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PHORBOL MYRISTATE ACETATE-ACTIVATED KERATINOCYTES STIMULATE PROLIFERATION OF RESTING PERIPHERAL BLOOD MONONUCLEAR LYMPHOCYTES VIA A MHC-INDEPENDENT, BUT PROTEIN KINASE C- AND INTERCELLULAR ADHESION MOLECULE-1-DEPENDENT, MECHANISM[1]

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Recent attention has focused on the role keratinocytes (KC) may play in the induction of T cell-mediated inflammatory responses in skin, particularly because KC, when activated by immunologic stimuli, express MHC class II Ag and secrete immunomodulatory cytokines. We tested the capacity of normal human KC that were stimulated with PMA to induce PBMC proliferation. PMA-treated, but not untreated, KC induced proliferation of allogeneic as well as autologous PBMC; in addition, when purified CD4$^+$ or CD8$^+$ T cells were used as responders, each subset proliferated. PBMC proliferation was not due to direct action of PMA on PBMC, nor to contamination of KC cultures with Langerhans cells (LC) or dermal APC. Pretreatment with different protein kinase C inhibitors abrogated the capacity of PMA-stimulated KC to induce proliferation. Paraformaldehyde-fixed PMA-KC stimulated PBMC proliferation, whereas supernatants from PMA-treated KC failed to do so, indicating that a membrane-associated activity on PMA-KC contributes to the induction of PBMC proliferation. PMA induced intercellular adhesion molecule-1 (ICAM-1) expression on KC; furthermore, mAb against ICAM-1 or against its ligand lymphocyte function-associated Ag (LFA-1) (CD11a/CD18) significantly, but incompletely, reduced the stimulatory capacity of PMA-treated KC, indicating that ICAM-1/LFA-1 interaction contributed to PBMC proliferation. IFN-γ or TNF-α also induced ICAM-1 on KC, but these KC failed to stimulate proliferation, suggesting that PMA induces additional signals from KC, which act in concert with ICAM-1 to promote proliferation. Finally, mAb against HLA-ABC or HLA-DR did not inhibit proliferation. We conclude that PMA can activate KC to stimulate T cell proliferation in a MHC-independent fashion. This activation is mediated by protein kinase C and in part by the induction of ICAM-1 expression on KC.

KC[3] make up the vast majority of cells within epidermis. These cells secrete a variety of cytokines which can modulate immune responses that occur in skin (1, 2). KC have also been shown to express MHC class II Ag in inflammatory dermatoses that are characterized by the presence of infiltrating lymphocytes (3, 4), raising the possibility that class II-Ag-bearing KC participate in the induction of T cell-mediated immune responses. However, normal KC that express class II MHC Ag after stimulation with IFN-γ are, at best, weak stimulators of alloreactive T cell lines (5, 6). In fact, such KC fail to induce proliferation of resting allogeneic lymphocytes (5–10). Furthermore, class II-positive, hapten-modified, mouse KC will induce tolerance in a hapten-specific CD4-positive T cell clone (11). Phorbol esters are established activators of PKC, a key enzyme of cellular signal transduction (12, 13). Their topical application on skin causes dermal edema, neutrophil influx, and epidermal hyperplasia (14, 15). Effects of phorbol esters on KC have been studied extensively and include activation of PKC and ornithine decarboxylase, increased release of arachidonic acid, PG, and IL-1, and the induction of terminal differentiation (14–17).

In this paper we characterize the influence of the phorbol ester PMA on the capacity of normal human KC to stimulate proliferation of resting PBMC. Our data indicate that PMA-treated KC can induce PBMC proliferation via a MHC-independent mechanism that appears to be regulated through PKC and is dependent on the concurrent induction of ICAM-1 expression on KC.

MATERIALS AND METHODS

Media, chemicals, and cytokines. Serum-free modified MCDB 153, KBM, was supplemented with bovine insulin (5 μg/ml), hydrocortisone (500 ng/ml), and murine epidermal growth factor (10 ng/ml) (all from Clonetics, San Diego, CA), and 1% penicillin-streptomycin (GIBCO, Grand Island, NY). RPMI 1640 (GIBCO) was supplemented with 10% heat-inactivated FCS, 25 mM HEPES (Sigma Chemical Co., St. Louis, MO), 1 mM nonessential amino acids (GIBCO), 2 mM L-glutamine (GIBCO), 1 nM 2-Me (Sigma), and 1% penicillin-streptomycin (GIBCO). MEM (GIBCO) was supplemented

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[3] Abbreviations used in this paper: KC, keratinocyte; EC, epidermal cells; ICAM-1, intercellular adhesion molecule-1; KBM, keratinocyte basal medium; LC, Langerhans cell; LFA-1, lymphocyte function-associated Ag; PKC, protein kinase C; SN, supernatant; H7, 1-(5-isoquinolinylsulfonyl)-methylpiperazine.

with 1.282 dornase U/ml DNAse I (ICN Biomedicals, Costa Mesa, CA), 10% FCS, and 1% penicillin-streptomycin (GIBCO). PMA, phorbol-12,13-dibutyrate, and *trans*-D-erythro-2-amino-4-octadecene-1,3-diol were all purchased from Sigma. The PKC inhibitors D-sphingosine, H7, and staurosporine were obtained from Sigma or Boehringer Mannheim Biochemicals (Indianapolis, IN), respectively. Radiolabeled PMA [20-^{3}H(H)] ([^{3}H]-PMA) (0.1 mCi/ml) was obtained from NEN (Boston, MA) and [^{3}H]TdR (6.7 Ci/mM) from ICN Radiochemicals (Irvine, CA). Human rIFN-γ (sp. act. 10^6 U/ml) and human rTNF-α (sp. act. 10^5 U/ml) were purchased from Genzyme Corp. (Boston, MA).

mAb. mAb are listed in Table I.

EC suspensions. Skin was obtained from recently deceased human cadavers (<24 h from the time of death, age 18 to 42 yr) (Skin Transplant Services Center, UT Southwestern Medical Center at Dallas), from neonatal foreskin discarded after circumcision (<24 h after operation), or from suction blisters raised on normal adult skin with a Dermovac suction device (Medko Medical, Espoo, Finland). Keratomized cadaver or neonatal skin was cut into 1 × 1 cm pieces, and floated in 0.5% dispase (Neutrale protease grade II, Boehringer Mannheim) for 60 min at 37°C; and epidermis was separated from dermis. Epidermal sheets or blister-roofs from suction blisters were placed in 0.3% trypsin (type XI, Sigma) in a buffer solution (0.17% glucose, 0.88% NaCl, and 0.04% KCl) for 20 min at 37°C. Stratum corneum, hair, and other debris were removed by filtration through Sera-Sepra columns (Evergreen Scientific, Los Angeles, CA). Disaggregated EC were then washed in MEM and resuspended in supplemented KBM. EC viability was estimated by trypan blue exclusion to range from 85 to 97%.

EC cultures and stimulation. Bulk EC suspended in supplemented KBM were seeded into 96-well, flat bottom plates (Corning Glass Works, Corning, NY) at 5 × 10^4 cells/dish. After 5 days of incubation at 37°C with 5% CO_2, cells were stimulated for 16 h with PMA (5 ng/ml), IFN-γ (300 U/ml), or TNF-α (500 U/ml) (all diluted in supplemented KBM). In some experiments KC were treated with PMA (5 ng/ml) for shorter time periods. The PKC inhibitors, D-sphingosine, H7, or staurosporine, were added to some panels at the indicated concentrations 3 min before stimulation with PMA (5 ng/ml). Cells incubated with supplemented KBM alone served as control. The viability of cells after 5 days of culture and 16 h of stimulation ranged from 50 to 80%, with no significant differences noted between the viability of control cultures and those stimulated with PMA, PKC-inhibitors + PMA, IFN-γ, or TNF-α.

Isolation of PBMC and T cell purification. PBMC were enriched from heparinized peripheral blood obtained from human volunteers by density centrifugation over 1.077 Histopaque (Sigma). Cells recovered from the interface were washed and resuspended in supplemented RPMI 1640. PBMC viability was >90%, as determined by trypan blue exclusion. T cells were purified as described elsewhere (18). Briefly, PBMC were layered on glass petri dishes (1 h, 37°C) and the nonadherent cells were rosetted with neuraminidase-treated SRBC and rosetting cells were isolated by density centrifugation. The SRBC were then lysed with isotonic ammonium chloride. Cells were passed over nylon wool columns (Robbins Scientific, Mountain View, CA). Residual monocytes, B cells, and NK cells were removed by panning with an array of mAb (B73.1, 63D3, L243; Table I). Finally, T cell populations were enriched for either $CD4^+$ or $CD8^+$ cells by panning with anti-CD8 or anti-CD4 mAb, respectively (Table I) (negative selection). The resulting $CD4^+$ or $CD8^+$ populations were >95% pure as determined by flow microfluorimetry analysis.

KC/PBMC coculture. KC were incubated for 16 h with PMA, D-sphingosine + PMA, IFN-γ, or TNF-α and then washed five times to prevent carryover into the subsequent cocultures with PBMC. Initially, KC were γ-irradiated (3000 rad, ^{137}Cs); however, because γ-irradiated and unirradiated KC showed the same background proliferation and acted equally as stimulators of PBMC proliferation, γ-irradiation was omitted after several experiments. PBMC were added to KC cultures at 5 × 10^4 cells/dish and incubated for 48 h at 37°C, 5% CO_2; all cocultures were performed in supplemented RPMI 1640. Cocultures were pulsed for an additional 18 h with [^{3}H]TdR (1 μCi/well) and then collected onto nitrocellulose filter paper with a MASH II cell harvester. Incorporated [^{3}H]TdR was determined by liquid scintillation spectroscopy. All cultures were carried out in triplicate and results were calculated as the mean cpm ± SD. Data are expressed as Δ cpm (mean cpm of cocultures containing both KC and PBMC minus the sum of mean cpm for KC and PBMC cultured separately). In blocking experiments, mAb against adhesion molecules, MHC-Ag, or appropriate isotype control mAb were added to cocultures at the indicated concentrations, remaining in the cocultures throughout the incubation period; alternatively, PMA-KC were preincubated with mAb for 2 h at 4°C and washed extensively before coculture with PBMC.

KC supernatants. After PMA stimulation KC were washed five times and then incubated for another 48 h in supplemented KBM, or RPMI 1640. SN from PMA-treated panels were collected and added to PBMC (5 × 10^4 cells/dish) at a final dilution of 1/4; SN from untreated KC served as the control. After 48 h of incubation at 37°C and 5% CO_2, PBMC proliferation was assessed by measuring [^{3}H]TdR incorporation as described above.

Fixation of KC. After stimulation with PMA (16 h, 5 ng/ml), KC were fixed with paraformaldehyde with the technique described by Kurt-Jones et al. (19). Briefly, KC were incubated with selected concentrations of paraformaldehyde at room temperature for 15 min, washed four times in HBSS, resuspended in fresh media, and then incubated at 37°C, 5% CO_2, for 4 h to allow release of residual paraformaldehyde or soluble cytokines from the fixed cells. Thereafter, KC were washed twice with HBSS and then cocultured with PBMC in RPMI 1640 as described above. Alternatively, KC were fixed in a modified procedure as described by Moreno et al. (20); briefly, KC were incubated with selected concentrations of paraformaldehyde for 1 h at 4°C, after which the reaction was stopped by the addition of cold 0.06% glycyl-glycine (Aldrich Chemical Co., Milwaukee, WI). Thereafter, KC were washed in HBSS and then cocultured with PBMC as described above. To assure that our fixation protocol was effective, we tested whether it would inhibit KC proliferation. After fixation, untreated or PMA-stimulated KC were resuspended in KBM and seeded at 2.5 × 10^4 cells/dish into 96-well/flat bottom microtiter plates. KC proliferation was determined at 96 h by [^{3}H]TdR incorporation.

[^{3}H-N]PMA pulsing of keratinocytes. We wished to exclude the possibility that significant amounts of PMA were carried over from the KC cultures into the cocultures. EC, cultured for 5 days in supplemented KBM (96 well plate, 10^4 cells/dish), were incubated with radiolabeled [^{3}H-N]PMA (5 ng/ml) for 16 h, and then washed five times. KC were collected onto nitrocellulose filter paper with a MASH II cell harvester and the amount of residual [^{3}H-N]PMA was determined by liquid scintillation spectroscopy (see above). Results are expressed as mean cpm ± SD of 18 wells. The concentration of residual PMA was calculated by comparing cpm obtained from such KC cultures after five washes with standards of known [^{3}H-N]PMA concentrations.

Immunofluorescence staining. EC suspended in supplemented KBM were seeded into 6-well, flat bottom wells (Costar, Cambridge, MA) at 3 × 10^6 cells/dish. After 5 days of culture (37°C, 5% CO_2) cells were stimulated for 16 h with PMA (5 ng/ml), D-sphingosine (30 μM) + PMA (5 ng/ml), IFN-γ (300 U/ml), TNF-α (500 U/ml), or KBM alone. Cells were harvested with a rubber policeman and washed three times with supplemented HBSS. Viability, as determined by trypan blue exclusion, ranged from 50 to 80%. Cells were aliquoted at 3 to 6 × 10^5 into polystyrene tubes and stained for 20 min at 4°C, first with several different primary antibodies (Table I) and then with FITC-conjugated F(ab')$_2$ fragments of rabbit anti-mouse IgG secondary antibody (Zymed Laboratories, Inc., San Francisco, CA), diluted 1/40. As controls, cells were labeled with an irrelevant mAb of the same isotype followed by the secondary antibody. Cells were analyzed by flow cytometry using a FACSstar/Consort 30 (Becton Dickinson, Sunnyvale, CA). Propidium iodide (100 μg/ml) was added to each sample to exclude dead cells.

TABLE I
mAb

Specificity	Clone	Isotype	Source[a]
CD11a	25.3.1.	mIgG1	AMAC
CD18	BL 5	mIgG1	AMAC
ICAM-1	84 H 10	mIgG1	AMAC
CD45	HLe-1	mIgG1	BD
CD1a	OKT6	mIgG1	ORTHO
CD14	63.D.3.	mIgG1	ICN
CD4	OKT4	mIgG1	ORTHO
CD8	OKT8	mIgG1	ORTHO
HLA-A, B, C	W6/32	mIgG2a	ICN
HLA-DR	L234	mIgG2a	BD
CD16	B73.1	mIgG1	ICN
Keratins:			
I (40, 48, 50K) II (58, 65, 67K)	AE2:AE3	mIgG1	ICN

[a] AMAC, AMAC, Inc., Westbrook, ME; BD, Becton Dickinson, Sunnyvale, CA; ORTHO, ORTHO Diagnostics, Inc., Raritan, NJ; and ICN, ICN ImmunoBiologicals, Lisle, IL.

RESULTS

PMA-treated KC stimulate proliferation of PBMC. Normal human KC, grown in supplemented KBM for 6

days, failed to induce proliferation of allogeneic PBMC (Fig. 1*A*). Indeed, PBMC proliferation observed after 48 h of coculture with unstimulated KC did not exceed background proliferation of either KC or PBMC cultured alone. However, KC stimulated with PMA (5 ng/ml) for 16 h, washed extensively (five times) to minimize PMA-carryover, and then cocultured with allogeneic PBMC, acquired the capacity to induce strong PBMC proliferation. Experiments with shorter PMA stimulations revealed that although a 4-h pulse with PMA was sufficient to enable KC to induce PBMC proliferation (Table II), optimal induction of PBMC proliferation occurs after 16 h of PMA stimulation; thus, in all subsequent experiments KC were PMA-stimulated for 16 h. To determine whether proliferation was due to an allogeneic response, PMA-treated KC were cocultured with autologous PBMC (Fig. 1*B*). Again, PMA-stimulated KC induced substantial proliferation of autologous PBMC, whereas unstimulated KC had no effect on PBMC proliferation.

Proliferation of PBMC is not due to residual PMA on KC. We considered the possibility that five washes did not completely remove PMA from the KC surface or from the wells thereby leading directly to PBMC proliferation. To address this issue, we determined residual PMA on KC by incubating KC for 16 h with the radioactive probe [20-^{3}H(N)]PMA (5 ng/ml) and washing five times. The recovered radioactivity was compared with known concentrations of labeled PMA and was just above the level of detectibility (Fig. 2*A*). Realizing that we are operating at the lowest level of sensitivity in our assay, the amount of residual PMA on KC was calculated to be less than 5 $\times 10^{-4}$ ng/ml (0.001% of the original concentration). To test whether such small concentrations of PMA would induce proliferation of PBMC, a dose response curve for [20-^{3}H(N)]PMA was generated. No proliferation of PBMC from two different donors was observed at a concentration of 5×10^{-4} ng/ml; in fact, PBMC proliferation rose only above background levels at PMA concentrations 3 logs higher (Fig. 2*B*).

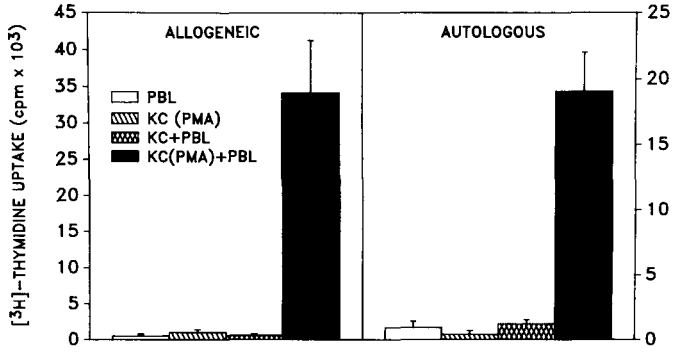

Figure 1. PMA-treated KC stimulate proliferation of PBMC. KC were cultured for 16 h in supplemented KBM with or without PMA (5 ng/ml), washed five times, and cocultured at 5×10^4 cells/dish with allogeneic or autologous PBMC (5×10^4 cells/dish). After 60 h, PBMC proliferation was measured by [^{3}H]TdR incorporation. Bars represent the means of triplicate measurements of cpm ± SD. These results are representative of 15 separate experiments which used 20 different sources of KC and 10 different sources of PBMC.

TABLE II

Effects of the duration of PMA stimulation on the ability of PMA-KC to induce PBMC proliferation[a]

PMA Exposure (h)	PBMC Proliferation (% of control)
16	100
6	75
4	69
2	13

[a] KC were stimulated with PMA (5 ng/ml) for the indicated time periods, washed extensively, and then cocultured with PBMC as described in Figure 1. PBMC proliferation was determined at 72 h by [^{3}H]TdR incorporation. All measurements were performed in triplicate and data are expressed as percentage of positive control (proliferation induced by KC stimulated with PMA for 16 h; Δ cpm = 9477 ± 732). Results are representative of two separate experiments.

KC cultures are not contaminated by epidermal or dermal APC. To rule out the possibility that PBMC proliferation was caused by "contaminating" bone marrow-derived APC such as epidermal LC or dermal dendritic cells, we determined the number of such APC in our KC cultures by immunofluorescence staining, by using an array of mAb (Tables I and III). EC cultured for 5 days in supplemented KBM and then incubated for an additional 16 h in KBM alone or KBM supplemented with either PMA or D-sphingosine + PMA, contained less than 0.5% LC (HLA-DR$^+$, CD1a$^+$, CD45$^+$) or dermal APC (CD45$^+$, 63D3$^+$, HLA-DR$^+$) (Table III). In IFN-γ-stimulated cultures approximately 4% of cells expressed HLA-DR but less than 0.5% were CD1a$^+$, CD45$^+$, 63.D.3$^+$, indicating that most of these cells were HLA-DR$^+$ KC rather than LC or dermal APC. Furthermore, more than 95% of cells stained with the keratin-specific markers AE1:AE3 (Table III). We conclude from these experiments that PBMC proliferation is induced by PMA-activated KC rather than by other epidermal or dermal bone marrow-derived cells. Further support for this conclusion derives from our observation that flow microfluorimetry-purified or second passage KC pretreated with PMA induced PBMC proliferation to a similar degree as EC grown in KBM for 5 days (data not shown).

The capacity of PMA-treated KC to induce proliferation of PBMC is PKC-dependent. Because PMA is a potent activator of PKC (12, 13), we questioned whether the ability of PMA-treated KC to stimulate PBMC proliferation was mediated by PKC. Pretreatment of cultures with each of three PKC inhibitors, H7, staurosporine, or D-sphingosine, reduced the capacity of PMA-treated KC to stimulate PBMC proliferation in a dose-dependent manner (Table IV). A cytotoxic effect on KC was excluded on the basis of comparable viabilities for KC after treatment with PMA or PKC-inhibitors plus PMA. These findings indicate that PMA treatment confers upon KC the capacity to stimulate PBMC proliferation through a PKC-dependent mechanism.

SN from PMA-treated KC do not induce PBMC proliferation. We next asked whether PMA-treated KC released soluble factors that stimulate PBMC proliferation. To address this question, we tested the capacity of SN from PMA-activated KC, to induce PBMC proliferation. No differences in proliferation were observed, whether PBMC were cultured for 48 h in SN from either PMA-stimulated, or unstimulated KC or in media alone (Fig. 3).

Paraformaldehyde-fixed PMA-KC retain their capacity to induce PBMC proliferation. The previous experiments were consistent with the possibility that soluble cytokines released from PMA-treated KC alone were not sufficient to induce PBMC proliferation. To test this hypothesis, we prevented the release of soluble cytokines from PMA-KC by fixing these cells with paraformaldehyde before coculture with PBMC. Fixed PMA-KC were fully capable of inducing PBMC proliferation (Table V).

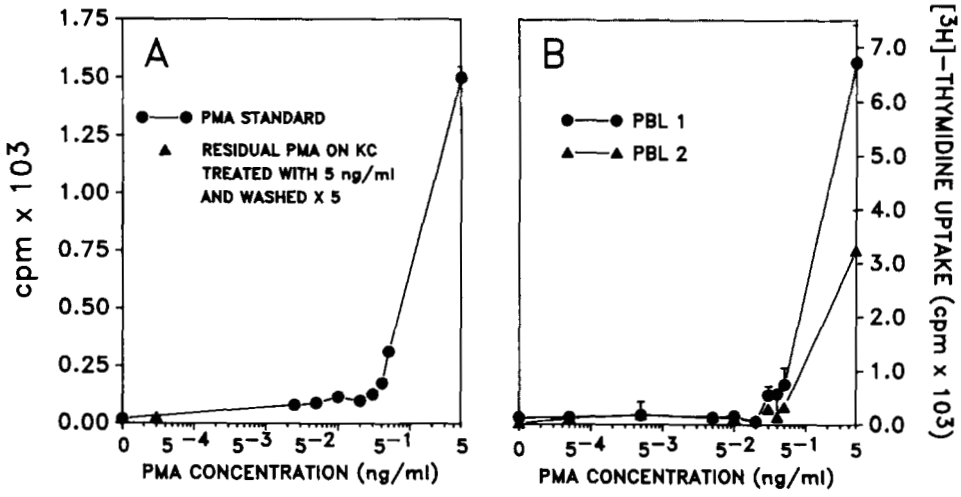

Figure 2. Residual PMA does not directly cause PBMC proliferation: *A*, KC were incubated for 16 h with [^{3}H]PMA (5 ng/ml) and then washed five times. Residual [^{3}H]PMA (▲) was measured by liquid scintillation spectroscopy. The concentration of residual PMA ($<5 \times 10^{-4}$ ng/ml) was calculated by comparison with a standard of known PMA concentrations (●). *B*, Dose response curve for [^{3}H]PMA. PBMC from donor 1 (●) or donor 2 (▲) (5×10^4 cells/dish) were incubated with varying concentrations of [^{3}H]PMA. After 60 h proliferation was assessed by [^{3}H]TdR incorporation. PBMC proliferation reached background levels at a concentration of 0.2 ng/ml. Each data point represents the mean of 18 measurements of cpm ± SD, representative of three separate experiments.

TABLE III

KC cultures are not contaminated by LC or dermal APC[a]

mAb against	Pretreatment of KC			
	Media	PMA	IFN-γ	TNF-α
HLA-DR	0.1	0.3	4.1	1.3
CD1a	0	0	0	0
CD45	0.5	0.6	0.3	0.5
63.D.3	0	0.1	0	0.2
AE1:AE3	95	95	94	97

[a] KC were treated for 16 h with or without PMA (5 ng/ml), IFN-γ (300 U/ml), TNF-α (500 U/ml), washed, and then stained with the listed mAb and analyzed by flow cytometry. Data are expressed as percentage of positive cells.

TABLE IV

Capacity of PMA-treated KC to induce PBMC proliferation is PKC-dependent

PKC Inhibitor	Concentration (μM)	Percent Inhibition of PBMC Proliferation
None		0
H7	2	38
	20	69
	100	92
Staurosporine	2	58
	20	69
	100	96
D-Spingosine	0.3	30
	3	45
	30	88

[a] The PKC inhibitors were added to KC cultures at the indicated concentrations 3 min before PMA stimulation (5 ng/ml). After 16 h, KC were washed five times and then cocultured at 5×10^4 cells/dish with autologous PBMC (5×10^4 cells/dish) and proliferation was determined after 60 h. Data are expressed as percentage inhibition of positive control (proliferation induced by PMA-treated KC without sphingosine, 15,120 ± 1,190 cpm). Identical treatment of KC that had not been stimulated with PMA did not alter their inability to induce PBMC proliferation (data not shown). Results are representative of three separate experiments.

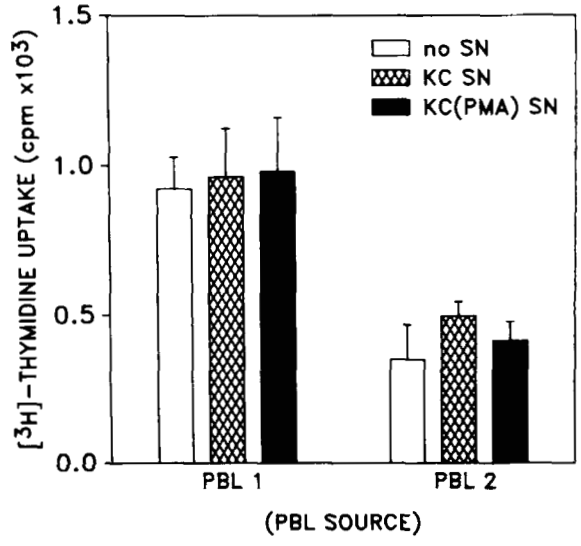

Figure 3. SN from PMA-treated KC do not induce PBMC proliferation: KC were cultured for 16 h in supplemented KBM with or without PMA (5 ng/ml), washed five times, and then incubated in fresh media. After 48 h SN were harvested and added to PBMC (5×10^4 cells/dish) of two different donors at a final dilution of 1/4, and proliferation was determined after 60 h. Bars represent the means of triplicate measurements of cpm ± SD. Positive control: PBMC cocultured with PMA-treated KC, proliferated vigorously (cpm donor 1: 25,340 ± 1,540; cpm donor 2: 15,050 ± 780). Data are representative of three separate experiments.

To confirm the effectiveness of our fixation protocol, we examined its effect on KC proliferation. Both fixation protocols inhibited KC proliferation in a dose-dependent manner (Table V). These results suggested that the ability of PMA-KC to induce PBMC proliferation requires a membrane-bound activity and is not solely due to release of a soluble factor.

PMA induces ICAM-1 on KC. We observed by light microscopy that PMA-stimulated, but not unstimulated KC would form clusters with PBMC (data not shown). Because binding of PBMC to KC may be mediated by the interaction of ICAM-1 on KC with LFA-1 on PBMC (21–23), we questioned whether PMA could induce ICAM-1 expression on KC. As shown in Figure 4, treatment with 5 ng/ml of PMA in KBM (16 h) did induce ICAM-1 expression in approximately 16% of KC; no ICAM-1 expression was observed in KC treated with KBM alone.

mAb against ICAM-1 block the capacity of PMA-treated KC to induce proliferation of PBMC. To evaluate whether the interaction of PMA-induced ICAM-1 on KC with its ligand LFA-1 (CD11a/CD18) on lymphocytes contributed to PBMC proliferation, mAb against these adhesion molecules were employed to block proliferation induced by PMA-treated KC. Treatment of cocultures with mAb against ICAM-1, CD11a, or CD18 reduced proliferation of allogeneic PBMC in a dose-dependent manner (Fig. 5). In these experiments mAb against CD11a were most effective (78% inhibition) followed by anti-ICAM-1 (74%) and anti-CD18 (61%) (Fig. 5). An irrelevant control mAb of the same isotype did not cause a significant reduction of PBMC proliferation, ruling out nonspecific blocking effects by mouse IgG1 mAb. Similarly, mAb

TABLE V

Paraformaldehyde-fixed PMA-KC retain their capacity to induce PBMC proliferation[a]

Fixation Protocol	Paraformaldehyde Concentration (%)	Percent of Positive Control	
		PBMC proliferation	KC proliferation
15 min at room temperature	0	100	100
	0.125	86	95
	0.25	103	89
	0.5	95	56
	1	110	41
1 h at 4°C	0	100	100
	0.125	86	80
	0.25	81	65
	0.5	86	42
	1	95	38

[a] After stimulation with PMA (16 h, 5 ng/ml), KC were fixed with paraformaldehyde (by using protocols described in *Materials and Methods*), and then cocultured with PBMC as described in Table III. PBMC proliferation was determined at 72 h of coculture by [^{3}H]TdR incorporation. All measurements were performed in triplicate and data are expressed as percentage of positive control (PMBC proliferation induced by PMA-KC without paraformaldehyde fixation, 15,120 ± 1,190 cpm). To measure KC proliferation after fixation, KC were resuspended in KBM and seeded at 2.5×10^4 cells/dish in 96-well/flat bottom microtiter plates. KC proliferation was determined at 96 h by [^{3}H]TdR incorporation. Data represent the mean of six separate measurements and are expressed as percentage of positive control (proliferation of KC without paraformaldehyde fixation, 4,350 ± 120). Results are representative of two separate experiments.

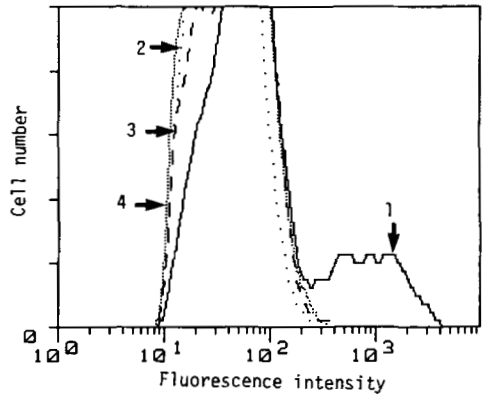

Figure 4. PMA induces ICAM-1 expression on KC. KC were cultured for 16 h in supplemented KBM with or without PMA (5 ng/ml), stained with mAb against ICAM-1, and analyzed by flow cytometry. *1*, PMA-treated KC, anti-ICAM-1; *2*, PMA-treated KC, control; *3*, untreated KC, anti-ICAM-1; *4*, untreated KC control. PMA (5 ng/ml) induced ICAM-1 expression on approximately 16% of KC. Data are representative of three separate experiments.

against adhesion molecules also reduced the capacity of PMA-activated KC to stimulate proliferation of autologous T cells (Fig. 5). Again, anti-CD11a caused the most marked inhibition of proliferation (77%), followed by anti-ICAM-1 (64%) and anti-CD18 (63%), whereas control IgG1 mAb had no significant effect (Fig. 5). To confirm ICAM-1 on PMA-KC as the target of the blocking mAb, we selectively preincubated PMA-KC with anti-ICAM-1 mAb before coculture with PBMC. This treatment resulted in a significant inhibition of PBMC proliferation (Table VI); inhibition was more pronounced when the mAb was present continuously throughout the coculture. The latter observation is most likely due to the longer incubation period with anti-ICAM-1. However, we cannot exclude the possibility that the mAb, when present continuously throughout the coculture, also blocks ICAM-1-mediated homotypic interaction of T cells occurring during late stages of coculture. These findings suggest that the capacity of PMA-treated KC to stimulate PBMC proliferation is mediated in part by the interaction of ICAM-1 on KC with LFA-1 on PBMC.

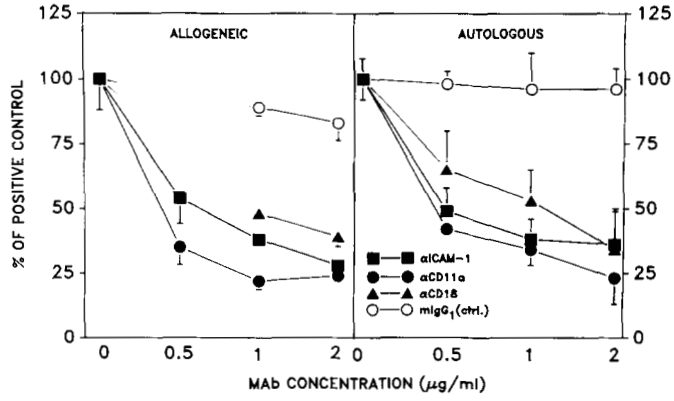

Figure 5. mAb against adhesion molecules block the capacity of PMA-treated KC to induce proliferation of PBMC. KC were cultured for 16 h in supplemented KBM with or without PMA (5 ng/ml), washed, and cocultured at 5×10^4 cells/dish with either allogeneic or autologous PBMC (5×10^4 cells/dish). mAb at the indicated concentrations were added to the cultures together with PBMC. After 60 h, PBMC proliferation was assessed. All cultures were performed in triplicate. Data are expressed as percentage of positive control (proliferation induced by PMA-treated KC in the absence of mAb) ± SD, obtained from three separate experiments.

TABLE VI

ICAM-1 expressed on PMA-KC is the target for the blocking effects of anti-ICAM-1 mAb[a]

mAb	Blocking Protocol	mAb Concentration (μg/ml)	Percent Inhibition
Anti-ICAM-1	Continuous	0.25	52
		1	79
		5	96
Control IgG1		5	12
Anti-ICAM-1	Preincubation	0.25	13
		1	24
		5	59
Control IgG1		5	9

[a] Anti-ICAM-1 mAb or appropriate isotype controls were added together with PBMC at the indicated concentrations and were present continuously throughout the incubation period; alternatively PMA-KC were preincubated with mAb for 2 h at 4°C, washed extensively, and thereafter cocultured with PBMC. KC-PBMC cocultures were performed as described in Figure 1; PBMC proliferation was measured at 72 h by [^{3}H]TdR incorporation. All measurements were performed in triplicate and data are expressed as percentage of positive control (proliferation induced by PMA-KC without mAb blocking; Δ cpm = 12,420 ± 590). Results are representative of two separate experiments.

IFN-γ and TNF-α induce ICAM-1 expression on KC but do not confer upon such KC the capacity to induce PBMC proliferation. The demonstrated role of ICAM-1 on PMA-treated KC for the induction of PBMC proliferation prompted us to ask whether treatment of KC with other inducers of ICAM-1 would also cause proliferation. IFN-γ (300 U/ml), TNF-α (500 U/ml), and PMA (5 ng/ml) each induced ICAM-1 expression on KC (11.2%, 7.0%, and 12.4%, respectively), whereas only PMA induced PBMC proliferation (Table VII). Moreover, IFN-γ, TNF-α, or PMA each induced comparable amounts of ICAM-1 per cell, as judged by the mean fluorescence intensity of the stained cells, and cytotoxic effects of IFN-γ or TNF-α on KC were excluded by comparable KC viabilities (data not shown). We conclude that, although ICAM-1 evidently contributes to PBMC proliferation, ICAM-1 expression on KC alone is not sufficient to stimulate this response. We propose that PMA (but not IFN-γ or TNF-α) generates additional costimulatory signals which, in concert with the signal delivered by ICAM-1/LFA-1 induces optimal proliferation.

PMA-treated KC stimulate PBMC proliferation via a

TABLE VII

IFN-γ or TNF-α induces ICAM-1 on KC but fails to induce PBMC proliferation[a]

Treatment of KC	Positive Cells (%)		PBL Proliferation (Δ cpm ± STD)
	ICAM-1	HLA-DR	
None	0	0.1	22 ± 17
IFN-γ	11.2	1.1	182 ± 42
TNF-α	7	1.5	12 ± 16
PMA	12.4	0.3	5020 ± 170

[a] KC were cultured for 16 h with or without PMA (5 ng/ml), IFN-γ (300 U/ml), TNF-α (500 U/ml). One part was stained with the listed mAb and analyzed by flow cytometry (data expressed as percentage of positive cells). The other part was cocultured with allogeneic PBMC. After 60 h of coculture, PBMC proliferation was assessed by [^{3}H]TdR incorporation (Δ cpm was calculated from means of triplicate measurements ± SD).

TABLE VIII

PMA-treated KC stimulate PBMC proliferation via a MHC-independent mechanism[a]

mAb against	Activity (cpm ± SD) at:			
	0.5 μg/ml	1 μg/ml	2 μg/ml	10 μg/ml
None	7690 ± 450[b]			
HLA-A, B, C	7190 ± 140	8720 ± 830	7780 ± 300	7520 ± 70
HLA-DR	7870 ± 290	8140 ± 2430	7055 ± 430	7980 ± 410
Control IgG	7500 ± 1290	8660 ± 2811	6410 ± 1410	7230 ± 150

[a] KC were cultured for 16 h in supplemented KBM with or without PMA (5 ng/ml), washed and cocultured at 5 × 10^4 cells/dish with allogeneic PBMC (5 × 10^4 cells/dish). mAb (listed in Table I) were added to the cultures together with PBMC at the indicated concentrations; such mAb effectively blocked proliferation in an allogeneic MLR or mixed epidermal cell leukocyte reaction. After 60 h, PBMC proliferation was assessed.

[b] Represents PBMC proliferation induced by PMA-treated KC in absence of mAb. Data are expressed as cpm ± SD obtained from triplicate measurements.

MHC-independent mechanism. To determine whether the capacity of PMA-treated KC to stimulate PBMC proliferation is MHC-restricted, we tested whether mAb against HLA-A,B,C or HLA-DR would inhibit this response. mAb against either MHC class I or class II molecules, which effectively blocked an allogeneic MLR or allogeneic mixed EC leukocyte reaction (data not shown), did not affect the ability of PMA-treated KC to stimulate proliferation of allogeneic PBMC (Table VIII).

PMA-treated KC stimulate proliferation of either CD4$^+$ or CD8$^+$ T cells. To begin to elucidate which cells within the PBMC fraction are activated by PMA-treated KC, we asked whether the responding cells were of T cell lineage. KC were cocultured with unfractionated PBMC or with CD4$^+$ or CD8$^+$ cells that had been purified by negative selection panning (Table IX). When stimulated with PMA-activated KC, the proliferative responses of CD4$^+$ and CD8$^+$ cells were significantly enhanced over that of unfractionated PBMC (Table IX). Again, untreated KC did not stimulate proliferation. These results suggest that PMA-treated KC have the capacity to activate resting T cells. Furthermore, PBMC, CD4$^+$ and CD8$^+$ T cells did not proliferate when treated with PMA (5 ng/ml, 48 h) in the absence of KC (Table IX). This finding further supports our contention that the described T cell proliferation is due to PMA effects on KC rather than to direct effects of trace amounts on T cells.

TABLE IX

PMA-treated KC stimulate proliferation of CD4$^+$ and CD8$^+$ T cells[a]

KC	Responder Cells	Stimulation	Activity (cpm ± SD)
+	None	Media	180 ± 80
+	PBMC	Media	350 ± 130
+	CD4	Media	160 ± 100
+	CD8	Media	190 ± 30
+	None	PMA	750 ± 60
+	PBMC	PMA	4,060 ± 890
+	CD4	PMA	11,180 ± 1,290
+	CD8	PMA	13,140 ± 4,850
−	PBMC	Media	370 ± 30
−	CD4	Media	300 ± 40
−	CD8	Media	140 ± 20
−	PBMC	PMA[b]	870 ± 160
−	CD4	PMA	870 ± 70
−	CD8	PMA	550 ± 150

[a] KC were cultured for 16 h in supplemented KBM with or without PMA (5 ng/ml), washed five times and then cocultured at 5 × 10^4 cells/dish with allogeneic unfractionated PBMC or purified CD4$^+$ or CD8$^+$ T cells (5 × 10^4 cells/dish). After 72 h, PBMC proliferation was assessed by [^{3}H]TdR incorporation. Data are expressed as cpm ± SD obtained from triplicate measurements.

[b] Alternatively, responder cells were cultured without KC in the continuous presence of PMA (5 ng/ml).

DISCUSSION

KC secrete immunomodulatory cytokines (1, 2) and express MHC class II Ag in inflammatory skin diseases (3, 4) and after in vitro treatment with IFN-γ (24, 25); this has led to the hypothesis that KC may possess the capacity to induce and modulate T cell-mediated immune responses. IFN-γ-stimulated, class II-positive KC have been shown to induce proliferation of highly activated alloreactive T cells or of allogeneic PBMC in the presence of added IL-2 (5, 6, 8). However, such KC are incapable of stimulating the proliferation of resting lymphocytes (5–10). In fact, class II positive-KC, conjugated with hapten, have been shown to induce long-lasting unresponsiveness in a hapten-specific murine CD4$^+$ clone of the TH$_1$ type (11). In the present study we demonstrate that PMA-treated KC can induce proliferation of resting T cells. This capacity is attributable to an effect of PMA on KC and not to direct PBMC activation by PMA nor to contamination of KC cultures with other APC such as LC or dermal dendrocytes.

Effects of tumor-promoting phorbol esters, such as PMA, on KC have been studied extensively. Topical application of phorbol esters to skin produces histologic alterations: dermal edema, an influx of neutrophils, and epidermal hyperplasia. These, in turn, are associated with biochemical alterations: activation of PKC and ornithine decarboxylase, release of arachidonic acid, PG and IL-1, and induction of terminal differentiation by KC (14–17). Based on in vitro responses to phorbol esters, two populations of KC have been identified: a major population that undergoes growth arrest and terminal differentiation, and a smaller population that proliferates markedly, without differentiation (16, 26–31). Our working hypothesis is that this latter population, which retains features of basal KC and which is highly active metabolically, is responsible for inducing proliferation of PBMC.

Phorbol esters activate PKC, a calcium- and phospholipid-dependent serine/threonine kinase that plays a central role in signal transduction (12, 13). Not only has PKC been isolated from epidermis (17), several phorbol-induced changes in skin are also antagonized by the PKC inhibitor D-sphingosine (14). In the present study, three different PKC inhibitors (H7, staurosporine, D-sphingosine) each abrogated the capacity of PMA-activated KC to induce PBMC proliferation in vitro, indicating that prolif-

eration is most probably mediated through PKC. Fisher et al. (17) showed that PMA-stimulation of KC leads to an initial increase in their PKC activity followed by a rapid down-regulation. We have yet to determine whether the capacity of PMA-KC to induce PBMC proliferation results from up-regulation or down-regulation of PKC activity.

Treatment with phorbol esters will transform KC from a "resting" to an "activated" state in which they express additional IL-1R and increase their release of IL-1 (2, 32, 33). Because binding of IL-1 to IL-1R induces KC to secrete relevant amounts of biologically active cytokines (1, 32, 34–38), we tested culture SN from PMA-treated KC for their capacity to induce PBMC proliferation. SN from PMA-treated KC failed to stimulate PBMC proliferation; furthermore, paraformaldehyde-fixed PMA-KC were fully capable of inducing PBMC proliferation. These results suggest that a membrane-associated activity on PMA-KC is important to trigger PBMC proliferation, and that KC-derived soluble cytokines alone are not sufficient. However, these findings do not exclude the possibility that soluble cytokines amplify other activation signals, or that membrane-bound cytokines play a role in PBMC activation.

Recent reports indicate that KC bind T cells via the interaction of ICAM-1 (on KC) with the LFA-1 heterodimer (CD11a/CD18) (on lymphocytes) (21–23). In addition to promoting cell adhesion, the interaction of ICAM-1 with LFA-1 may also deliver costimulatory signals which are required for efficient T cell activation (39–48). PMA-induction of ICAM-1 expression on KC has been shown by several laboratories (49) including our own. This finding encouraged us to ask whether the interaction of ICAM-1 on KC with LFA-1 on lymphocytes contributed to PBMC proliferation. Indeed, addition of mAb against ICAM-1, CD11a, or CD18 to KC/PBMC cocultures inhibited significantly the proliferation of allogeneic and autologous PBMC, suggesting that the ICAM-1/LFA-1 complex does deliver costimulatory signals. Furthermore, selective blocking of ICAM-1 on PMA-KC also inhibited PBMC proliferation, confirming that ICAM-1 on PMA-KC was indeed the target for the blocking. However, since PBMC proliferation was never abrogated completely, it remains possible that not all ligands involved in LFA-1- or ICAM-1-mediated signaling (i.e., ICAM-2 for LFA-1 (50), or both CD11a and CD18 for ICAM-1) were blocked, or that PMA induces an additional, yet unidentified, activation signal that is responsible for this residual proliferation.

To investigate whether the capacity to induce PBMC proliferation was a general property of ICAM-1-positive KC, we examined other known inducers of ICAM-1 (21, 51–53). Although IFN-γ, TNF-α and PMA each induced ICAM-1 expression on KC, only PMA-treatment led to PBMC proliferation: this disparity could not be attributed to differences in the number of ICAM-1-positive KC, in the amount of ICAM-1 per KC, or in cell survival. Thus, ICAM-1 expression on KC is necessary, but not sufficient, to induce PBMC proliferation.

Several explanations may account for this unique effect of PMA. First, PMA itself may contribute to lymphocyte activation, by delivering a second signal that acts in concert with the ICAM-1/LFA-1 interaction. Indeed, immobilized mAb against LFA-1 has been reported to stimulate T cells when PMA is also present at a concentration of 10 ng/ml (54). Although only $<5 \times 10^{-4}$ ng/ml of residual PMA were found in our KC cultures, it remains possible that small amounts of PMA, bound to KC membranes, could provide an additional activation signal. To address this question by a different method, we tested whether KC treated with PDB (a hydrophilic phorbol ester, which can be completely removed from cell membranes) would also stimulate PBMC proliferation. Unfortunately, over a broad dose range (0.05 to 50 ng/ml) PDB was cytotoxic to KC (cell viability <18%) and KC treated in this way failed to stimulate PBMC (data not shown). Thus, at present we are unable to exclude further the possibility that trace amounts of membrane-bound PMA contribute to PBMC activation. Second, PMA may produce a unique conformational change within the ICAM-1 molecule, which enhances its capacity to deliver costimulatory signals. Such PMA-induced changes have been reported for the LFA-1 molecule (55–57). Third, PMA may be unique in producing aggregation of cell surface ICAM-1 molecules, thus enhancing the capacity to cross-link with LFA-1 and to deliver costimulatory signals. This possibility is also not without precedent; PMA and FMLP both increase CD11b expression on neutrophils, but only PMA induces aggregation of CD11b molecules, which in turn mediates clustering and activation (57, 58). Finally, PMA alone may trigger a second signal in the form of an as yet unidentified cell surface ligand or a membrane bound cytokine, which acts in concert with ICAM-1 to induce lymphocyte proliferation.

Our studies also demonstrate that PMA-treated KC induce PBMC proliferation via a MHC class I and II-independent mechanism, because mAb against HLA-ABC and HLA-DR failed to block PBMC proliferation. The related finding that PMA-treated KC activate both $CD4^+$ and $CD8^+$ T cells (purified by negative selection panning) raises at least two possibilities. 1) Both $CD4^+$ and $CD8^+$ T cells respond to PMA-KC in a fashion that does not require interaction of classical MHC molecules with the TCR complex. 2) The response is due to small numbers of $CD4^-$, $CD8^-$ cells, which would not have been depleted by the negative selection panning. Because NK cells were effectively eliminated by the initial panning with mAb B73.1, it is possible that the proliferating cells are class I and class II MHC-unrestricted γ/δ T cells, a hypothesis that is currently being tested in our laboratory.

We conclude that PMA treatment confers upon KC a state of activation that enables them to stimulate T cell proliferation via a MHC-independent mechanism that appears to be regulated through PKC and is in part dependent on the concurrent induction of ICAM-1 on KC. These findings raise two intriguing hypotheses concerning the pathogenesis of inflammatory (e.g., psoriasis) and malignant (e.g., cutaneous T cell lymphoma) skin diseases that are characterized by persistent lymphocytic infiltrates: 1) PMA treatment in vitro mimics the KC activation process that occurs in such chronic skin disorders, and 2) KC activated in this fashion may acquire the capacity to induce the clonal expansion of lymphocytes that migrate to epidermis.

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