Phorbol ester effects on splenic lymphocyte composition and cytotoxic T cell activities of SSIN mice: a strain deficient in CD8+ T cells

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SSIN mice are considerably more sensitive to the effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) in two-stage skin carcinogenesis protocols than are other strains and stocks of mice. Experiments were performed to determine whether there was an immunological basis for this sensitivity. SSIN mice were haplotyped and found to be H-2b. T cells represented ~31% of the splenic cellularity of non-treated SSIN mice, but ~44% in BALB/c, C57BL/6, B6.C5F1 and SENCAR mice. Splenic CD4+/CD8+ T cell ratios were ~4.2, 2.9, 2.4, 1.8 and 1.7 in SSIN, SENCAR, BALB/c, B6.C5F1 and C57BL/6 mice, respectively. The unusually high ratio in SSIN spleens was the consequence of reductions in CD8+ T cells. The ratio of CD4+/CD8+ T cells in SSIN thymocytes was similar to that measured in the spleen. The splenic cytotoxic T lymphocyte (CTL) activities of the various murine strains inversely correlated with their splenic CD4+/CD8+ ratios and their sensitivities in two-stage skin carcinogenesis protocols. Repeated in vivo topical treatment of SSIN mice with TPA caused significant decreases in splenic T cell contents, but affected neither the splenic CD4+/CD8+ T cell ratio nor the development of a CTL response upon allogeneic tumor challenge. SSIN mice also had very low splenic natural killer cell activities. Furthermore, relative to the other strains of mice, SSIN mice were poor responders upon alloantigen challenge in mixed lymphocyte response assays. These findings demonstrate that SSIN mice differ markedly from other strains of mice. This information was used to develop protocols (1). Of the many studied to date, probably the best characterized is the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA*).

Multiple components of the immune system are altered by topically applied TPA. In the immediate area of treatment mast cells are induced to degranulate (2) and neutrophil, macrophage and T cell contents increase as a consequence of recruitment from peripheral areas (3–5). Much of the cutaneous inflammation and epidermal hyperplasia associated with TPA treatment appears to be associated with the recruitment of these latter cell types (3–5). In addition, the morphology and functional properties of Langerhans cells (LC) are altered in the treated areas (6). LC are resident dendritic cells involved in cutaneous immunosurveillance processes. Specifically, they are involved in the recognition and processing of cutaneous antigens and the initiation of cell-mediated immune responses to the antigens. Exposure to TPA in vivo alters the ability of LC to present antigen to T cells (7) and suppresses the development of contact hypersensitivity, a cell-mediated immune reaction (8–11). It is unclear as to whether the modulation of LC function by TPA is germane to the mechanism of promotion. However, it has been reported that LC recognize cutaneous tumors and initiate cell-mediated immune responses (e.g., the generation of cytotoxic T cells) that ultimately contribute to rejection of the tumors (12–14).

Cytotoxic T cells are a subset of lymphocytes that express the surface antigens CD3 and CD8. They are involved in class I MHC-restricted recognition and destruction of a variety of infectious agents and tumor types (15–17). Indeed, modulation of CD8+ cytotoxic T lymphocyte (CTL) activities has been directly correlated with the rejection/growth of several tumor types (17). We recently discovered that the splenic CD8+ T cell content of SSIN mice is very low, relative to the values reported in the literature for other strains and stocks that are refractory to TPA promotion (unpublished data). The SSIN strain was derived by the inbreeding of SENCAR mice, a mouse stock that is exceptionally sensitive to a variety of chemical promoters in the two-stage skin carcinogenesis model (18). SSIN mice are even more sensitive to chemical tumor promoters than the parental SENCAR stock, based upon analyses of tumor multiplicities and the kinetics of papilloma appearance at varying doses of promoter (18,19).

Assessment of CTL activities in SENCAR and SSIN mice, and how these activities are affected by TPA, has been hindered by the absence of a characterization of their haplotypes. In the current investigation we determined the MHC haplotype of SSIN mice. This information was used to develop protocols to assess in SSIN mice, and other strains and stocks of mice that vary in their sensitivities to chemical tumor promoters, the in vivo development of a CTL response to an allogeneic tumor challenge. Analyses were also made of the effects of topically applied TPA on CTL development, since treatment of cultured human lymphocytes with low concentrations of TPA has been reported to suppress the development of HLA-dependent CTL activity (20). Lastly, we quantitated basal

Introduction

The murine skin two-stage carcinogenesis model has contributed greatly to the concept that the ontogeny of cancer is a multistage process. It has also proven to be a useful tool in the bioassaying of putative initiators and tumor promoters and unraveling their mechanisms of action. Indeed, dozens of chemicals have been identified as promoters in the two-stage skin carcinogenesis protocol (1). Of the many studied to date, probably the best characterized is the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA*).
natural killer (NK) cell activities in the same strains of mice. Unlike CTL reactions, NK recognition and lysis of tumor cells are MHC independent (21,22). Collectively, NK and CTL reactions constitute two of the major pathways by which the immune system controls tumor growth. Our data indicate that both pathways are depressed in SSIN mice and that there is an inverse relationship between sensitivities in two-stage carcinogenesis protocols and CTL activities.

Materials and methods

Chemicals
TPA and mitomycin C were purchased from 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). [Methyl-3H]thymidine (6.7 Ci/mmol) and sodium iodide (Na2111CrO4, 400–1200 Ci/μg) were purchased from NEN (Wilmington, DE). Acetone (Baker analyzed reagent) was purchased from J.T.Baker (Phillipburg, NJ). Hamster anti-mouse CD3-e-fluorescein isothiocyanate (FITC) (Boehringer-Mannheim, Indianapolis, IN), mouse anti-mouse Thy 1,2-phycocerythrin (PE) (Caltag, South San Francisco, CA), goat anti-mouse (gM+M+G-A)-FITC (Cappel Laboratories, Durham, NC), rat anti-mouse CD8–PE (Pharminex), rat anti-mouse L3T4–FITC and rat anti-mouse Ly2–FITC (the last two from Becton-Dickinson, San Jose, CA) were all purchased as direct conjugates to either FITC or PE fluorophores. The murine mastocytoma cell line P815 (derived from DBA/2 mice, H-2b) and the A/Sn mouse-derived T cell lymphoma cell line YAC-1 were purchased from ATCC (Rockville, MD).

Animals and haplotype
Female Super SENCAR Inbred (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 SENCAR, DBA/2, C57BL/6 and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Animals were quarantined for a minimum of 1 week before the initiation of any treatments. Mice were housed in microisolator cages and food and water were provided ad libitum. Animals were maintained on a 12 h light:12 h dark photocycle. At the time of shipping vendor-provided mice were confirmed to be free of the following infectious agents: Sendai virus, PVM, MHV, Mycoplasma pulmonis, Theiler's mouse encephalomyelitis virus, MVM, OPV (recently renamed mouse parvovirus), reovirus, encephalomyelitis virus, polyoma virus, LCM and EDIM. Sentinel mice were randomly chosen from shipments received from the vendors and housed in conventional caging. They were tested quarterly for the first seven infectious agents in the above list. Analyses were uniformly negative throughout the 2 year period required to perform the reported studies.

The SSIN were haploptyped as a consequence of a contractual service provided by Harlan UK Ltd. Haplotyping of the SSIN mouse was performed by the laboratory of Dr J.Klein of the Max Planck Institut für Biologie (Tubingen, Germany). Class I MHC typing was achieved by PCR utilizing primers designed specifically to amplify H-2K locus alleles. PCR products obtained from three splenic DNA preparations coded for several closed and different sequences. One of the sequences, present in all mice, was identical to the K locus of haplotype q. Class II MHC typing was achieved by PCR utilizing primers specific for the flanking sequences of exon 2 of the A locus. The sequence obtained revealed the presence of only one allele in all three mice and corresponded to haplotype q. Collectively, the sequencing analyses indicated that SSIN mice are H-2b. The H-2 haplotypes of C57BL/6, DBA/2, B6CF1 and BALB/c mice are b, d, bk and d respectively (23).

Animal treatments
The dorsal trunks of 6–8 week old mice were shaved with surgical clippers, 2–4 days prior to initiation by topical applications of 200 μl acetone or 2 μg TPA dissolved in acetone. Animals that had been nicked during the shaving process or in hair regrowth at the time of the first treatment were excluded from the study. Control animals were shaved and manipulated to simulate treatment, but not treated with acetone or TPA. With the exception of the mice used in CTL assays, mice were treated with acetone or TPA on Tuesdays and Saturdays.

The treatment regimens used in the in vivo CTL studies are summarized in Figure 1. Mice were treated topically before, after or before and after tumor injection with 200 μl acetone or 2 μg TPA dissolved in acetone. Animals that had been nicked during the shaving process or in hair regrowth at the time of the first treatment were excluded from the study. Control animals were shaved and manipulated to simulate treatment, but not treated with acetone or TPA. With the exception of the mice used in CTL assays, mice were treated with acetone or TPA on Tuesdays and Saturdays.

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Culture conditions
YAC-1 cells were grown in medium A (RPMI-1640 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) at 37°C in a humidified chamber containing 5% CO2. P815 cells were grown in medium B (DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine) at 37°C in a humidified chamber containing 5% CO2. To load YAC-1 cells with 31Cr, exponentially growing cells were pelleted, washed and resuspended in medium A (5–6×10^6 cells/400 μl) and incubated with 100 μCi Na2111CrO4 (400–1200 Ci/μg) for 1.5 h at 37°C in a humidified 5% CO2 chamber. Labeled YAC-1 cells were washed three times with modified medium A (FCS raised to 20%) and then resuspended and diluted with medium A to 10^7 cells/ml. To load P815 cells with 31Cr, exponentially growing cultures were washed and resuspended in medium B (5–6×10^6 cells/100 μl) and incubated with 200 μCi Na2111CrO4 for 1.5 h at 37°C in a humidified 5% CO2 chamber. Labeled P815 cells were washed three times with modified medium A and then resuspended and diluted with medium A to 10^8 cells/ml.

Preparation of single cell suspensions from tissues
Unless specified noted, mice were killed by cervical dislocation 2 days after the last topical application of acetone or TPA. Single cell suspensions were prepared from aseptically removed spleens, thymus and popliteal lymph nodes as described by Updyke et al. (24). Briefly, tissues were dispersed by gently pressing them through a stainless steel screen into RPMI-1640 medium supplemented with 10% heat-inactivated FBS. Cell suspensions were centrifuged at 300 g for 5 min and contaminating erythrocytes were lysed by washing cell suspensions in Tris-buffered 0.15 M ammonium chloride solution, pH 7.2. Cells were washed three times and then resuspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (complete medium). Nucleated cells were counted with a hemocytometer and viabilities were assessed by Trypan blue exclusion (routinely >90%).

Natural killer (NK) cell assay
The procedure described by Updyke et al. (25) was used to measure NK activity. Single cell suspensions of splenocytes prepared from control mice functioned as effector cells. 31Cr-Loaded YAC-1 cells were used as target cells. Analyses were performed in triplicate in U-bottomed 96-well microtiter plates containing 100 μl labeled target cells (10^6 cells) and 100 μl varied numbers of effector cells to achieve effector:target cell ratios of 25:1, 50:1 or 100:1. After 4 h incubation at 37°C in a humidified 5% CO2 incubator, the plates were spun at 400 g for 10 min and 100 μl supernatant fluid was removed for determination of radioactivity. As a control, 31Cr-labeled target cells were incubated with either medium (spontaneous release) or 1.0 N HCl.
been noted in other strains of mice and attributed to increases in cellularity of the spleen was elevated in mice treated four times with TPA (Table I). Increases in splenic cellularity was little effect on thymic cell contents. In contrast, the overall percentage of T cells in the spleen decreased following repeated topical applications of TPA (Table II). This decrease reflected the loss of both CD4+ and CD8+ T cells. Both T cell subsets were comparably affected, since the ratio of CD4+ to CD8+ T cells did not change following TPA application. The reductions in splenic T cell composition noted after eight TPA treatments were offset by increases in splenic B cell contents (Table II).

The thymus of SSIN mice contained primarily CD4+CD8+ double positive T cells, and virtually no B cells (Table II), CD4+ and CD8+ T cells constituted ~13-15% of the total thymic cellularity and were present at ratios similar to those measured in the spleen (compare Tables I and II). Neither the cellular composition of the thymus nor the thymic CD4+/CD8+ ratio were affected by repeated topical applications of TPA (Table II).

Splenic lymphoid phenotyping in various stocks and strains

Surface marker phenotyping of splenocytes was simultaneously performed on several strains and a stock of mice that differed in their sensitivities to phorbol ester promotion in two-stage carcinogenesis protocols (Table III). On a percentage basis the spleens of SSIN mice contained more B cells and fewer T cells than the other mice examined. They also had a significantly higher CD4+/CD8+ T cell ratio. This higher ratio reflected preferential reductions in CD8+ T cells (Table III). The CD4+/CD8+ ratios reported in Table III for C57BL/6, B6.C5F1 and BALB/c mice are very similar to values reported by other investigators (26,29-31).

The cellular contents of the spleens of the murine stock and strains listed in Table III differed from one another by more than 15% (unpublished data).

Cytotoxic T cell activities in various strains of mice

T cells expressing CD8+ are primarily responsible for the class I MHC-dependent cytotoxic/cytolytic activities of T lymphocytes and the activities measured in CTL assays (15,16). The strength of a CTL response is influenced by the haplotypes of the effector and target cells. Specifically, the CTL activities of effector/target cell pairings having a similar haplotype are generally lower than those achieved with pairings having dissimilar haplotypes. SSIN, SENCAR, C57BL/6 and B6.C5F1 all have haplotypes that differ from the P-815 tumor (H-2d) used in the CTL assay. Given their lower splenic T cell content and higher CD4+/CD8+ ratio, one would predict that splenocytes from SSIN mice would have CTL activities lower than those obtained with comparable numbers of splenocytes from SENCAR, C57BL/6 and B6.C5F1 mice. This prediction proved to be correct (Figure 2). The relative CTL activities of these mice ranked C57BL/6 > B6.C5F1 > SENCAR > SSIN and correlated directly with their CD4+/CD8+ ratios (compare Table III with Figure 2). The low CTL activity of BALB/c mice was expected since it, unlike the other mice tested, had the same haplotype (H-2d) as the P815 tumor used to elicit the development of cytotoxic T cells.
Table I. Effects of TPA on the cellularity of lymphoid tissues from SSIN mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of treatments</th>
<th>Number of mice</th>
<th>Total number of cells ($\times 10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Spleen</td>
<td>4</td>
<td>13</td>
<td>43.3 ± 3.9</td>
</tr>
<tr>
<td>Thymus</td>
<td>4</td>
<td>13</td>
<td>110.4 ± 14.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>8</td>
<td>13</td>
<td>52.8 ± 2.7</td>
</tr>
<tr>
<td>Thymus</td>
<td>8</td>
<td>13</td>
<td>98.1 ± 28.7</td>
</tr>
</tbody>
</table>

Nucleated cells were isolated and counted 2 days after the final topical application of 200 μl acetone or 2 μg TPA dissolved in acetone. Control mice were not treated with anything. Data represent means ± SE of 13 mice pooled from three experiments.

*Statistically greater than control and acetone-treated mice, $P < 0.05$.

Table II. Flow cytometric analyses of lymphocyte cell types isolated from TPA-treated SSIN mice

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tissue</th>
<th>Percent tissue population bearing surface marker</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Four treatments</td>
<td>Eight treatments</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Acetone</td>
</tr>
<tr>
<td>Total CD3+</td>
<td>Spleen</td>
<td>30.8 ± 1.1</td>
</tr>
<tr>
<td>CD4+</td>
<td>25.6 ± 1.5</td>
<td>23.5 ± 1.8</td>
</tr>
<tr>
<td>CD8+</td>
<td>6.2 ± 0.4</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>4.1 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Total B cell</td>
<td>52.2 ± 2.0</td>
<td>56.3 ± 2.7</td>
</tr>
<tr>
<td>Total Thyl.1+</td>
<td>98.1 ± 0.3</td>
<td>98.3 ± 0.3</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>81.0 ± 0.6</td>
<td>80.7 ± 1.8</td>
</tr>
<tr>
<td>CD8+</td>
<td>11.8 ± 0.4</td>
<td>11.9 ± 1.1</td>
</tr>
<tr>
<td>CD4+</td>
<td>2.5 ± 0.1</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>4.6 ± 0.2</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Total B cell</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

Nucleated cells were isolated and counted 2 days after the final topical application of 200 μl acetone or 2 μg TPA dissolved in acetone. Control mice were not treated. Data represent means ± SE of nine mice for the four and eight times splenocyte analyses and six mice for the thymocyte analyses.

*Statistically less than control group, $P < 0.05$.

Table III. Splenic B and T cell composition of different mouse strains and stocks

<table>
<thead>
<tr>
<th>Strain/stock</th>
<th>n</th>
<th>Percent of splenocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ig+</td>
</tr>
<tr>
<td>SSIN</td>
<td>12</td>
<td>51.9 ± 1.3a</td>
</tr>
<tr>
<td>SENCAR</td>
<td>5</td>
<td>32.9 ± 1.4</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>5</td>
<td>39.9 ± 0.9</td>
</tr>
<tr>
<td>B6.C3H/Fl</td>
<td>5</td>
<td>35.3 ± 1.5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>5</td>
<td>31.8 ± 0.7</td>
</tr>
</tbody>
</table>

Data represent the means ± SE of 5–12 untreated mice.

aSignificantly different from all other mice, $P < 0.01$.

bSignificantly less than all other mice except C57BL/6, $P < 0.05$.

Reported by all other mice, $P < 0.01$.

Effects of in vivo TPA treatment on the development of cytotoxic T cells

The treatment protocols depicted in Figure 1 were used to examine the effects of topically applied acetone and TPA, and the timing of treatment relative to tumor injection, on the in vivo development of cytotoxic T cells. The data in Table IV summarize the results obtained in the six protocols depicted in Figure 1. Although TPA appears to slightly stimulate CTL activities in several protocols, such stimulations were neither statistically significant nor reproducible. Furthermore, in several instances the effects of TPA could be attributed to the solvent used for its application. Consequently, it appears that application of TPA prior to, after, and prior to and after tumor injection has very little effect on the development of CTL activities.

Our results are in agreement with the studies of Murray et al. (32). These authors reported that s.c. administered TPA, at doses 300-fold in excess of the dose topically administered.
Alloantigen induced-lymphocyte proliferation obtained with the other allogeneic pairings. This prediction MLR responses were statistically significant, the magnitudes pairing would produce a MLR response less than that, it was anticipated that the BALB/c-DBA/ mice are also H-2d were used as the stimulator population. Since BALB/c 2d MLR assays are commonly used to approximate the early restricted interaction of CD8 + T cells, T cell activation and a class I MHC-restricted interaction of CD8 + T cells with antigen. In vitro MLR assays are commonly used to approximate the early stages in this process (33). The studies summarized in Figure 3 represent one-way MLR assays in which DBA/2 mice (H-2b) were used as the stimulator population. Since BALB/c mice are also H-2d, it was anticipated that the BALB/c-DBA/2 pairing would produce a MLR response less than that obtained with the other allogeneic pairings. This prediction was basically correct. However, although the differences in MLR responses were statistically significant, the magnitudes by us, had minimal effects on the development of CTL activities in B6.C3F1 mice.

**Alloantigen induced-lymphocyte proliferation**

Several processes are involved in the development of an in vivo CTL reaction. These include antigen recognition, processing and presentation to CD4+ T cells, T cell activation and release of cytokines, CD8+ T cell activation and a class I MHC-restricted interaction of CD8+ T cells with antigen. In vitro MLR assays are commonly used to approximate the early stages in this process (33). The studies summarized in Figure 3 represent one-way MLR assays in which DBA/2 mice (H-2b) were used as the stimulator population. Since BALB/c mice are also H-2d, it was anticipated that the BALB/c-DBA/2 pairing would produce a MLR response less than that obtained with the other allogeneic pairings. This prediction was basically correct. However, although the differences in MLR responses were statistically significant, the magnitudes of the differences between SENCAR, SSIN and BALB/c mice were very small (Figure 3). The MLR responses occurring in SENCAR and SSIN mice were statistically indistinguishable from one another and significantly less than those occurring in either B6.C3F1 or C57BL/6 mice (Figure 3). Overall, the

<table>
<thead>
<tr>
<th>Table IV. Effects of topically applied TPA on CTL activities in SSIN mice</th>
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<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>25:1</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>25:1</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
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<td>E</td>
</tr>
<tr>
<td>25:1</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>25:1</td>
</tr>
<tr>
<td>50:1</td>
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</tbody>
</table>

SSIN mice were topically treated with 200 μl acetone or 2 μg TPA dissolved in acetone at various times relative to i.p. injection of P815 tumor cells. Control mice received no topical treatments, but were injected with tumor cells. Spleens were removed 10 days after tumor injection and used in in vitro CTL assays. The data represent 11 separate experiments, each involving four to five mice per treatment group, and are expressed as means ± SE.

*Treatment protocols are depicted in Figure 1.

**Fig. 2.** Strain/stock-dependent variations in splenic cytotoxic T cell activities. Mice were injected i.p. with P815 tumor cells 10 days prior to being killed and the harvesting of spleens for use in CTL assays. Data represent means ± SE of a minimum of five mice of each strain or stock. *Significantly greater than control group, P < 0.05. Statistically greater than acetone group, P < 0.05. Statistically less than control group, P < 0.05.

**Fig. 3.** MLR responses of splenocytes from various murine strains. Cultured splenocytes from five different strains/stock were exposed to either DBA/2 splenocytes (allogeneic response) or their own splenocytes (autologous response) Data represent the differences of the allogeneic and autologous responses and are means ± SE of a minimum of five mice. The results of two independent experiments are presented. *Significantly less than the response obtained with C57BL/6 and B6.C3F1 and SENCAR mice, P < 0.05.бал/с were not analyzed at the 50:1 effector:target ratio.

**Fig. 4.** Effects of topically applied TPA on CTL activities in SSIN mice.
The spleens of SSIN mice contained lower percentages of both CD4+ and CD8+ T cells than the other murine strains investigated in this study. However, the largest percentage reduction occurred in the CD8+ T cell population. Since the ratio of CD4+ to CD8+ T cells in the thymus was virtually identical to that measured in the spleen (Table II), it would seem that the observed splenic CD4+/CD8+ ratio in SSIN mice reflects the preferential selection/maturation of thymic CD4+CD8+ double positive T cell precursors to CD4+ T cells, as opposed to a preferential trafficking of thymic CD4+ T cells to the spleen. This preferential selection/maturation of thymic CD4+CD8+ double positive T cell precursors to CD4+ T cells in SSIN mice appears to have developed as a consequence of the failure of the immune system to recognize and eliminate an emerging neoplasm (39). Studies designed to examine the role of immunosurveillance processes in two-stage carcinogenesis protocols have yielded equivocal results (reviewed in 40). For example, comparative analyses of athymic and euthymic mice in initiation–promotion protocols have both implicated (41) and discounted (42) a role for T cell-mediated processes in regulating papilloma development/rejection. Furthermore, assessment of the relationships between T cell function and promotion processes has been confounded by the absence of information (e.g. knowledge of haplotype) critical to the design of studies intended to examine these relationships and the preponderant use of outbred stock of mice (e.g. SENCAR and CD-1) in initiation–promotion protocols. Although inbreeding of the SENCAR stock to produce the SSIN strain eliminated potential complications arising from histocompatibility differences between mice, there was still the issue of haplotype. In this study we report that the haplotype of the SSIN mouse is H-2k and used this information to assess CTL activities, an important component of the immune surveillance system, in control and TPA-treated mice.

SSIN mice developed a weak CTL response, relative to other murine strains, upon challenge with allogeneic tumor cells. Indeed, the CTL activities of the mice used in this investigation ranked SSIN < SENCAR < BALB/c < C57BL/6 < B6C3F1. The inverse of this ranking defines the relative susceptibilities of these same mice to TPA in two-stage skin carcinogenesis protocols (43–46). Hence, mice with the lowest cytotoxic T cell activities are the most sensitive in two-stage skin carcinogenesis protocols. However, the functional significance of this inverse relationship is dependent upon several unresolved issues. First, the development and activation of CD8+ T cells is dependent upon the antigenicity and co-stimulating activities of their targets (17). Second, the CTL must be recruited to the site of the developing tumor and remain active upon infiltrating the tumor. To date, there are no published data on the antigenicity/co-stimulating activities of tumors developing in two-stage skin carcinogenesis protocols or their T cell contents or the functional status of the infiltrating T cells. These are areas that we are actively pursuing.

The low CTL activities detected in SSIN mice may be the consequence of several factors. CD8+ T cells constituted ~6% of the splenic cells of SSIN mice, but ~15% in the other strains/stock investigated. Consequently, in any given number of splenocytes, SSIN mice would contain considerably fewer CD8+ T cells than are found in the other strains/stock. Alternatively, processes involved in T cell activation may be muted in SSIN mice. The development of an in vivo CTL reaction is dependent upon antigen recognition, processing and presentation to CD4+ T cells, T cell activation and release of cytokines, CD8+ T cell activation and a class I MHC-restricted interaction of CD8+ T cells with antigen. One-way MLR assays are commonly used to approximate the early stages in this process and indicated that T cells from SSIN mice are markedly less responsive to alloantigen than several other strains of mice having higher CTL activities (see Figure 3). Performance of CTL assays with enriched populations of CD8+ T cells should facilitate assessment of whether the low in vivo CTL activities of SSIN mice reflect the paucity of CD8+ T cells and/or a defect in processes involved in CD8+ T cell activation or their function.

Discussion

Two theories have evolved which evoke different roles for the immune system in the process of tumor development. In the ‘immunostimulation/immunofacilitation’ theory it is proposed that components of the immune system actually contribute to the process of promotion by phorbol esters. Systemic or local injections of anti-IL-1 (36,37) and anti-GM-CSF (38) cytokines, CD8+ T cell activation or their function. One-way MLR assays are commonly used to approximate the early stages in this process and indicated that T cells from SSIN mice are markedly less responsive to alloantigen than several other strains of mice having higher CTL activities (see Figure 3). Performance of CTL assays with enriched populations of CD8+ T cells should facilitate assessment of whether the low in vivo CTL activities of SSIN mice reflect the paucity of CD8+ T cells and/or a defect in processes involved in CD8+ T cell activation or their function.

The spleens of SSIN mice contained lower percentages of both CD4+ and CD8+ T cells than the other murine strains investigated in this study. However, the largest percentage reduction occurred in the CD8+ T cell population. Since the ratio of CD4+ to CD8+ T cells in the thymus was virtually identical to that measured in the spleen (Table II), it would seem that the observed splenic CD4+/CD8+ ratio in SSIN mice reflects the preferential selection/maturation of thymic CD4+CD8+ double positive T cell precursors to CD4+ T cells, as opposed to a preferential trafficking of thymic CD4+ T cells to the spleen. This preferential selection/maturation of thymic CD4+CD8+ double positive T cell precursors to CD4+ T cells in SSIN mice appears to have developed as a consequence of the inbreeding protocol, since the parental stock (SENCAR mice) has a markedly different splenic CD4+/CD8+ T cell
ratio. The SENCAR–SSIN pair may prove to be very useful in elucidating the processes involved in thymic T cell matura-
tion and selection.

A variety of local and systemic immune processes are
differentially affected by topically applied TPA (reviewed in
40). For example, splenic and lymph node NK cell activities
are suppressed in mice treated topically with TPA (24), as are
the abilities to develop CHS reactions (8–11). In contrast,
topical treatment of SSIN mice with TPA prior to sensitization/
immunization with antigen does not suppress the subse-
quent development of either a delayed hypersensitivity reaction
(DHS) (11) or humoral immunity (47). Indeed, in our DHS
studies it appeared that topically applied TPA may have
actually stimulated the development of DHS. The differential
effects of TPA on CHS and DHS are of particular interest
since they suggest that distinct subsets of T cells are associated
with these processes. The differential effects of methylpredni-
solone (48) and IL-10 (49) on the development of CHS
and DHS reactions have led other investigators to a similar
conclusion. Our current studies corroborate the observation
by Updyke et al. (28) that the spleens of mice treated topically
with TPA have a lower percentage of T cells than the
spleens of mice treated with acetone. However, we demonstra-
tion additional that TPA does not alter the splenic CD4+/CD8+
T cell ratio (see Table II). Furthermore, we show that control
and TPA-treated SSIN mice develop comparable CTL
responses upon i.p. injection of allogeneic tumor cells. Indeed,
there may even be a small stimulation of CTL activities in
TPA-treated mice if one considers that the spleens of mice
treated topically with TPA contain fewer CD8+ T cells (eight
times treatment data in Tables I and II). However, the relevance
of the data generated in our CTL assays to the process of
two-stage skin carcinogenesis is questionable. Our protocol
examined primarily the systemic, as opposed to local, con-
sequences of topical TPA application on CTL development.
Given the prevalence of multiple systems in both the skin and
serum capable of inactivating TPA (50–52), it is quite probable
that circulating levels of TPA are very low in mice treated
topically. If so, and if TPA is actually capable of suppressing
the development of CTL responses, our protocol does not
model events that might occur locally in skin following topical
application.

NK cells are also involved in tumor rejection. However,
unlike cytotoxic T cells, they are neither MHC nor antigen
restricted (21,22). Updyke et al. (25) reported that SENCAR
mice had splenic NK activities that were ~50% those measured
in B(6)C3F1 mice. The relative basal NK activities of the mice
used in this study ranked B(6)C3F1 > C57BL/6 > SENCAR >
SSIN > BALB/c. If one excludes BALB/c mice, the rank
order of basal NK activities in the mice we investigated inversely
correlates with their susceptibility in two-stage carcinogenesis
protocols. Additional experimentation is required before one
can determine whether this correlation is coincidental in nature
or reflects a genuine relationship.

The SSIN mouse has several immunological features that
distinguish it from many other immune-competent murine
strains and stocks. Most notably, it has low NK and CTL
activities and is not particularly responsive in MLR assays. It
is also very poor at developing primary and secondary antibody
responses following immunization with T cell-dependent anti-
gens (47). Although an alteration in any one of these parameters
is unusual, it is unusual for all of these parameters to be
low in a naïve, presumably immune-competent mouse. The
basis for the immunological profile of the SSIN mouse is not
known, however, all of the aforementioned parameters either
directly involve T cells or are dependent upon cytokines
produced by T cells (e.g. IL-2). We are currently attempting
to determine the underlying basis for the immunological profile
of the SSIN mouse.

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