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Molecular Mechanism of 7,12-Dimethylbenz[a]anthracene-Induced Immunosuppression: Evidence for Action via the Interleukin-2 Pathway

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SUMMARY

Previous studies in this laboratory have demonstrated that exposure of mice to the carcinogenic polycyclic aromatic hydrocarbon 7,12-dimethylbenz[a]anthracene results in suppression of both humoral and cell-mediated immunity. This suppression is unaccompanied by any significant alteration in splenic lymphocyte subpopulation composition, suggesting that the immune deficit was due to a modulation of lymphocyte function. Additional studies implicated the T helper lymphocyte as the probable target for 7,12-dimethylbenz[a]anthracene-induced immunosuppression, apparently through an inhibition of interleukin 2 production. The purpose of the present study was to examine the mechanism of 7,12-dimethylbenz[a]anthracene-induced T lymphocyte dysfunction at the molecular level and to determine the consequences of 7,12-dimethylbenz[a]anthracene exposure on the interleukin 2 pathway. *In vitro* exposure of Con A-activated splenocytes to 7,12-dimethylbenz[a]anthracene resulted in suppression of the mitogenic response, suppressed interleukin 2 production, and reduced the expression of the high affinity receptor for interleukin 2. In contrast, expression of the low affinity interleukin 2 receptor was not affected. In addition, interleukin 2-dependent lymphoblasts and long term cultured splenocytes exhibited a dose-dependent decrease in proliferation following *in vitro* exposure to 7,12-dimethylbenz[a]anthracene. These results suggest that 7,12-dimethylbenz[a]anthracene-induced immunosuppression may be mediated, at least in part, through the interleukin 2/interleukin 2 receptor pathway.

PAHs are ubiquitous environmental contaminants generated as by-products of the incomplete combustion of fossil fuels, wood, and other organic materials and many are carcinogenic (1, 2). A number of the carcinogenic PAHs have been shown to be immunosuppressive, whereas their noncarcinogenic congeners have no immunosuppressive effect (3, 4). Previous studies conducted in this laboratory with the prototype carcinogenic PAH DMBA have shown a suppression of both humoral (5) and cell-mediated immunity (6). The following parameters were affected in DMBA-exposed mice: proliferative response to T cell mitogens, unidirectional mixed lymphocyte response, generation of CTL, and natural killer cell tumor cytolysis. Although spleen cellularity was markedly reduced after *in vivo* exposure to DMBA (6), the distribution of lymphoid subpopulations remained unaltered, suggesting a DMBA-induced perturbation of intrinsic immune function rather than any effect on lymphocyte viability or proliferation.

Exposure to DMBA has been demonstrated to suppress CTL induction by disruption of T helper cell function, specifically by inhibition of IL-2 production (7). This suppression was reversible *in vitro* by the addition of naive T helper cells or exogenous IL-2. A hypothesis for the mechanism of DMBA-induced immunosuppression may be the alteration of T helper lymphocyte function at the level of IL-2 production. The purpose of the present study was to evaluate the interaction between DMBA and the IL-2 pathway by using an *in vitro* Con A-mediated lymphocyte activation model, in which lymphoproliferation, IL-2 production, Il-2 responsiveness of Con A-induced blasts, and IL-2-dependent proliferation of long term cultured splenocytes were measured.

Materials and Methods

Mice. Specific pathogen-free female B6C3F1 mice were obtained at 4 to 6 weeks of age from Charles River Breeding Laboratories (Raleigh, NC). Animals were maintained in a mass air-displacement room (Bioclean; Hazleton Systems Inc., Vienna, VA) and housed in polypropylene

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; DMSO, dimethyl sulfoxide; CM, complete medium; DMBA, 7,12-dimethylbenz[a]anthracene; Con A, concanavalin A; IL-2, interleukin 2; IL-2R, interleukin 2 receptor; FBS, fetal bovine serum; LA, leucoagglutinin; CTL, cytotoxic T lymphocytes; PBS, phosphate-buffered saline.

cages with filter tops. Mice were found to be free of known rodent pathogens throughout the study. Experiments were performed when mice were approximately 8 weeks of age.

Preparation of cell suspensions. Spleens were teased apart in RPMI 1640 medium that contained 24 mM HEPES buffer (Cellgro, Herndon, VA), 2 nM *l*-glutamine, 50 μg/ml gentamycin, 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 5% heat-inactivated FBS (Gibco Life Technologies, Grand Island, NY). This medium will be referred to as CM. Cell debris was allowed to sediment and the cells were washed once in CM. Cells were resuspended in CM before chemical exposure.

Chemical exposure of cells. DMBA (Aldrich, Milwaukee, WI) was dissolved in DMSO (Sigma) at a stock concentration of 40 μM. Dilutions of the DMBA stock solution were made in DMSO to yield a final concentration of DMSO in culture of 0.1%. Cells were always treated with DMBA for 1 hr before any stimulation or culture. Cell viability in all cultures was approximately 95% at the time of assay, indicating a lack of direct cytotoxicity to splenocytes of DMBA or DMSO.

Lymphoproliferative response to Con A. The effect of DMBA exposure on the proliferation of splenocyte in response to the T cell mitogen Con A (Sigma) was performed using a microculture assay (3). DMBA was added to cells, which were then cultured (2×10^5 cells/well) for 24 or 48 hr at an optimal concentration of Con A. This concentration was determined by titration for each lot of Con A used (usual range of 3 to 5 μg/ml). The cultures were pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Cambridge, MA) per well 8 hr before harvesting with a semiautomatic cell harvester (Skatron, Sterling, VA). Lymphoproliferation was determined by measuring the amount of incorporated label by liquid scintillation fluoroscopy.

Bioassay for IL-2 production. IL-2 concentration in supernatant fluid from DMBA-exposed splenocytes was determined by using a modification of the method of Gillis and colleagues (8). Splenocytes from B6C3F1 mice were treated with various concentrations of DMBA and subsequently incubated at 2×10^6 cells/ml in CM for 24 or 48 hr. Culture supernatants were collected and assayed for ability to support proliferation of the IL-2-dependent CTLL-2 indicator cell line (American Type Culture Collection, Bethesda, MD). CTLL-2 proliferation was measured by incorporation of [^{3}H]thymidine during the last 6 hr of a 24-hr incubation. Culture supernatants containing Con A (5 μg/ml) have been shown to have no effect on CTLL-2 proliferation (9). DMBA alone did not affect CTLL-2 proliferation at any concentration utilized in this assay.

Stimulation of T lymphocyte with LA. LA, a step one ligand at submitogenic levels, induces the expression of functional IL-2R without inducing the production of IL-2 (10). This property allows the direct measurement of the effects of experimental treatments on IL-2R expression or function independent of the IL-2 autocrine pathway. Naive murine splenocytes were cultured with 1 μg/ml LA (Pharmacia Biotechnology, Piscataway, NJ) and various concentrations of DMBA in flat-bottomed 96-well tissue culture plates at 4×10^4 cells/well. IL-2 (Electronucleonics, Silver Spring, MD; specific activity 640 units/ml) was subsequently added at concentrations ranging from 1 to 5 units/ml and culture was continued for 72 hr. Lymphoproliferation was determined by [^{3}H]thymidine incorporation, as described above.

Fluorescence staining with anti-IL-2R antibody. To examine the effect of DMBA treatment on the expression of IL-2R, the rat monoclonal antibody 7D4 (11) was utilized (American Type Culture Collection). The hybridoma was grown in static phase culture in serum-free HB-101 medium (Hana Biologics, Berkeley, CA) and purified by gel filtration on an ACA 22 column (Pharmacia LKB, Piscataway, NJ). For fluorescence staining, the cells were washed twice in CM and were resuspended in PBS, with a predetermined optimum concentration of 7D4, for 30 min at 4°. The cells were washed three times in PBS that contained 0.1% NaN_3 and 2% FBS. These cells were then stained (30 min at 4°) with excess purified fluorescein-conjugated monoclonal mouse anti-rat κ chain (ICN Biomedicals, Lisle, IL), washed three times in PBS (containing 0.1 NaN_3 and 2% FBS), and then analyzed by a fluorescence-activated cell sorter (Coulter EPICS V; Coulter Electronics, Hialeah, FL).

Receptor binding assay. The high affinity IL-2R was assayed by a modification of the method of Robb and associates (12). Cells were washed twice with CM and incubated for 1 hr at 37° to remove endogenous IL-2 bound to IL-2R. Several dilutions of radiolabeled IL-2 (^{125}I-IL-2; specific activity, >600 Ci/mmol; Amersham, Arlington Heights, IL) were incubated with 5×10^5 cells in a total volume of 100 μl of CM for 15 min at 37°. The tubes were mechanically rotated about their longest axis. After this incubation period, 1 ml of ice-cold CM was added to each tube and the cells were centrifuged at 9,000 × *g* for 1 min in an Eppendorf microfuge. The cell pellet was resuspended in 100 μl of CM and was centrifuged through 200 μl of an oil mixture (84% silicone oil; Aldrich, Milwaukee, WI; and 16% paraffin oil, Fisher Scientific, Raleigh, NC) at 12,000 × *g* for 1 min. The tips of the tubes containing the cell pellet were removed and counted directly using a Packard Multi-Prias γ counter. Nonspecific binding was determined by adding a 200-fold excess of unlabeled IL-2 (Amgen, Thousand Oaks, CA). Scatchard analysis of the results was used to calculate the number of binding sites per cell and the affinity of the receptor.

IL-2-mediated blast proliferation. Splenocytes from B6C3F1 mice were exposed to DMBA and cultured in tissue culture flasks (Costar, Cambridge, MA) with Con A for 24 hr (defined as 24-hr blasts) or 48 hr (48-hr blasts). After this incubation period, the cells were washed in CM, treated with α-methyl-mannoside (Sigma), and washed again in CM. The cells were distributed in 96-well tissue culture plates (2×10^5 cells/well) and cultured in the presence of purified IL-2 (0 to 50 units/ml) for 24 hr. The cultures were pulsed with 1 μCi of [^{3}H]thymidine/well 6 hr before harvesting.

Long term cultured splenocytes. To investigate the interaction of DMBA with the IL-2 pathway, a long term cultured splenocyte assay was employed (12). Con A-stimulated splenocytes were harvested after 72 hr of culture in CM, resuspended in the presence of IL-2 (5–10 units/ml), and cultured for an additional 12 days. IL-2 was added only during the first 8 days, so that by day 12 no proliferative activity was detected by [^{3}H]thymidine incorporation. These long term cultured splenocytes were then exposed to DMBA and plated in flat-bottomed 96-well tissue culture plates at 2×10^5 cells/ml, and IL-2 was added at a concentration of 0 to 50 units/ml. After 30 hr, the cells were harvested and the proliferation was determined by incorporation of [^{3}H]thymidine during the last 6 hr of culture.

Statistical analysis. Dunnet's multicomparison modification of Student's *t* test was used to assess the statistical significance of experimental data. Experimental data were considered significantly different from control values at $p < 0.05$.

Results

Lymphoproliferation following *in vitro* DMBA exposure. *In vitro* exposure to DMBA (0–40 μM) resulted in a decreased Con A-induced lymphoproliferation by up to 41% after 24 hr of incubation and 75% after 48 hr (Table 1). DMBA-mediated suppression of proliferation was dose related over the range of concentrations employed in the assay.

IL-2 production. IL-2 production was decreased, in a dose-related manner, up to 40% following DMBA exposure (Fig. 1). This decrease was significantly different from controls at DMBA concentrations of 20 and 40 μM. Relevant concentrations of DMBA alone had no appreciable effect on IL-2-mediated proliferation of the CTLL-2 indicator cells (data not shown).

Inhibition of LA-induced, IL-2-dependent proliferation. Splenocytes exposed *in vitro* to DMBA showed a dose-dependent inhibition of LA-induced, IL-2-dependent prolifer-

TABLE 1

Inhibition of Con A-induced lymphoproliferation following *in vitro* exposure to DMBA

Results are expressed as the percentage of vehicle control. Values represent mean ± standard error of three independent experiments, five cultures per group.

DMBA Concentration	Proliferation	
	24-hr incubation	48-hr incubation
μM	% of control	
10	99 ± 4	90 ± 2*
20	88 ± 4*	75 ± 5*
30	77 ± 6*	43 ± 6*
40	59 ± 6*	25 ± 7*

* Significantly different from respective controls at $p < 0.05$.

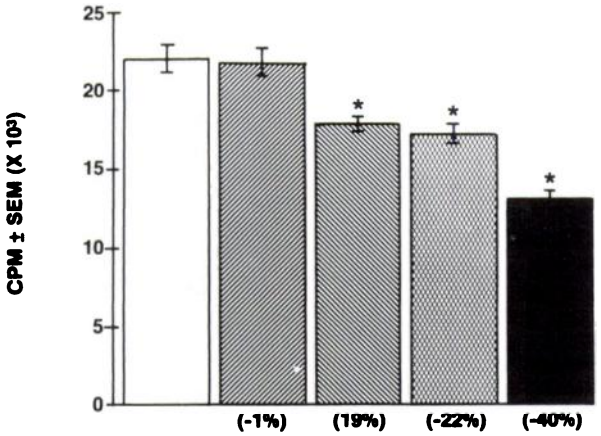

Fig. 1. IL-2 production by Con A-stimulated splenocytes following a 24-hr *in vitro* exposure to DMBA. Values represent incorporation of [^{3}H] thymidine by CTLL-2 cells expressed as cpm ± standard error. *Bars* indicate control (□), 10 μM DMBA (▨), 20 μM DMBA (▩), 30 μM DMBA (■) and 40 μM DMBA (■). *Asterisks* indicate significant difference from control values at $p < 0.05$. Results of a representative experiment as shown.

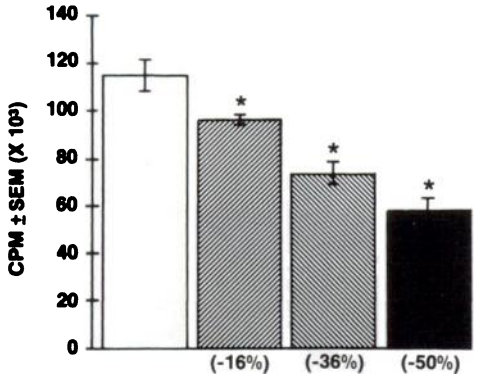

Fig. 2. Stimulation of T lymphocytes with LA and IL-2 following *in vitro* DMBA exposure. Values represent incorporation of [^{3}H]thymidine in cpm ± standard error. *Bars* indicate control (□), 4 μM DMBA (▨) 20 μM DMBA (▩), and 40 μM DMBA (■). Concentration of IL-2 in all cultures was 5 units/ml. *Asterisks* indicate significant difference from control values at $p < 0.05$. Results of a representative experiment, five cultures per group, are shown.

ation up to 50% of control, which was observed at all IL-2 concentrations examined. Fig. 2 shows the result for 5 units/ml IL-2, which was representative of all concentrations examined. Incorporation of [^{3}H]thymidine was essentially at background levels when stimulated lymphocytes were cultured without IL-2, confirming the IL-2 dependence of the model.

Evaluation of IL-2R expression. Fluorescence staining of Con A-induced splenocytes with monoclonal antibody 7D4 was performed at 24 and 48 hr of incubation with DMBA. At 24 hr of exposure, there was a slight nonsignificant enhancement in both the percentage of IL-2R-positive cells and the mean fluorescence intensity for DMBA-treated cells (Table 2). At 48 hr of exposure, there was no significant change in either the percentage of IL-2R-positive cells or the mean fluorescence intensity at any dose of DMBA examined.

High affinity IL-2R measurements. The number of IL-2R per cell and the receptor affinity were evaluated at 24 and 48 hr of incubation in Con A-activated splenocytes previously treated *in vitro* with DMBA at concentrations of 0–40 μM. The number of IL-2R per cell was decreased dose dependently at both 24 (up to 61%) and 48 (up to 82%) hr of incubation (Figs. 3 and 4). There was a slight but significant decrease in the IL-2R affinity at the 40 μM DMBA dose (data not shown). This result was observed at both 24 and 48 hr.

Con A-induced blast proliferation. To further investigate the mechanism of DMBA interaction with the IL-2 pathway, the IL-2 responsiveness of Con A-induced blasts (24 and 48 hr) was examined following DMBA exposure. When exogenous IL-2 was added, a significant enhancement of proliferation was induced at all IL-2 concentrations used. This was consistent for both 24- and 48-hr blasts (data not shown). Blast responsiveness to IL-2 was significantly inhibited at DMBA concentrations of 30 and 40 μM for 24-hr blasts. This effect was noted with all IL-2 concentrations tested (Fig. 5). IL-2 responsiveness of 48-hr blasts was affected at DMBA concentrations of 30 and 40 μM, but only when the highest concentration of IL-2 (50 units/ml) was used (Fig. 5).

High affinity IL-2R expression on Con A-induced blasts. Studies were conducted with cells cultured with exogenous IL-2, at a concentration of 50 units/ml, to assess the expression of high affinity IL-2R on Con A-induced blasts. High affinity IL-2R expression was decreased significantly following exposure to 40 μM DMBA (Table 3). This result was found at both the 24 and 48 hr timepoints.

IL-2-dependent proliferation of long term cultured splenocytes. To examine DMBA-induced suppression of IL-2 responsiveness independent of other activation signals, an IL-2-dependent long term splenocyte culture model was used. After a 30-hr exposure to DMBA (10–40 μM) and IL-2 (5–50 units/ml), the proliferation of long term cultured splenocytes was found to be inhibited at all DMBA and IL-2 concentrations tested (Fig. 6). These cells showed background level of incor-

TABLE 2

IL-2R expression on Con A-activated lymphocytes following *in vitro* exposure to DMBA

Values represent mean ± standard error of three independent experiments. No significant difference from vehicle control was noted.

DMBA Concentration	24-hr Incubation		48-hr Incubation	
	Positive cells*	MFI†	Positive cells	MFI
μM	%		%	
0	67 ± 4	66 ± 4	82 ± 5	84 ± 6
20	73 ± 5	66 ± 10	83 ± 8	86 ± 12
30	79 ± 4	71 ± 5	83 ± 5	84 ± 8
40	78 ± 4	70 ± 4	85 ± 5	85 ± 5

* Percentage of cells bearing the p55 IL-2R subunit, as assessed by specific staining with monoclonal antibody 7D4.

† Mean fluorescence intensity, expressed in arbitrary units.

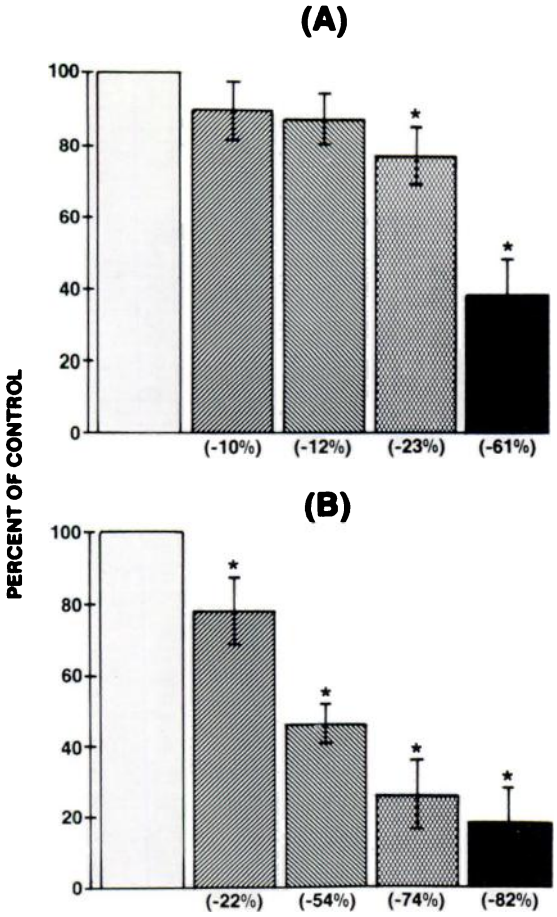

Fig. 3. High affinity receptor expression on Con A-activated lymphocytes following a 24-hr (A) and 48-hr (B) *in vitro* DMBA exposure. All values are expressed as the percentage of respective control cultures. *Bars* indicate control (□), 10 μM DMBA (▨), 20 μM DMBA (▩), 30 μM DMBA (■), and 40 μM DMBA (■). *Asterisks* indicate significant difference from control values at $p < 0.05$. Results are mean ± standard error of three independent experiments.

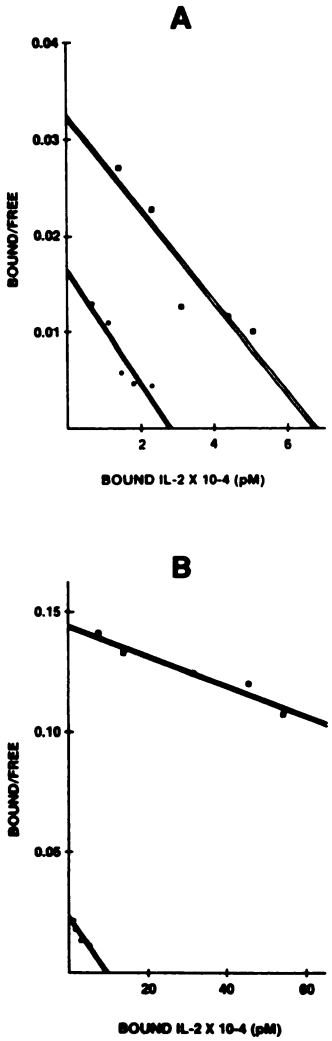

Fig. 4. Scatchard plot analysis of the binding of ^{125}I-labeled IL-2 to Con A-activated lymphoblasts following a 24-hr (A) and 48-hr (B) *in vitro* DMBA exposure. Values on the *x* axis represent bound IL-2, in pM × 10^{-4}. Values on the *y* axis represent the ratio of bound IL-2 to free IL-2. ■, vehicle control; ●, 40 μM DMBA treatment. The specific radioactivity of the probe was used to convert the level of bound radioactivity to pM, the maximum level of IL-2 bound was used to calculate the number of IL-2 binding sites per cell, and the slope of the line was used to calculate the dissociation constant (K_D), in pM. Results of a representative experiment are shown.

poration of [^{3}H]thymidine before they were cultured with DMBA and IL-2 (data not shown).

Discussion

Previous studies in this laboratory have demonstrated a significant and persistent suppression of both humoral and cell-mediated immunity following exposure of murine lymphocytes to DMBA (5–7). This suppression correlated with an increased susceptibility to challenge with infectious agents and transplantable tumors (5, 6). The relative proportions of lymphocyte subpopulations in exposed animals was not altered, as analyzed by cell-surface marker analysis, despite a significant decrease in spleen cellularity (6, 13). Further studies demonstrated that DMBA-induced immunosuppression occurred independently of the *Ah* locus and associated metabolic processes (9). Moreover, the functionality of the T helper subset of lymphocytes was shown to be disrupted through the alteration of IL-2 production.

Resting T cells do not produce IL-2, nor are they capable of responding to IL-2 when it is supplied exogenously. Signals from the T cell antigen receptor complex, which may be triggered by Con A, induce the transcriptional activation of both the IL-2 gene and the gene encoding the IL-2R (14, 15). The subsequent interaction of IL-2 with its high affinity membrane receptor results in cellular proliferation and expansion of the designated cell population (15). IL-2 concentration, IL-2R density, and duration of the IL-2R interaction with its ligand are all variables that determine the T cell cycle progression (16, 17). Thereafter, the level of IL-2R declines progressively and most of the cells revert to G_0 or early G_1 by 12 to 14 days after initiation of the culture (18).

To clarify the mechanism of DMBA interaction with the IL-2 pathway, we employed an *in vitro* activation model utilizing Con A to induce proliferation. This model was adequate, because Con A is known to trigger expression of the IL-2 gene in T lymphocytes via the T cell antigen receptor (14). Con A-induced lymphoproliferation was inhibited following *in vitro* DMBA exposure. Significant suppression was obtained after 24 hr and was markedly enhanced after 48 hr of incubation. IL-2 was measured in 24-hr supernatants from DMBA-treated,

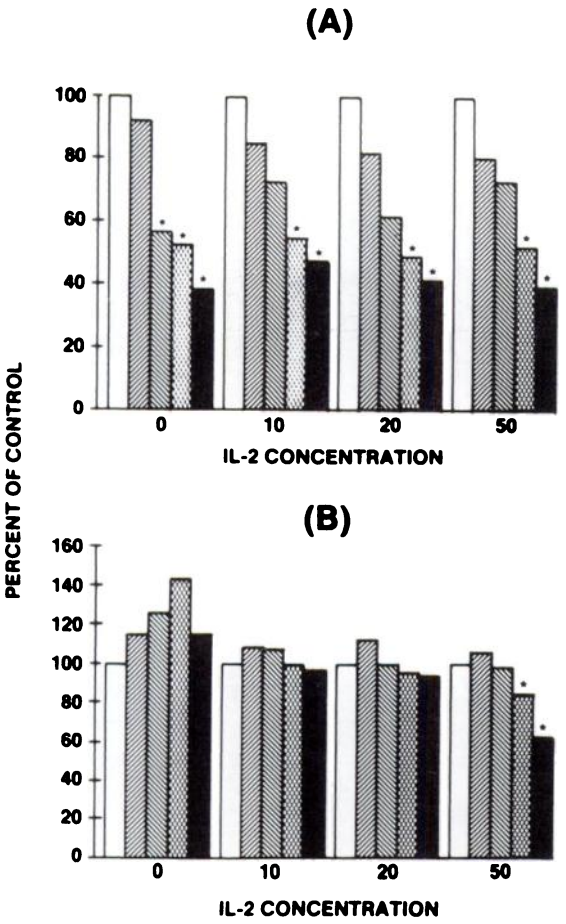

Fig. 5. IL-2 responsiveness of Con A-induced blasts at 24 hr (A) and 48 hr (B) of DMBA exposure. Values on the *x* axis represent IL-2 concentration and values on the *y* axis represent percentage of control. *Bars* indicate control (□), 10 μM DMBA (▨), 20 μM DMBA (▧), 30 μM DMBA (■) and 40 μM DMBA (■). *Asterisks* indicate significant difference from control values at $p < 0.05$. Values represent the mean ± standard error of three independent experiments.

TABLE 3

High affinity IL-2R expression on DMBA-exposed, Con A-induced blast cells cultured with exogenous IL-2

Values represent number of IL-2R per cell. Values in parentheses represent percent change from control. Final concentration of IL-2 in culture was 50 units/ml. Results of a representative experiment, five cultures per group, are shown.

DMBA Concentration	IL-2R Number	
	24-hr blasts	48-hr blasts
μM		
0	1734	3705
10	1614 (↓7%)	3705
20	1583 (↓9%)	3354 (↓3%)
30	1563 (↓10%)	3374 (↓9%)
40	792 (↓54%)	1359 (↓63%)

Con A-induced splenocytes, because normal lymphocytes enter the cell cycle asynchronously and produce maximal levels of IL-2 in late G_1 (18, 19). Significant suppression of IL-2 production was obtained with DMBA-treated cells, suggesting that decreased IL-2 production might participate in DMBA-induced immunosuppression.

We next evaluated high affinity IL-2R function and expression using the "step one" ligand LA. IL-2-dependent proliferation of LA-induced lymphocytes was decreased, indicating a

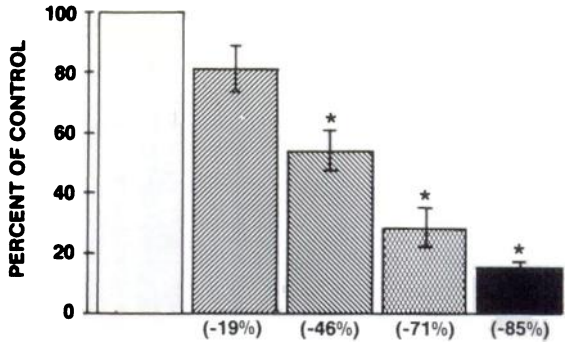

Fig. 6. Inhibition of IL-2-dependent proliferation by long term cultured splenocytes following *in vitro* DMBA exposure. Values are presented as percentage of control. *Bars* indicate control (□), 10 μM DMBA (▨), 20 μM DMBA (▧), 30 μ m DMBA (■), and 40 μM DMBA (■). Cells were cultured with 50 units/ml IL-2. *Asterisks* indicate significant difference from control values at $p < 0.05$. Results represent the mean ± standard error of three independent experiments.

possible involvement of the IL-2R in the mechanism of immunotoxicity. IL-2R expression was measured by fluorescence staining with the 7D4 anti-IL-2R antibody. The high affinity IL-2R consists of two subunits, p55 and p75, where only p75 is able to mediate the transduction of the signal (20, 21). 7D4 is a rat monoclonal antibody specific for the p55 subunit (11) and is capable of blocking IL-2-dependent T cell proliferation without affecting the binding of IL-2 to high affinity IL-2R (20). DMBA treatment did not induce any significant modification in either the percentage of cells bearing IL-2R or the mean fluorescence intensity. These results were valid for both 24- and 48-hr incubation cultures.

These results yielded only a partial resolution of the problem, because it is generally accepted that the high affinity form of the IL-2R mediates most of the physiological responses to IL-2 through the p75 receptor subunit (21, 22). Direct ligand binding measurements represent the most accurate means for determining the capacity of a cell population to interact with IL-2 (12). Therefore, we measured IL-2R expression by quantitating the number of receptors per cell, in addition to their functionality (i.e., receptor affinity), in DMBA-treated Con A-induced blasts. We found that DMBA treatment affected the expression of high affinity IL-2 binding sites in a dose-dependent manner after a 24-hr exposure, and an even greater suppression was noted at 48 hr. The greater degree of suppression noted at the later time point may possibly reflect the higher number of IL-2R per cell following a longer culture with exogenous IL-2 (17). IL-2R affinity of DMBA-treated cells was significantly decreased only following exposure to the highest concentration of DMBA (40 μM). These data, therefore, suggest that impairment by DMBA of Con A-mediated splenocyte proliferation is at least partly due to inhibition of the expression of high affinity binding sites of the IL-2R. IL-2R affinity itself does not appear to play a significant role, because splenocyte proliferation is decreased at DMBA concentrations of 30 and 40 μM without any significant modification of IL-2R affinity.

Studies of mitogen stimulation of T cells indicated that IL-2R expression is transient (18). The highest receptor levels are observed when the cells undergo optimal proliferation, after which the level of cell surface IL-2R rapidly declines (15, 18). It was, therefore, of interest to determine how the modulation of the expression of high affinity IL-2R affected the ability of the DMBA-treated cells to respond to IL-2. IL-2 responsiveness

of 24- and 48-hr Con A-induced blasts was differentially affected by *in vitro* DMBA treatment. IL-2 responsiveness of 24-hr blasts was affected at all IL-2 concentrations, whereas IL-2 responsiveness of 48-hr blasts was decreased only at the highest IL-2 concentration. It is possible that exposure of these cells to a high concentration of IL-2 leads to an accelerated internalization of the IL-2R, thus enhancing the sensitivity of the model. A possible explanation for this is the lipophilicity of DMBA, which may account for its immunomodulatory effect through the disruption of normal membrane function (23). To analyze this discrepancy, we evaluated the IL-2R expression and function of these blast cells in the presence of 50 units/ml IL-2. We found that the IL-2R number did not precisely correlate with the proliferation results (i.e., IL-2 responsiveness), suggesting that the decrease in IL-2R was not solely responsible for the DMBA-induced inhibition of proliferation. To verify this hypothesis, we developed a cell culture model based on IL-2 dependency for proliferation. Using this model we were able to assess the function of the IL-2 pathway independent of other activational processes. When DMBA was added to these cells; IL-2-dependent proliferation was inhibited in a dose-dependent manner; further, this suppression could not be restored by increasing the IL-2 concentration.

Activation of the T cell antigen receptor complex renders lymphocytes competent to receive the cell cycle progression signals provided by IL-2. Inhibition of IL-2 production and IL-2R expression in DMBA-treated cells demonstrated the interference of this compound with lymphocyte activation mediated through the T cell antigen receptor. However, the lack of production of an "activation mediator" (e.g., IL-1 or IL-6) could not be ruled out, because IL-1 production has been shown to be unaffected by DMBA treatment.[4] DMBA also disrupted the progression of the cells into the cell cycle. This phenomenon could be partially explained by the decrease in IL-2R number expressed on the membrane of DMBA-treated lymphocytes. In fact, within an activated T cell population, IL-2R density varies by a factor of 1000 among the individual cells and a critical threshold of triggered IL-2R is required before the cell can totally replicate DNA (18). In accordance with this mechanism, DMBA may prevent a certain number of these cells from entering into S phase of the cell cycle by decreasing the IL-2R expression on activated cells. Our IL-2 responsiveness experiments performed with Con A-induced lymphoblasts and long term cultured splenocytes suggest that IL-2R number is not the only parameter involved in DMBA-induced alteration of lymphocyte expansion. Thus, DMBA was able to alter the IL-2-mediated signal transduction by either a direct action on the receptor (e.g., internalization) or action on the second messenger cascade linked to the IL-2R. In this regard it is important to point out that DMBA, due to its high lipophilicity, might interact at the membrane level and disrupt transduction of signals and conformation of receptors. However, studies conducted in this laboratory (24) have shown that responsiveness to T cell-replacing factor, production of interferon-γ, and activation of antigen-induced polyclonal CTL are not affected by *in vitro* exposure to DMBA. Therefore, these data suggest that DMBA displays a defined specificity for certain lymphokine/receptor systems.

In summary, the present study indicates that DMBA-induced suppression of cell-mediated immunity may result from a disruption of both the activation signal, as shown by the decrease in IL-2R expression and IL-2 production, and proliferative signals, as shown by the inhibition of IL-2 responsiveness.

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