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The skin is generally not thought of as an immune organ. However, both the epidermal and dermal components of the skin contain several types of immune cells (Baxter et al., 1989). Furthermore, the skin is capable of producing a variety of cytokines and factors capable of modulating both the local and systemic immune systems. For example, interleukin-1α (IL-1α)3 is constitutively produced by murine keratinocytes and stored in the differentiated cells (Robertson et al., 1995). Exposure of murine skin to the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) causes the release of stored IL-1α and stimulates the de novo synthesis and release of IL-1α, GM-CSF, and TGF-β by various cell types in the skin (Akhurst et al., 1988; Lee et al., 1993; Robertson et al., 1994; Robertson et al., 1995). TPA also stimulates the synthesis and release of PGE2 by keratinocytes and monocytes/macrophages (Fischer et al., 1987; Lewis and Adams, 1986). Some of the cutaneously derived cytokines presumably find their way into the circulatory system since serum IL-1 and GM-CSF contents are markedly elevated in treated animals (Robertson et al., 1995; Updyke et al., 1991a), and there is a rapid recruitment of circulating PMNs into the treated skin (Baxter et al., 1989; Robertson et al., 1994).

Subsets of CD4+ T cells are involved in the development and regulation of immunological reactions. Specifically, T helper (Th) cells designated Th1 and Th2 produce lymphokines required for the development of cell-mediated and humoral immune responses, respectively (Abbas et al., 1991). They also secrete lymphokines that induce a functional anergy in one another (Abbas et al., 1991; Street and Mosmann, 1991). As a consequence of this latter property, the preferential activation of one Th cell type can potentially suppress the other Th cell type and modulate the development, duration...
Two factors produced by TPA-treated skin are known modulators of Th1 and Th2 function. IL-1 is costimulatory for Th2 cells, but not for Th1 cells (Williams et al., 1991; Manetti et al., 1994). PGE\(_2\) has little effect on lymphokine production by Th2 cells. However, it inhibits Th1 production of lymphokines required for cell-mediated immune responses and IFN-\(\gamma\), a lymphokine that suppresses cytokine production by Th2 cells (Betz and Fox, 1991). Consequently, topical treatment of mice with TPA results in the production of factors that should support the development of humoral immunity, but suppress cell-mediated immune responses. Indeed, the development of several prototypical cell-mediated immune responses (e.g., delayed hypersensitivity, contact hypersensitivity (CHS), graft rejection) is suppressed in various strains of mice treated topically with TPA (Halliday et al., 1988; Kodari et al., 1991; Zhao and Cao, 1994).

In the current study we initiated an investigation of the effects of topically applied TPA on humoral immune development and lymphoproliferation in Super SENCAR Inbred (SSIN) mice. In addition, efforts were specifically made to control for the effects of the solvents (e.g., acetone and ethanol) commonly used to dissolve and apply TPA. In general, protocols intended to assess the effects of topically applied chemicals utilize comparisons to solvent-treated controls, but exclude comparisons to naive animals. This exclusion of naive controls appears to be derived from the tacit assumption that topically applied acetone and ethanol do not evoke biologically significant reactions. While it is true that both solvents are neither carcinogenic when applied chronically to rodent skin (Stenbäck, 1969; Kuratsune et al., 1971; Zakova et al., 1985; Wei et al., 1990) nor promoters when assessed in murine skin multistage carcinogenesis protocols (Stenbäck, 1969; Kuratsune et al., 1971; Slaga et al., 1981), chronic topical administration of acetone induces a weak but long-lasting epidermal hyperplasia (Iversen et al., 1981). Furthermore, Müller-Decker et al. (1994) recently reported that cultured human keratinocytes release IL-1\(\alpha\) and arachidonic acid, and stimulate the synthesis of PGE\(_2\), upon exposure to acetone or ethanol. The results of the current study demonstrate that topically applied ethanol and acetone evoke a systemic modulation of the immune system and emphasize the potential for erroneous conclusions if naive controls are not included to correct for solvent-dependent effects.

**MATERIALS AND METHODS**

**Chemicals.** Lipopolysaccharide from *Escherichia coli* 055:B5 (LPS), concanavalin A (Con A), 2,4,6-trinitrobenzenesulfonic acid (TNBS), TPA, and mitomycin C were purchased from the Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium, penicillin, and streptomycin were obtained from GIBCO Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Sheep red blood cells (SRBC) in Alsever's solution were obtained from the Colorado Serum Co. (Denver, CO) and TNP-LPS was from List Biological Laboratories (Campbell, CA). [methyl-\(^3\)H]thymidine (6.7 Ci/mmol) was purchased from NEN (Wilmington, DE). Acetone (Baker analyte reagent) was purchased from J. T. Baker (Phillipsburg, NJ) and ethanol (200 proof absolute, USP grade) was obtained from AAPER Alcohol and Chemical Co. (Shelbyville, KY). Hamster anti-mouse CD1-\(\varepsilon\)-FITC (Boehringer-Mannheim, Indianapolis, IN), mouse anti-mouse Thy 1.2-PE (Callag, South San Francisco, CA), goat anti-mouse IgM + G + A-FITC (Cappel Laboratories, Durham, NC), rat anti-mouse CD8-PE (Pharmingen), rat anti-mouse L3T4-PE, and rat anti-mouse Ly2-FITC (last two antibodies were from Becton-Dickinson, San Jose, CA) were all purchased as direct conjugates to either FITC or PE fluorophores.

**Animals.** Female SSIN mice, 5–6 weeks old, were purchased from the Science Park-Veterinary Division, The University of Texas M. D. Anderson Cancer Center (Bastrop, TX). Female DBA/2 and B\(_6\)C\(_3\)F\(_1\) mice were purchased from the National Cancer Institute (Frederick, MD). Animals were quarantined a minimum of 1 week before the initiation of any treatments. Mice were housed in microisolation cages and food and water were provided ad libitum. Animals were maintained on a 12-hr light:12-hr dark photocycle.

**Animal treatments.** The dorsal trunks of 6–8-week-old mice were shaved with surgical clippers 2–4 days prior to the initiation of topical applications of solvent or TPA. Animals that had been nicked during the shaving process or in hair regrowth at the time of the first solvent treatment were excluded from the study. The size of the shaved areas was ~7.5 ± 1.5 cm\(^2\). Mice were treated with 50, 100, 200, or 300 \(\mu\)l of solvent or 2 \(\mu\)g of TPA dissolved in acetone on the shaved area either once or twice weekly for 2 or 4 weeks. Applications of 200 and 100 \(\mu\)l, separated by ~5 min, were used to achieve a final volume of 300 \(\mu\)l. Control animals were shaved and manipulated to simulate treatment, but not treated with solvent or TPA.

**Preparation of single cell suspensions.** Unless specifically noted, mice were euthanized by cervical dislocation 2 days after the last topical application of solvent. Single cell suspensions were prepared from aseptically removed spleens as described by Updyke et al. (1989). Washed cells were suspended in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 2 \(\mu\)M glutamine (complete medium). Nucleated cells were counted with a hemocytometer and viabilities were assessed by trypan blue exclusion (routinely >90%).

**Hemagglutination assay.** The procedure described by Zaidi et al. (1990) was used to estimate serum hemagglutinating antibody titers to sheep red blood cells. Blood was drawn from the retroorbital plexus by using glass capillary tubes. Sera were heat inactivated at 56°C for 30 min. Twofold dilutions of the sera were added 25 \(\mu\)l of a 1% suspension of SRBC in PBS. Plates were scored after 2 hr at room temperature for the highest dilution that caused complete agglutination of the RBC.

**Mitogen-induced lymphocyte proliferation assay.** The proliferative responses of spleen lymphocytes to the T cell mitogen Con A and the B cell mitogen LPS were assessed as described by Updyke et al. (1991b) with slight modifications. Single cell suspensions in complete medium were dispensed in triplicate into 96-well flat-bottom microtiter plates (2 x 10\(^5\) cells/well in 100 \(\mu\)l). Con A and LPS were diluted in complete medium and were added to each well such that final amounts were 1 and 5 pg/ml, respectively. Plates were incubated at 37°C in a humidified, 5% CO\(_2\) atmosphere for a total of 72 hr. Each well was pulsed with 1 \(\mu\)Ci of [\(^3\)H]thymidine 18 hr prior to harvesting. Cells were harvested onto glass fiber filter paper and processed with an automatic sample harvester unit (SKATRON Combi Cell Harvester, Sterling, VA). Radioactivity was measured by liquid scintillation counting. Non-mitogen-treated cultures incorporated <100 dpm per well. LPS- and Con A-treated splenocytes isolated from nontreated mice incorporated >25X and >70X the number of dpm incorporated by non-mitogen-exposed cultures, respectively.
Mixed lymphocyte response (MLR). Single cell suspensions of splenocytes from each SSIN mouse (responder cells, R) were dispensed in triplicate into 96-well flat-bottom microtiter plates (2 x 10⁵ cells/well in 100 μl). Mitomycin C-treated splenocytes from either naive syngeneic SSIN mice (Ro) or allogenic DBA/2 mice (stimulator cells, S) were added to each well containing R cells (R:S or R:Ro = 1:2 ratio). Plates were incubated at 37°C in a humidified, 5% CO₂ atmosphere for a total of 72 hr. Cultures were pulsed with 1 μCi [³H]thymidine for 18 hr prior to harvesting and the determination of incorporated radiolabel, as described above. Alloantigen-specific stimulation of T cell proliferation was calculated as the difference between cultures being stimulated with syngeneic (Ro) and allogenic stimulator (S) cells.

Plaque-forming assay for the T-dependent antigen SRBC. Mice were immunized ip 1–168 hr after the final topical solvent treatment with ~0.9 x 10⁶ SRBC (diluted in PBS, 0.2 ml injection). Mice were euthanized 4 days after immunization for quantitation of IgM antibody-forming cells, unless it is specified otherwise in the text. In order to study IgG antibody-producing cells, a second immunization was performed 7 days after the first injection with the same number of SRBC, and spleens were collected two days later. Previous studies have demonstrated that the IgG response to SRBC following the second immunization is optimal two days later (Koller and Kovacic, 1974; Koller et al., 1976; Singh et al., 1982). A modification of a procedure described by Cunningham and Szenberg (1968) was used for the quantitation of IgM- and IgG-secreting cells. Briefly, 100-μl suspensions of 20% SRBC and 25 μl of undiluted guinea pig complement (Pel-freez, Rogers, AR) were added to 350 μl of splenocytes (2.45 x 10⁵ to 5.6 x 10⁶ cells) in complete media. The volume was adjusted to 500 μl with 25 μl of medium (for IgM determinations) or 25 μl of a 1:10 dilution of rabbit anti-mouse IgG (for IgG determinations) (Pel-freez, Rogers, AR). The number of IgG-secreting B cells was calculated by subtracting the number of IgM plaques from the IgM + IgG total plaque count. Cunningham chambers were loaded with 30 μl of the assay mixture and sealed with petroleum jelly. After a 45-min incubation at 37°C plaques were counted at room temperature with the aid of a microscope.

Phenotyping of Splenocytes

The SSIN strain was derived by the inbreeding of SENCAR mice, a murine stock commonly used in the screening of putative carcinogens, promoters, and chemoprotective agents (Fischer et al., 1987). Initially, analyses were made following four or eight topical applications of 200 μl of solvent or 2 μg of TPA dissolved in acetone. Control mice were not treated. Data represent the means ± SE of 13 mice per treatment group, except for the ethanol treatment groups, where n = 6 mice.

RESULTS

The overall cellularity of the spleen was not significantly affected by repeated topical application of either solvent or eight applications of TPA (Table 1). However, splenic cell content was significantly increased in mice treated four times with TPA (Table 1).

Surface marker phenotyping of splenocytes demonstrated that the relative percentages of B cells and T cells were similar in control mice and animals treated topically four or eight times with solvents (Table 2). The ratio of CD4⁺ to CD8⁺ T cells was also not affected by solvent treatment (Table 2). In contrast, the relative T cell composition of the spleen decreased following repeated topical applications of

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tbody>
<tr>
<td><strong>Effects of TPA and Solvents on the Cellularity of the Spleen</strong></td>
</tr>
<tr>
<td><strong>Number of treatments</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
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</table>

Note. Nucleated splenocytes were isolated and counted 2 days after the last of either four or eight topical applications of 200 μl of solvent or 2 μg of TPA dissolved in acetone. Control mice were not treated. Data represent the means ± SE of 13 mice per treatment group, except for the ethanol treatment groups, where n = 6 mice.

* Statistically greater than control or acetone-treated mice, p < 0.05
TABLE 2
Flow Cytometric Analyses of Splenocytes Isolated from TPA- and Solvent-Treated Mice

<table>
<thead>
<tr>
<th>Cell type or phenotype marker</th>
<th>4x (%)</th>
<th>8x (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total T cells</td>
<td>Control</td>
<td>Acetone</td>
</tr>
<tr>
<td>Total T cells</td>
<td>34.3 ± 1.7</td>
<td>29.5 ± 1.7</td>
</tr>
<tr>
<td>CD4+</td>
<td>29.3 ± 1.5</td>
<td>24.3 ± 2.1</td>
</tr>
<tr>
<td>CD8+</td>
<td>6.6 ± 0.4</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>CD4+/CD8+ ratio</td>
<td>4.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Total B cells</td>
<td>52.2 ± 2.0</td>
<td>56.4 ± 2.7</td>
</tr>
</tbody>
</table>

Note. Nucleated splenocytes were isolated and counted 2 days after the last of either four or eight topical applications of 200 μl of solvent or 2 μg of TPA dissolved in acetone. Control mice were not treated. Data represent the means ± SE of either 12 mice (4x treatment) or 9 mice (8x treatment).

* Statistically less than acetone-treated group, p < 0.05.
Statistically less than TPA-treated group, p < 0.05.

TPA (Table 2). These reductions did not reflect the preferential loss of either CD4+ or CD8+ T cells since the overall ratio of CD4 to CD8 T cells was not affected by TPA treatment. The relative reductions in splenic T cell content were accompanied by small, nonstatistically significant increases in splenic B cell contents. Although such increases theoretically accounted for the altered composition seen after eight TPA treatments, they did not account for the changes noted after four TPA treatments.

Humoral Responses to SRBC, a T-Cell-Dependent Antigen

Plaque-forming assays were used to assess the effects of solvents on the development of humoral immunity. The overall kinetics of appearance/disappearance of splenic IgM-producing B cells occurring in control SSIN mice (Figs. 1A and 1B) were similar to those reported in other strains of mice following a primary immunization with SRBC (Koller and Kovacic, 1974; Koller et al., 1976; Singh et al., 1982). Responses measured in mice treated 8x with solvents were indistinguishable from those measured in control mice (Fig. 1B). In contrast, a retardation in the development of splenic B cells secreting IgM against SRBC occurred in mice treated 4x with either solvent (Fig. 1A). However, the rate of loss of plaque-forming cells (PFCs) was not affected by either solvent (Figs. 1A and 1B).

A delay in the development of PFCs, coupled with normal kinetics of loss, would effectively decrease the overall duration of IgM synthesis in solvent-treated animals. Since neither the overall cellularity of the immunized spleen (Figs. 2B and 3B) nor the relative percentage of B cells in the spleen (Table 2) was affected by repeated topical applications of 200 μl of acetone or ethanol, the relative capacity of solvent-treated mice to produce Ig to SRBC should be proportional to the numbers of PFCs. Hemagglutination assays were used to indirectly quantitate serum Ig titers to SRBC. The kinetics of appearance of hemagglutinating activities in the serum paralleled the appearance of IgM-secreting PFCs (compare Figs. 1A and 1C, and 1B and 1D), and hemagglutinating titers reflected overall PFC contents. Indeed, hemagglutination titers were identical in control mice and animals immunized after eight solvent treatments (Fig. 1D). However, serum isolated from mice immunized after the last of four solvent treatments had ~50% of the hemagglutinating activity of serum isolated from immunized control mice (Fig. 1C). Consequently, a functional suppression of the development of humoral immunity occurred in mice treated topically 4x with either acetone or ethanol.

The appearance of PFCs was retarded in mice treated 4x with TPA (Fig. 1A) and paralleled by reductions in serum hemagglutination titers (Fig. 1C). However, these effects of TPA were basically indistinguishable from those of the solvent used for its application.

Plaque formation was assessed in animals treated 1, 4 or 8 times with 50, 100, 200, or 300 μl of solvent (Figs. 2 and 3) in order to determine the effects of solvent dose and duration of treatment on the development of humoral immunity. Plaque formation 4 days after immunization was statistically and significantly suppressed in SSIN mice treated one, four, or eight times with 300 μl of acetone (Fig. 2A). In contrast, suppression of PFC development by the other doses of acetone appeared to be schedule-dependent. This was most obvious in animals treated with 200 μl of acetone. Whereas plaque numbers were not significantly affected in mice immunized shortly after the eighth application of this volume of solvent, there was a dramatic suppression when immunization followed the fourth application of this volume.
None of the changes in plaque numbers were accompanied by changes in splenic cellularity. Indeed, varying the dose or duration of acetone treatment had no effect on overall splenic cellularity in immunized mice (Fig. 2B).

The development of IgM-secreting PFCs was also affected by topically applied ethanol (Fig. 3A). Specifically, four treatments with ethanol lead to a volume-dependent reduction in PFCs. However, similar effects did not occur after eight applications of ethanol. As with acetone, varying the dose or duration of ethanol treatment had little effect on the overall cellularity of the spleen (Fig. 3B).

The studies depicted in Figs. 1–3 employed a protocol in which the mice were immunized with SRBC within 1 hr of the last solvent treatment. In order to determine how long the suppressive state is maintained in solvent-treated mice, the time of immunization was varied after the last solvent application (Fig. 4). Plaque formation was reduced in 4X acetone-treated (Fig. 4A) or 4X ethanol-treated (Fig. 4B) mice immunized as late as 168 hr after the last solvent treatment. Indeed, the level of suppression seen at 168 hr was similar to that occurring when mice were immunized 1 hr after solvent treatment. In contrast, no reductions in plaque-forming cells occurred in 8X solvent-treated mice when immunized 1–48 hr after the last solvent application. These latter studies suggest that a component or process involved in or affecting the development of IgM-producing B cells becomes refractory to the solvent’s effects between the fourth and eighth applications.

Varying the time of immunization after the last TPA treatment modulated IgM PFC development in a fashion that was indistinguishable from the results obtained with acetone alone (Fig. 4A). Consequently, the effects of TPA on IgM PFC development appear to reflect primarily the activities of the solvent used for its application.

In order to determine if solvents and TPA could affect the development of a secondary humoral response, indirect plaque assays were used to assess the relative numbers of IgG-producing PFC in solvent- and TPA-treated mice. Splenic IgG-producing PFCs were reduced in mice treated 4X with 200 μl of either acetone (~45% reduction) or ethanol (~55% reduction), but recovered to control levels after eight topical applications of either solvent (Fig. 5). Consequently, the development of IgG- and IgM-producing PFC was comparably affected by either solvent (compare Figs. 1 and 5).

Splenocytes derived from 4X TPA-treated mice and control mice developed similar numbers of IgG-secreting PFCs per 10^6 splenocytes (Fig. 5). Thus, exposure to TPA counteracted
the effects of acetone on the development of a secondary humoral immune response. However, additional applications of TPA did not elevate the numbers of IgG-secreting PFCs above those scored in immunized control mice (Fig. 5).

Solvent Effects in B6C3F1 Mice

Unlike SSIN mice, which are exceptionally sensitive in murine skin two-stage carcinogenesis protocols to the effects of topically applied tumor promoters (DiGiovanni et al., 1991), B6C3F1 mice are refractory to topically applied tumor promoters (Eastin, 1989). In order to determine whether solvent modulation of humoral immunity was strain specific or correlated with susceptibility to two-stage carcinogenesis, B6C3F1 mice were treated 4X with 50 or 200 µl of ethanol and immunized with SRBC 10 hr after the last solvent treat-
CUTANEOUS EXPOSURE AND IMMUNOMODULATION

1 10 100 1000

Hours After Last Solvent Treatment

FIG. 4. Influence of the time of immunization, relative to the last solvent or TPA application, on the development of B cells producing IgM. SSIN mice were ip immunized with SRBC 1–168 hr after the last of one (A), four (●), or eight (■) topical applications of 200 μl of acetone (A) or ethanol (B) to shaved dorsal trunks or four (○) or eight (■) topical applications of 2 μg TPA dissolved in 200 μl of acetone (A). Nontreated mice were similarly immunized. All mice were euthanized 4 days after immunization, and splenocytes were isolated, counted, and processed for quantitation of IgM-secreting B cells in plaque-forming assays. Plaque data are presented as percentages of control response and represent the means ± SE of one to three separate experiments each involving a minimum of five animals per control and treatment group. *Statistically different from control, p < 0.05.

Mitogen- or Alloantigen-Induced Splenocyte Proliferation

Chronic oral ingestion of ethanol suppresses the development of humoral immunity to T-cell-dependent antigens. This suppression is characterized by reductions in in vitro splenic proliferative responses to alloantigen or the T cell mitogen Con A (Jerrells et al., 1990; Chang and Norman, 1991, 1992; Domiati-Saad and Jerrells, 1993), but not the B cell mitogen LPS (Jerrells et al., 1990; Holtsapple et al., 1993; Domiati-Saad and Jerrells, 1993). Relative to responses measured in control mice, in vitro proliferative responses to LPS were not significantly affected in splenocytes prepared from mice treated topically 4× with ethanol. However, significant increases occurred in splenocyte preparations isolated from mice treated topically 4× with acetone, or 8× with either solvent (Fig. 6A). In vitro proliferative responses to Con A were also elevated in these latter solvent-treated animals (Fig. 6B). In contrast, in vivo exposure to solvents had no significant effect on alloantigen-induced MLR (Fig. 6C).

The in vitro proliferative responses of splenocytes isolated from TPA-treated mice to T and B cell mitogens were markedly greater than those observed in either control or acetone-treated mice (Figs. 6A and 6B). The capacity of alloantigen to stimulate T cell proliferation in a MLR assay was enhanced, but not significantly, by topical TPA pretreatment of the mice providing the responding cells used in the assay (Fig. 6C).

DISCUSSION

Acetone and ethanol are commonly used as vehicles for the topical application of chemicals in animal studies and in particular murine skin carcinogenesis protocols (Slaga and Fischer, 1983). This use stems from their functioning as good solvents and their uniform spreading on hairless cutaneous surfaces. Protocols intended to assess the effects of topically applied chemicals generally employ comparisons to solvent-treated animals, but not naive controls. Although such protocols control for solvent effects, they do not evaluate the effects of the solvent. This exclusion of naive controls may be dictated by economics, limitations imposed by experimental design, or the assumption that topically applied acetone/ethanol does not evoke biologically significant reactions. Our demonstration that topically applied acetone and ethanol modulate the development of humoral immunity, and the proliferative responses of splenic B and T cells to mitogens, certainly argues against the validity of the latter assumption. Furthermore, our data emphasize the need for

FIG. 5. Influence of topically applied acetone, ethanol, and TPA on B cells producing IgG against SRBC. SSIN mice were ip immunized with SRBC 1 hr after the last of four or eight topical applications of 200 μl of either acetone or ethanol to shaved dorsal trunks. A second immunization was performed 7 days after the first. Nontreated mice were similarly immunized. All mice were euthanized 2 days after the last immunization, and splenocytes were isolated and processed for quantitation of IgG-secreting B cells in plaque-forming assays. Numbers of plaques are presented per 10⁶ splenocytes for nontreated (solid), acetone-treated (shaded), ethanol-treated (horizontal bar), or TPA-treated (diagonal bar) mice. Data represent the means ± SE of 15–20 mice *Statistically different from control, p < 0.05.

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TABLE 3
Effects of Acetone, Ethanol, and TPA on the Development of B Cells Producing IgM against TNP-LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of applications</th>
<th>10⁶ Splenocytes</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4X</td>
<td>281 ± 28</td>
<td>15,783 ± 1871</td>
</tr>
<tr>
<td>Acetone</td>
<td>4X</td>
<td>277 ± 20</td>
<td>14,443 ± 1405</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4X</td>
<td>284 ± 23</td>
<td>13,654 ± 1420</td>
</tr>
<tr>
<td>TPA</td>
<td>4X</td>
<td>300 ± 23</td>
<td>13,429 ± 1317</td>
</tr>
</tbody>
</table>

Note SSIN mice were ip immunized with TNP-LPS 1 hr after the last of four topical treatments of the shaved dorsum with 200 μl of either solvent or 2 μg of TPA dissolved in acetone. Nontreated, control mice were similarly immunized. All mice were euthanized 3 days after immunization and splenocytes were isolated and processed for quantitation of IgM-secreting B cells in plaque-forming assays. Data represent the means ± SE of 10 mice per treatment group.

Analyzing solvent effects in cutaneous application protocols. Had we not corrected for solvent contributions in the current study, we would have mistakenly concluded that TPA stimulates IgG PFC development, as opposed to antagonizing the suppressive effects of the solvent used in its application.

The numbers of PFCs that developed in solvent-treated, immunized mice generally decreased with increasing volumes of applied solvent. However, reductions in PFC numbers were not directly proportional to the applied volume and plateaued at ~50% of control values. Although speculative on our part, it is conceivable that this lack of proportionality reflects a limitation intrinsic to haired mice. Specifically, the area of cutaneous treatment is dependent upon both the volume of solvent applied and the size of the shaved area. Neither solvent spreads particularly well from a shaved area into a haired region. In our studies applications of 100 and ≥200 μl of solvent treated ~70 and 100% of the shaved areas, respectively. If the solvent effects on humoral development are a consequence of factors generated in the skin, then the area of treatment, as opposed to the volume applied, should be a critical determinant. The plateauing of the solvent response and the fact that 100–300 μl volumes gave basically similar results are consistent with this later hypothesis.

The duration of solvent treatment also affected the development of humoral immunity. In most cases volumes of solvents causing suppression following one or four applications lost their suppressive activities when the immunization was performed after the eighth solvent treatment. The basis for this loss is not known. However, it may reflect an adaptive response by the skin. Some species of rodents develop a pronounced epidermal hyperplasia and cutaneous inflammation following an initial topical application of a tumor promoter, but progressively become refractory to the induction of these processes upon chronic exposure (Sisskin and Barrett, 1981; Sisskin et al., 1982).

Solvent modulation of humoral immunity was also dependent upon the nature of the antigen used for immunization. Specifically, conditions suppressing the development of humoral immunity to the T-cell-dependent antigen SRBC were ineffective with the T-cell-independent antigen TNP-LPS. Antibody production to T-cell-dependent antigens requires both T cells and accessory cells (Hodgkin et al., 1994). In the case of orally ingested ethanol, suppression of the development of humoral immunity to T-cell-dependent antigens is generally paralleled by reductions in splenic proliferative responses to T cell mitogens or alloantigen (Jerrells et al., 1990; Chang and Norman, 1991, 1992; Domiati-Saad and Jerrells, 1993) and/or net decreases in splenic T cell content (Bagasra et al., 1987; Jerrells et al., 1990) and increases in the CD4<sup>+</sup> to CD8<sup>+</sup> ratio (Saad and Jerrells, 1991; Lopez et al., 1994). Our data clearly demonstrate that such...
changes do not occur in mice having retarded humoral responses as a consequence of four topical applications of either acetone or ethanol. Furthermore, the results obtained in the MLR assay indicate that splenic T cells of solvent-treated mice are capable of interacting with, and being activated by, normal antigen-presenting cells. Consequently, the capacity of the T cell to respond and function following several types of stimulation does not appear to be suppressed in mice treated topically with acetone or ethanol. However, stimulation of T cells is dependent upon functional accessory cells. Rodents maintained on oral doses of ethanol sufficient to suppress the development of humoral immunity contain macrophages with altered or impaired function (Bagasra et al., 1988; Dorio, 1990; Szabo et al., 1993). The protocols employed in the current study do not assess directly the effects of topically applied solvents on accessory cell function. This is an area that possibly merits further investigation.

A variety of immune processes are systemically affected by topically applied TPA (reviewed by Reiners et al., 1995). For example, peripheral blood polymorphonuclear leukocytes and splenic monocytes/macrophages appear to undergo priming in mice treated topically with TPA (Sirak et al., 1991; Updyke et al., 1989). Reciprocally, splenic and lymph node natural killer cell activities are suppressed in similarly treated mice (Updyke et al., 1988), as are the abilities to develop CHS and delayed hypersensitivity reactions (Kodari et al., 1991; Zhao and Cao, 1994). Our current studies demonstrate that topically applied TPA alters splenic cellularity, T cell content, and in vitro proliferative responsiveness to B and T cell mitogens and modulates the suppressive effects of solvents on IgG PFC development in SSIN mice. Increases in splenic cellularity following limited topical TPA treatment have also been noted in SENCAR and B6C3F1 mice (Updyke et al., 1989, 1991a) and attributed to granulocyte recruitment and proliferation in the spleen (Schoof and Baxter, 1986). Similarly, reductions in the relative T cell composition of the spleen have been noted in SENCAR and B6C3F1 mice following topical TPA treatment (Updyke et al., 1991a). The basis for this latter effect is not known. The enhanced in vitro responsiveness of splenocytes isolated from TPA-treated mice to T and B cell mitogens cannot be attributed to compositional increases in splenic B or T cell contents. Preliminary studies indicate that supplementation of splenocyte cultures prepared from naive mice with serum isolated from SSIN mice treated topically with TPA (either 4× or 8×) dramatically enhances proliferative responses to both T and B cell mitogens. The factor(s) in the serum is not mitogenic by itself to splenocytes. The enhanced in vitro responsiveness of splenocytes derived from TPA-treated mice may be a consequence of prior in vivo exposure to the factor(s) present in the serum.

Adsorbed and circulating TPA is probably not responsible for the systemic effects of topically applied phorbol ester. First, a considerable percentage of topically applied TPA is functionally inactivated in the epidermis as a consequence of its being conjugated to long-chain fatty acids yielding TPA-20 acylates (Roesser et al., 1991). Furthermore, there are esterases in both the skin and the serum capable of hydrolyzing TPA to an inactive metabolite (Berry et al., 1978; Kadner et al., 1985). Second, in vitro pretreatment of splenocyte cultures with TPA prior to the addition of alloantigen or T and B cell mitogens suppresses thymidine incorporation and cell proliferation (Mastro and Mueller, 1978; Mastro et al., 1979; Mastro and Pepin, 1980). Thus, splenocytes isolated from naive mice that are treated in vitro with TPA differ from splenocytes derived from mice treated topically with TPA in their in vitro responses to mitogen stimulation. Third, exposure of splenocytes isolated from naive mice to TPA in vitro suppresses the in vitro development of PFCs following immunization (Castagna et al., 1980; Shopp and Munson, 1984). We have never detected a suppression of in vivo PFC development in mice treated topically with TPA (once corrections are made for the contributions of solvent). The systemic immunomodulation noted in our studies is most likely initiated by cytokines and factors produced and released by the skin as a consequence of TPA exposure.

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