table 7).

Overall effect of A23187 and PMA on mitogen-stimulated cells

As seen in Table 6, IR inhibited, in a dose-dependent manner, the cell proliferation induced by mitogens. When cells were pre-treated with A23187 for 5 min and were then cultured with mitogens and PMA, their response was not inhibited at high IR doses (5 Gy). Thus, the joint effect of both drugs was greater than it was for each separate drug. This increase was more pronounced in the irradiated cells than in the controls. Moreover, the presence of A23187 (48 h) and PMA in the mitogen-stimulated cultures had a strong inhibitory effect on cell proliferation.

Table 4. Effects of IR on cell proliferation in mitogens stimulated cells with/without PMA (10 ng/ml)

	Mitogens					
IR	ConA (1 μg/ml)		ConA (3 μg/ml)		PHA (10 μg/ml)	
(Gy)	− PMA	+ PMA	− PMA	+ PMA	− PMA	+ PMA
0	88946 ± 6066	103786 ± 4662	110689 ± 4532	109908 ± 3859	55780 ± 2513	83774 ± 34
1	72967 ± 7112^{a}	94704 ± 2204^{i}	99593 ± 3053	110864 ± 3410	46840 ± 2732^{b}	79493 ± 1683^{ii}
3	35940 ± 2627^{c}	76290 ± 2674$^{iii, b}$	80367 ± 2977$^{iii, c}$	90227 ± 1644^{a}	22355 ± 1272^{c}	65890 ± 2005$^{iii, b}$
5	17338 ± 1789^{c}	42101 ± 63$^{iii, c}$	60168 ± 2274^{c}	80798 ± 3388$^{iii, c}$	14095 ± 150^{c}	65290 ± 1561$^{iii, c}$

	LPS (40 μg/ml)		LPS (80 μg/ml)		PWM (2%)	
	− PMA	+ PMA	− PMA	+ PMA	− PMA	+ PMA
0	21760 ± 1386	50460 ± 2300	18401 ± 1247^{c}	47273 ± 1867	22703 ± 1497	46105 ± 2344^{ii}
1	11490 ± 1006^{c}	35310 ± 1441$^{i, c}$	11311 ± 828^{c}	31328 ± 1341$^{i, c}$	14448 ± 669^{c}	40597 ± 917$^{iii, a}$
3	5834 ± 687^{c}	21673 ± 1316$^{iii, c}$	4794 ± 365^{c}	17331 ± 521$^{iii, c}$	8593 ± 255^{c}	26990 ± 1002$^{iii, c}$
5	3818 ± 370^{c}	11025 ± 1076$^{iii, c}$	3321 ± 279^{c}	11748 ± 1482$^{ii, c}$	5298 ± 265^{c}	18350 ± 794$^{iii, c}$

PMA was added at the beginnig of the culture and was present until the end. Arithmetic means ± SEM of counts *per* minute are shown; the number of mice was 10 in all experiments. Paired Student *t*-test was used to compare no irradiated *vs*. irradiated cells (^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$), and presence *vs*. absence of PMA (^{i} $p < 0.05$, ^{ii} $p < 0.01$, ^{iii} $p < 0.001$). ConA, concanavalin A; PHA, phyto-haemoglutinin A; PMA, phorbol myristate acetate; LPS, lipopolysaccharide; PWM, pokeweed mitogens.

Table 5. Interaction index values obtained between differents mitogens and PMA in order to IR dose

IR	ConA (μg/ml)		PHA (μg/ml)	LPS (μg/ml)		PWM
(Gy)	1	3	10	40	80	2 %
0	0.0	0.0	0.3	0.6	0.7	0.4
1	0.2	0.0	0.4	0.6	0.5	0.7
3	0.9	0.1	1.5	1.2	0.9	1.1
5	1.2	0.3	3.0	0.9	1.2	1.5

The number of mice was 10 in all experiments. (For abbreviations see Table 4).

Discussion

We have analyzed the effects of IR *in vitro* on the lymphocyte proliferation of BALB /c mice treated with PMA with/without A23187 (5 min or 48 h) in two situations: in quiescent cells and in mitogen-stimulated cells.

In the PMA-treated quiescent cells, PMA significantly increases cell proliferation. The high DRF value confers it a radioprotector nature, which becomes more intense the higher the IR dose becomes. It is known that IR induces increased transcription of some genes in mammalian cells and that PMA induces the expression of certain genes, which have different effects on irradiated cells (Banda et al. 2008). Thus NFκB found in the cytosol in quiescent cells and after activation by PMA induces migrates to the nucleus, where it modulates the active genes of cell survival and apoptosis (Meng et al. 2002; Smith-Garvin et al. 2009; Khalaf et al. 2013). In addition, some authors have demonstrated in Syrian hamster embryo (SHE) fibroblasts and human lymphocytes that IR significantly increases the mRNA expression of PKC, and that this increase also becomes more marked with a higher dose (Woloschak et al. 1990; Sikpi et al. 1999). Thus, the effect of PMA on irradiated quiescent (1–5 Gy) cells can be explained if an increased expression of PKC, depending on IR dose, occurs and results in reduced apoptosis, leading to increased cell survival.

In the quiescent cells treated with PMA and A23187, a mitogenic effect occurs, which peaks when the treatment with A23187 is long. Moreover, synergism is also demonstrated, which becomes greater at higher IR doses. The inhibition produced by IR is less intense when cells are cultured in the presence of A23187 (48 h) and PMA than when stimulated with mitogens. This suggests that the inhibitory effect of IR on the mitogen-induced proliferation is not fundamentally due to the blocking of the signals mediating PKC activation; it is also necessary to increase [Ca^{2+}] for the proliferation of early events to take place (Smith-Garvin et al. 2009). Hence the effect of IR can be explained, at least in part, by a defect in the binding of mitogens to the cell membrane and/or by a transmission of their signals. These results agree with those of other authors (Woloschak et al. 1990; Varadkar and

Table 6. Effects of IR on lymphoproliferative response after mitogens stimulation and A23187 and/or PMA treatment

			IR (Gy)			
	A23187	PMA	0	1	3	5
ConA (1 μg/ml)	–	–	76030 ± 1876	81267 ± 10414	55462 ± 6030**	42168 ± 6932***
	5 min	–	100087 ± 684	61466 ± 6901***	38620 ± 5594***	20588 ± 4022***
	48 h	–	39352 ± 7675	26769 ± 3622*	12951 ± 1856**	11248 ± 1510**
	–	+	111482 ± 6340	105212 ± 13077***	75700 ± 5716**	63338 ± 4952***
	5 min	+	100137 ± 4891	89539 ± 9512	94024 ± 7331	69521 ± 7328*
	48 h	+	44299 ± 3131	30424 ± 2843**	30839 ± 5488*	24174 ± 4607**
ConA (3 μg/ml)	–	–	84455 ± 3041	77619 ± 3908	67075 ± 7208*	50756 ± 5797***
	5 min	–	108456 ± 4112	86030 ± 7470*	84186 ± 6357*	66004 ± 6423***
	48 h	–	37930 ± 5433	23965 ± 3466**	15057 ± 2268***	8818 ± 1180***
	–	+	106225 ± 3727	82917 ± 5198***	83811 ± 8834	67497 ± 2016***
	5 min	+	115297 ± 7770	107281 ± 10126	102407 ± 9641	75102 ± 10457***
	48 h	+	23296 ± 4290	21290 ± 4818	13206 ± 2199**	9185 ± 1882***
PHA (20 μg/ml)	–	–	56385 ± 3732	41138 ± 3030***	33023 ± 2196***	16336 ± 2063***
	5 min	–	90324 ± 8658	76989 ± 6668*	69253 ± 5918***	41208 ± 4089***
	48 h	–	26020 ± 4034	16459 ± 4968*	12451 ± 3875**	5641 ± 1248**
	–	+	94140 ± 403	77598 ± 5713*	73357 ± 5916***	44000 ± 4928*
	5 min	+	96698 ± 8786	94796 ± 5713	94075 ± 4752	70925 ± 7010*
	48 h	+	27083 ± 8036	23546 ± 7730	24764 ± 10299	19937 ± 6842**

Arithmetic mean ± SEM of counts *per* minute are shown; the number of mice was 10 in all experiments. Paired Student t-test was used to compare responses in irradiated *vs.* no irradiated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (For abbreviations, see Table 4).

Table 7. Interaction index values obtained between mitogens and A23187 treatments (5 min and 48 h).

IR	ConA (1 μg/ml)		ConA (3 μg/ml)		PHA (20 μg/ml)	
(Gy)	5 min	48 h	5 min	48 h	5 min	48 h
0	0.3	− 0.6	0.3	− 0.6	0.6	− 0.6
1	− 0.3	− 0.7	0.1	− 0.7	0.9	− 0.6
3	− 0.3	− 0.8	0.3	− 0.8	1.1	− 0.6
5	− 0.5	− 0.7	0.3	− 0.8	1.5	− 0.7

The number of mice was 10 in all experiments. (For abbreviations, see Table 4).

Krishna 2004; Khalaf et al. 2013), who used fibroblasts, lymphocytes Swiss mice and Jurkat T-cells. Thus, this suggests that calcium plays an important role in regulating protein trafficking and lymphocytes signaling.

In the cells treated with PMA and mitogens, PMA significantly enhances cell proliferation induced by mitogens of not only T-cells (ConA, PHA), which activate two different populations, but also of B-cells (LPS and PWM), which are much more radiosensitive. This synergism between mitogens and PMA increases as the IR dose becomes higher. This fact can be explained by considering that, and as demonstrated by other groups (Woloschak et al. 1999; Sikpi et al. 1999), IR can increase the mRNA expression of PKC in the splenocytes from stimulated BALB/c mice in a dose-dependent manner.

In the cells treated with mitogens, A23187 and PMA, the pre-treatment lasting 5 min with A23187, followed by a culture with mitogens and PMA, the proliferation induced by ConA or PHA is enhanced. Furthermore, this increase becomes more intense in the irradiated cells than in the controls. Yet the presence of the calcium ionophore throughout the culture leads to a marked inhibitory effect on the proliferation of the non-irradiated control cells. Therefore in our work, pretreatment with A23187 in irradiated cells gave a significantly higher response than in the controls, which made us think that an initial increase in calcium serves to modulate its effects on proliferation.

These results agree with others who have employed lymphocytes from BALB/c mice (Planelles et al. 1992; Hernández-Godoy et al. 2013) and Swiss mice splenocytes (Agrawal et al. 2003). Also, the fact that this inhibition can be reversed by PKC activators, such as A23187 and PMA, suggests that inhibition may be related to alterations in specific membrane receptors and/or that the signals leaving them can activate PKC.

Our previous studies (Hernández-Godoy et al. 2008, 2010) and others (Sharma et al. 2010) have shown that the lymphocytes of mice irradiated *in vivo* are more sensitive to radiation than those irradiated *in vitro*. Our experiments about the proliferation induced by PMA/A23187 indicate that the stimulation effect of PMA/A23187 is less marked in lymphocytes from *in vivo* irradiated animals than in lymphocytes irradiated *in vitro*, which indicates that the inhibition effects of IR *in vivo* on cell proliferation are more intense than these described now *in vitro*. It has been suggested that this increased radiosensitivity can be attributed to the signals entering from outside the spleen, possibly cytokines or chemokines (Sharma et al. 2010; Khalaf et al. 2013) and distinct kinases (tyrosine, PKC and MAP) behave differently according to the *in vivo* or *ex vivo* radiation (Varadkar and Krishna 2004). Finally it should be noted that, in the cells of the same animal exposed to increasing doses of radiation, the intensity of synergism increased with the intensity of radiation.

In short, ionizing radiation produces inhibition of the lymphoproliferative response, which can be partly reversed by two PKC activators, PMA and calcium ionophore that potentiate each other with increasing radiation doses. All this suggests that radiation causes an alteration in the cellular activation mechanisms mediated by PKC and cell membrane receptors.

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