ABSTRACT

Although 12-O-tetradecanoylphorbol-13-acetate (TPA) can synergize with lectins to enhance lymphocyte proliferation, pretreatment of lymphocytes with TPA decreases their response. Pretreatment also inhibits the response to allogeneic cells in the mixed lymphocyte culture. In this study we determined that at least part of this inhibition was due to the generation of T-lymphocytes with the ability to suppress proliferation in vitro.

By using populations enriched in lymphocytes or macrophages we determined that the interaction of TPA with lymphocytes, but not macrophages, was required to mediate the suppression. The number of macrophages present in culture (range, 0.5-10%) was irrelevant to the generation of inhibitory activity. Moreover, the TPA-induced suppressor activity copurified with T-cells. Furthermore, when peanut lectin (agglutinin) (PNA) was used to separate T-cells after treatment with TPA, essentially all of the activity copurified with the PNA positive cells. When PNA separations were carried out before treatment with TPA, the suppressor activity arose from the PNA negative fraction. Therefore, TPA appeared to cause phenotypically PNA negative T-cells to gain the PNA positive marker, as well as to function as suppressor cells in vitro.

Suppressor activity was also found in the culture medium. Thus the suppression observed may be mediated through a soluble factor released by the TPA-treated cells. Although the suppressor cell activity induced by TPA can only partially account for its in vitro inhibition of lymphocyte proliferation, the development of suppressor cells merits further study with respect to lymphocyte phenotypic and functional differentiation. The results also suggest the possibility that similar processes could occur in vivo, possibly during the course of tumor promotion.

INTRODUCTION

TPA, the most potent tumor-promoting compound derived from croton oil (1), promotes skin tumors in mice which have been initiated with a carcinogen such as dimethylbenzanthrazine. Following a single application of dimethylbenzanthrazine, repeated treatments with TPA are required. While TPA has been shown to cause a necessary, but not sufficient, round of cell division (2), other, as yet not fully described activities, appear important in tumor promotion (3).

In an attempt to understand the biological and biochemical effects of TPA it has been tested on many cells in vitro (4). Much of this work has been done with mononuclear leukocytes (for reviews see Refs. 5 and 6). In particular, with lymphocytes, several in vitro effects on proliferation have been observed. For example, TPA can enhance proliferation with lectins and a number of other compounds. However, direct addition of TPA to allogeneic cells in culture inhibits the MLR of bovine, murine, and human cells (7-9). Furthermore, pretreatment of lymphocytes with TPA depresses their proliferation in response to mitogenic lectins, such as ConA, or to allogeneic cells (10).

Recently we demonstrated that some of this inhibition may be due to activation by TPA of cells with suppressor activity in vitro (11). This activity was assayed as the ability of TPA treated, irradiated LNC to inhibit the response of untreated autologous or allogeneic lymphocytes to mitogenic stimulation. This suppression was not due to carryover of TPA, release of thymidine, changes in thymidine pool size, or changes in ability of the cells to incorporate [3H]thymidine (11-13). Neither the induction of suppressor cells nor their activity was due to production of prostaglandins, the expression of IL-2 receptors, inhibition of IL-2 secretion, or depletion of IL-2 (11).

In this present study we attempted to identify and purify the cells responsible for suppressor activity. We also compared the effect of addition of cells with the direct addition of TPA on proliferation. For example, it was not known if macrophages mediated the inhibitory effects of TPA in either case. When purified macrophages and lymphocytes were pretreated with TPA and tested in mitogenic assays, only the treatment of the lymphocytes resulted in inhibitory or suppressor activity. Moreover, the suppressor activity was enriched in purified T-cell populations. Specifically, when T-cells fractionated with PNA were treated with TPA, the precursors were PNA negative T-cells, but the effectors were PNA positive T-cells. These results are consistent with the idea that suppressor cells differentiated from PNA negative precursor cells in the presence of TPA.

MATERIALS AND METHODS

Materials. Solutions of TPA and PDBu obtained from CCR (Eden, MN), were prepared as previously described (11). They were used at a final concentration of 10^-7 M, 0.1% dimethyl sulfoxide, in RPMI 1640 medium with 10% calf serum (CS), and gentamicin (50 µg/ml) (complete medium). PNA obtained as an electrophoretically pure, lyophilized powder (Sigma, St. Louis, MO; or PL-Pharmacia Milwaukee, WI) was dissolved in PBS (pH 7.2), to 1 mg/ml, and stored at -20° until used.

Preparation of Macrophage-depleted Lymphocytes and Purified Total T-Cells. Cells were isolated from retropharyngeal lymph nodes or from thymus of beef cattle taken at the time of slaughter as previously described (14). Typically the cells were more than 90% viable (range, 75-95%) as determined using trypan blue exclusion. Macrophages were depleted by incubating the LNC twice for at least 1 h each in plastic tissue culture dishes at 37°C, followed by one pass of the nonadherent cells over a cotton column (15, 16). Recovery from the columns was about 65% (range, 53-80%). These cells, hereafter referred to as "lymphocytes," contained on average 95% lymphocytes, 72% T-cells (57% Ea and 40% En), 27% B-cells, and less than 0.5% macrophages as assessed by rosetting and differential staining. For some experiments these macrophage-depleted lymphocytes, were further treated with a polyclonal antimacrophage serum (16).
TPA-INDUCED T-SUPPRESSOR CELL DIFFERENTIATION

For one experiment macrophages were removed using Sephadex G-10 columns (17). The recovery was 58% of the starting cells. The cells were 92% viable, and contained 98% lymphocytes, 75% T-cells (61% Eα and 39% Eν), and less than 0.5% macrophages.

Where indicated, B-cells were removed by a modification of the panning technique of Wysocki and Sato (18) using rabbit anti-bovine-IgM beads and light-chain specific antibody (Cappel, Cochransville, PA). The yield of nonadherent T-cells was 70–85% of the total lymphocytes. In order to quantitate the number of T-cells, the percentage of cells forming Enα and Eα rosettes was determined (19). The cells were greater than 95% lymphocytes and 90% T-cells (81% Eα and 63% Eν) by differential staining and rosetting assays.

Preparation of Macrophages. LNC at 2 x 10⁶/ml were placed in large rectangular plates (150 cm², NUNC), 50 μl per plate for 24 to 48 h. The nonadherent cells were removed, the plates were washed with complete medium, and 35 ml of Hank's balanced salt solution with 2.5 mM EDTA (pH 7.2), 37°C, was added. After a 30- to 45-min incubation with rocking, the cells were gently scraped from the surface with a rubber policeman, and collected. This process was repeated. The macrophages were pooled, centrifuged at 400 x g for 10 min and resuspended in complete medium to 1 x 10⁶ macrophages/ml. The recovery of macrophages (70–85% viable) was about 0.8–1.5 x 10⁶/plate. The cells were 87 ± 6% macrophages by differential staining. Where indicated the macrophages were added directly to the cells in the assay mixtures. Assays were done in duplicate for each sample and the means are reported.

Treatment of Cells with TPA and PDBu. LNC, lymphocytes, T-cells, and thymocytes were cultured at 1 x 10⁶/ml in complete medium plus or minus 10⁻⁷ M TPA or PDBu at 37°C. After the times indicated the cells were washed three times in PBS and resuspended to 6 x 10⁶/ml in complete medium. Previous studies with [³H]TPA and [³H]PDBu have shown that from 0.3–3% of the phorbol ester compounds remain in the cells after washing (11, 13). Adherent macrophages, treated with TPA were also washed three times with PBS prior to harvest.

Assay of the Mixed Lymphocyte Response and Suppression of the Mixed Lymphocyte Response. Two-way allogeneic MLC were prepared by mixing equal numbers of LNC (6 x 10⁶ cells/ml) from two animals. The cells were cultured in triplicate in round-bottom, 96-well plates at 0.2 ml/well, and incubated at 37°C. The MLR was measured by addition of [³H]thymidine, 1 μCi/ml, 6.7 Ci/mM, (ICN, Cleveland, OH) for 6 h on both days 4 and 5. The cells were harvested onto glass fiber filter papers using a Skatron multiwell harvester (Skatron, Sterling, VA). [³H]Thymidine incorporation was measured by liquid scintillation spectrophotometry (Model LS-5801, Beckman).

Suppressor cell activity was determined by adding 6 x 10⁶ autologous, treated, irradiated (1000 rads) cells to the MLC as described previously (11). For comparison, irradiated cells which were not treated were added to replicate cultures. As a further control, a MLC without added cells was also carried out. For the experiments presented herein, the MLR without added cells and that obtained with the addition of untreated cells were not significantly different. In all assays [³H]thymidine incorporation was also measured for cultures containing only autologous cells. Suppression was calculated by the formula where t indicates TPA-treated cells and u indicates untreated cells:

\[
%S = \left[ 1 - \frac{(cpm\ MLC + t) - (cpm\ autologous\ cells + t)}{(cpm\ MLC + u) - (cpm\ autologous\ cells + u)} \right] \times 100 \quad (A)
\]

Peanut Lectin Separation of LNC and Purified T-Cells. LNC were separated by agglutination with PNA (20). The LNC were washed with PBS, resuspended to 1 x 10⁶ cells in 1 ml of a PNA solution (PBS, 1 mg/ml), and incubated at 37°C for 10 min. They were layered over 10 ml of 50% CS in PBS for the Sigma PNA or 100% CS for the Connaught PNA, as was found necessary for optimum separation. Samples of larger than 1 ml could not be separated cleanly. Separation was carried out at unit gravity for 30 to 45 min at 37°C until the cells stopped "raining" through the serum. Cells floating at the serum interface were collected as "PNA ~" cells. Those immediately below the interface were discarded to minimize contamination. The "PNA ~" cells were collected by centrifugation of the remaining serum. The cells from both of the recovered fractions were incubated with a 0.2 μl galactose solution for 10 min at 37°C and washed by resuspension and centrifugation in PBS to remove the PNA. An average of 75 ± 12% of the starting LNC was recovered after separation. Of the recovered cells 50 ± 13% separated into the PNA ~ fraction and 47 ± 9% separated into the PNA + fraction. The viabilities of the cells in each fraction were typically greater than 85% and always greater than 72%.

Purified T-cells were also subjected to PNA separation. An average of 80% of the starting T-cells were recovered; the cells were greater than 80% viable. Of the T-cells recovered 31 ± 3% were in the PNA ~ cell fraction and 68 ± 2% were PNA +.

In order to determine the purity of the fractions, cells from some of the separations were also marked with fluoresceinated PNA (FITC-PNA, Sigma, St. Louis, MO). The cells, 0.1 ml, washed and resuspended in PBS to 1 x 10⁷/ml, were incubated with 0.1 ml of FITC-PNA (250 μg/ml in PBS) for 10 min in the dark at room temperature. They were washed twice by resuspension in cold (4°C) PBS for 5 min, after which the percentage of fluorescing cells was determined with a phase, epifluorescent microscope (E. Lietz, New York, NY). Cells (200–400) were counted for each determination. The LNC averaged 51 ± 7% PNA ~ cells. Cells from the PNA ~ fraction were 85–92% positive, and cells from the PNA + fraction were 12–22% positive (range for counts of cells from five experiments). The purity of the PNA ~ and PNA + fractions of TPA-treated and -separated cells were similar.

Treatment of LNC, PNA ~, and PNA + Cells with TPA before and after PNA Separation. LNC, PNA ~, and PNA + cells, 1 x 10⁷/ml, were incubated in complete medium plus or minus 10⁻⁷ M TPA for 24 h at 37°C. The recoveries of TPA-treated cells were equivalent to those of untreated cells for all populations. The cells were washed three times in PBS; and 1 x 10⁶ cells of each population was resuspended in complete medium for the MLR suppression assay. The remainder of the recovered LNC, PNA ~, and PNA + cells (5 x 10⁶ to 1 x 10⁷ cells) were separated (LNC) or re-separated (PNA ~, PNA +) with PNA. The separated cell fractions were washed and resuspended in complete medium for use in the suppressor cell assay. Recovery of the cells measured after both the TPA treatment and PNA separation was between 65 and 90% of the starting cell populations.

Assay for Soluble Suppressor Activity. Cells from the LNC, PNA ~, or PNA + fractions were incubated in complete medium plus or minus 10⁻⁷ M TPA for 24 h, washed, irradiated to 1000 rads, and held at 1 x 10⁶ cells/ml for 18 h. The supernatant, removed from the cells by centrifugation, was filtered through a 0.22-μm syringe filter (Millipore, Medford, MA) and added to MLC as 0, 10, 20, 30, 40, and 50% of the medium volume. The percentage of suppression was calculated by the following formula where t is the supernatant from TPA-treated cells and u is the supernatant from untreated cells:

\[
%S = \left[ 1 - \frac{(cpm\ MLC + t) - (cpm\ MLC + u)}{(cpm\ MLC + u)} \right] \times 100 \quad (B)
\]

The number of units of suppressor activity in the supernatants was calculated as the reciprocal of the 2-fold dilution required to cause 50% suppression of the MLR.

In a similar assay, 100 μl of culture media from TPA-treated cells was transferred directly from microwell cultures with a Handispense Plate Feeder (Sandy Spring Inst. Co., Ijamsville, MD) to an MLC containing 1.2 x 10⁶ cells/ml (100 μl), yielding an MLC with 6 x 10⁶ cells/ml and 50% conditioned supernatant.

RESULTS

Effect of TPA on Lymphocytes and Macrophages. Previously we demonstrated that LNC pretreated with TPA were unable to undergo an MLR (14) and were depressed as much as 60% in lectin-driven proliferation (10). In order to determine if the inhibitory effect of TPA on proliferation was due to a direct effect on the lymphocytes or, to an indirect effect through the macrophages in the culture, the two cell types were separated,
treated with TPA, and recombined in a ConA mitogenesis assay or in a two-way MLC. First, as previously observed (16), lymphocytes depleted of macrophages no longer proliferated in response to ConA (Fig. 1). However, addition of 0.5% macrophages allowed lymphocyte division as measured by \(^{3}H\) thymidine incorporation (Fig. 1). Addition of either untreated macrophages or macrophages treated with TPA to untreated lymphocytes restored proliferation of the lymphocytes to the same extent. In contrast, addition of either untreated or TPA-pretreated macrophages did not restore the response of TPA-treated lymphocytes to ConA. These results indicated that TPA acted directly on the lymphocytes rather than through the macrophages.

In a similar experiment TPA-treated or -untreated macrophages were added to two-way MLCs composed of purified, untreated lymphocytes (Fig. 2). The MLC did not occur in the absence of macrophages but was fully reconstituted by the addition of either TPA-treated or -untreated macrophages.

Effect of Macrophages on the Development of TPA-induced Suppressor Cells. Previously we found that TPA-treated LNC suppressed proliferation of lymphocytes undergoing an MLR (14). Therefore, we attempted to determine which cells in the LNC were responsible for this effect. We first examined the role of macrophages by comparing the induction of suppressor cell activity by TPA in the presence and absence of macrophages. There was no significant difference in the suppression induced in cultures of lymphocytes depleted of macrophages compared with the total LNC (Table 1). In fact, for five of seven experiments, the percentage of suppression was enhanced in the lymphocyte preparation as compared to the LNC.

To further test the role of macrophages, they were added back to lymphocytes which had been depleted of macrophages. Confirming the previous finding, there was no difference in the amount of suppression seen with LNC or macrophage-depleted cells. The addition of 1 or 2% macrophages to the LNC and macrophage-depleted lymphocytes had no effect on the development of suppressor activity by TPA (Table 2). Furthermore, when the percentage of macrophages was reduced more than 14-fold, from 7 ± 1% in the total LNC to less than 0.5%, there was no accompanying loss of suppressor activity. Finally, addition of macrophages to almost twice the number present in the LNC did not increase the amount of induced suppressor activity. These results taken together suggested that macrophages were not required for the generation of TPA-induced suppressor cells.

Enhancement of TPA Suppressor Activity in Preparations Enriched in T-Lymphocytes. In an attempt to further identify the cell population in which the TPA-induced suppressor activity resided, LNC were compared with total T-cell preparations purified from the LNC by removal of macrophages on cotton columns and by removal of B-cells by use of anti-IgM-coated plates. In some experiments thymocytes were also isolated and compared. The LNC, total T-cells, and thymocytes were treated with \(10^{-8}\) M TPA and assayed for suppression of the MLR.

There was an increase in the percentage of suppression induced with purified T-cells (69%) compared to the total LNC (36%) (Table 3). The activity induced from thymocytes (80%) was generally higher than that induced from purified T-cells (four of six experiments) even though there was no significant difference in the total percentage of T-cell (92 versus 90%, respectively). This result with thymocytes may reflect the fact that these cells mark predominantly both En\(^+\) and Ea\(^+\) (an average of 70 ± 6%, while the T-cells derived from LNC are more heterogeneous (an average of 54 ± 15% En\(^+\) and Ea\(^+\) with a range of 36 to 71%). In general, these data suggested that T-cells were responsible for the TPA-induced suppressor cell activity and further supported the finding that macrophages were not the suppressor cells nor required for their activity.

Relationship between En and Ea Rosetting Markers and the PNA Marker. In addition to the observation with the T-cells and thymocytes that the presence of Ea\(^+\) and En\(^+\) markers possibly correlated with the induction of the suppressor activity, it had also been observed that treatment of LNC with TPA caused a decrease in the percentage of En\(^+\) cells without affecting the percentage of Ea\(^+\) cells (21). We had hoped to use rosetting to further separate the subpopulations. However, rosetting was not practical for recovering large numbers of cells. Therefore, we tested three lectins, PNA Helix pomatia lectin, and wheat germ agglutinin, which bind to bovine T-cells (22, 23). It was observed that populations similar to those identified by En and Ea rosetting could be generated by separation with PNA. Starting with purified T-cells, a unit gravity flotation technique was used to separate PNA-treated cells (20). On average in four experiments 65 ± 10% of the T-cells segregated in the PNA\(^+\) fraction, and 34 ± 9% in the PNA\(^-\) fraction (Table 4). When the cells in the PNA\(^+\) fraction were evaluated by microscopy for binding of fluorescently labeled PNA, an average of 85 ± 5% (mean ± SD) were FITC-PNA\(^+\). These cells were also 25 ± 4% En\(^+\) and 85 ± 7% Ea\(^+\). Similar marker analysis for the PNA\(^-\) fraction gave 12 ± 5% FITC-PNA\(^-\), 96 ± 3% En\(^+\), and 95 ± 2% Ea\(^+\). Therefore, although the Ea marker segregated almost equally with the PNA\(^+\) and PNA\(^-\)
LNC were prepared as described in "Materials and Methods." The purified lymphocytes were prepared by macrophage depletion on cotton columns. The cells were washed, irradiated, and added 1:1 to two-way MLC. Differential cell counts on 300–400 cells per sample from the animals used in experiments 1 to 4 averaged 7 ± 1% macrophages for LNC and less than 0.5% macrophage for purified lymphocytes. Rosetting showed no significant difference in percentage of T-cells for LNC (70 ± 6%) and purified lymphocytes (73 ± 8%).

Use of PNA to Segregate TPA-induced Suppressor Cells. PNA separation and marking was used to search for the population of cells involved both in the induction phase and the effector phase of the TPA-induced suppressor activity. In the first approach LNC were treated with TPA prior to separation into PNA* and PNA− populations. Each fraction was tested for suppressor activity (Fig. 3). It was seen that almost all of the suppressor activity segregated with the PNA* fraction (Fig. 3, I) when the cells were first treated with TPA and then separated. The LNC averaged 66% suppression while the PNA* showed 83% suppression. In contrast, the PNA− cells showed almost no activity. This lack of suppression was not due to a loss of suppressor activity. It was observed that when the separated PNA* and PNA− fractions were incubated with TPA (Fig. 3) almost all of the suppressor activity segregated with the PNA* fraction (Fig. 3, II).

Therefore in the converse experiment, LNC were first separated into PNA* and PNA− fractions, and secondly treated with TPA (Fig. 3, II and III). These cells were separated again after the TPA treatment. At each stage they were tested for suppressor activity. It was observed that when the separated PNA* and PNA− fractions were incubated with TPA (Fig. 3) almost all of the suppressor activity was induced in the cells from the PNA− fraction (82%) compared with the PNA* fraction (4%). Again

### Table 1 Comparison of TPA-induced suppressor cell activity in total LNC or in lymphocytes depleted of macrophages

<table>
<thead>
<tr>
<th>Experiment</th>
<th>MLR of LNC</th>
<th>MLR + untreated LNC</th>
<th>MLR + TPA- treated LNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>37,568 ± 8,915</td>
<td>52,055 ± 6,234</td>
<td>44,797 ± 19,818</td>
</tr>
<tr>
<td>2a</td>
<td>4,497 ± 1,010</td>
<td>9,052 ± 4,980</td>
<td>6,635 ± 935</td>
</tr>
<tr>
<td>3a</td>
<td>27,341 ± 1,038</td>
<td>26,534 ± 1,227</td>
<td>15,925 ± 806</td>
</tr>
<tr>
<td>4a</td>
<td>21,337 ± 2,025</td>
<td>13,721 ± 1,091</td>
<td>9,879 ± 1,206</td>
</tr>
<tr>
<td>5a</td>
<td>13,654 ± 650</td>
<td>38,216 ± 5,055</td>
<td>3,667 ± 2,799</td>
</tr>
<tr>
<td>6a</td>
<td>36,541 ± 3,212</td>
<td>22,515 ± 1,816</td>
<td>8,464 ± 1,015</td>
</tr>
<tr>
<td>Total</td>
<td>36,541 ± 3,212</td>
<td>28,015 ± 1,611</td>
<td>7,205 ± 1,131</td>
</tr>
</tbody>
</table>

Suppression of the MLR (%) = (Control cpm - Experimental cpm) / Control cpm × 100

### Table 2 Effect of addition of macrophages to LNC and to lymphocytes depleted of macrophages on the induction of suppressor-cell activity by TPA

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Macrophages added (%)</th>
<th>Macrophages* counted (%)</th>
<th>Suppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNC</td>
<td>6.2 ± 1.7</td>
<td>75 ± 9</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (by cotton depletion)</td>
<td>1% 8.7 ± 2.3</td>
<td>81 ± 12</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (by G-10 depletion)</td>
<td>1% 2.3 ± 2.5</td>
<td>72 ± 12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>65 ± 10*</td>
<td>86 ± 5</td>
<td>25 ± 4</td>
</tr>
</tbody>
</table>

* Average percentage ± SD.

### Table 3 Comparison of TPA-induced suppressor cell activity in total LNC, purified T-cells, or thymocytes

<table>
<thead>
<tr>
<th>Cell population</th>
<th>MLR of T-cells</th>
<th>MLR + untreated T-cells</th>
<th>MLR + TPA-treated T-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNC</td>
<td>37,568 ± 8,915</td>
<td>52,055 ± 6,234</td>
<td>44,797 ± 19,818</td>
</tr>
<tr>
<td>purified T-cells</td>
<td>36,541 ± 3,212</td>
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<td>7,205 ± 1,131</td>
</tr>
<tr>
<td>purified thymocytes</td>
<td>36,541 ± 3,212</td>
<td>28,015 ± 1,611</td>
<td>7,205 ± 1,131</td>
</tr>
</tbody>
</table>

Suppression of T-cells (%) = (Control cpm - Experimental cpm) / Control cpm × 100

### Table 4 PNA binding and En and Ea resetting markers of bovine T-cells separated by peanut lectin agglutination

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Experiment</th>
<th>Distribution of cells in each fraction (%)</th>
<th>FITC-PNA* cells (%)</th>
<th>Cells forming En* rosettes (%)</th>
<th>Cells forming Ea* rosettes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA*</td>
<td>1</td>
<td>55</td>
<td>92</td>
<td>26</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>57</td>
<td>79</td>
<td>18</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>70</td>
<td>88</td>
<td>30</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>76</td>
<td>83</td>
<td>27</td>
<td>81</td>
</tr>
<tr>
<td>Total</td>
<td>34 ± 9</td>
<td>12 ± 5</td>
<td>96 ± 3</td>
<td>95 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

* Average percentage ± SD.
In other experiments the percentage of FITC-PNA\(^*\) cells was measured in the LNC, PNA\(^*\), and PNA\(^-\) fractions after incubation for 24 h plus or minus TPA (Table 5). TPA treatment of LNC caused a slight increase in FITC-PNA\(^*\) cells. TPA treatment of the PNA\(^-\) fraction caused about a 30% increase in the percentage of FITC-PNA\(^*\) cells; and TPA treatment of the PNA\(^*\) fraction caused essentially no change in the percentage of FITC-PNA\(^*\) cells. These findings confirmed the distribution of activity seen after PNA separation of the TPA-treated LNC, PNA\(^-\), and PNA\(^*\) fractions.

The result of these experiments suggested that the precursor cell induced by TPA to develop suppressor activity was a PNA\(^-\) T-cell which differentiated into a PNA\(^*\) suppressor, effector T-cell. Both the marker and functional change were consistent with maturation of the cells under the influence of TPA.

Comparison of Dose Response of LNC, PNA\(^*\), and PNA\(^-\) Cells Treated with TPA. To test whether the observed segregation of the suppressor activity could be explained by a shift in the dose response of a small subpopulation, treated LNC, PNA\(^*\), and PNA\(^-\) cells were added to the MLC at various ratios compared with the cells of the MLC. In preliminary experiments we found that a 1:1 ratio of treated cells to MLC participants was optimal for detection of suppressor activity. In these experiments cells from each preparation were added to the MLC in ratios of 0.2, 0.5, 1.0, and 2.0 treated, irradiated cells to 1.0 participant cell. The suppressor activity increased with increasing cell number up to a ratio of 1:1 where it appeared to level off (Fig. 4). The TPA-treated PNA\(^*\) fraction exhibited essentially all of the suppressor activity seen in the treated LNC population. The PNA\(^*\) fraction showed no activity at lower ratios, and only about 20% suppression at the highest ratio of treated cells to cells in MLC.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>LNC</th>
<th>LNC(^*)</th>
<th>PNA(^*)</th>
<th>PNA(^-)</th>
<th>PNA(^-)</th>
<th>PNA(^*)</th>
<th>PNA(^-)</th>
<th>PNA(^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>59</td>
<td>89</td>
<td>86</td>
<td>11</td>
<td>41</td>
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<tr>
<td>2</td>
<td>53</td>
<td>60</td>
<td>91</td>
<td>93</td>
<td>9</td>
<td>37</td>
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<tr>
<td>3</td>
<td>81</td>
<td>84</td>
<td>86</td>
<td>93</td>
<td>51</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>51 ± 3</td>
<td>59 ± 1</td>
<td>89 ± 3</td>
<td>88 ± 5</td>
<td>13 ± 5</td>
<td>43 ± 7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a}^*\)T cells incubated 24 h with or without TPA (10\(^{-4}\) M).

![Fig. 4. Comparison of the dose response of TPA-induced suppressor activity of LNC, PNA\(^*\), and PNA\(^-\) cells. LNC, PNA\(^*\), and PNA\(^-\) cells were treated for 24 h with 10\(^{-4}\) M TPA before addition to the MLC at 2:1, 1:1, 0.5:1, and 0.2:1 ratios (suppressor:MLC cells). Values, mean ± SD of the results of four experiments. The PNA\(^*\) population contained about 12% PNA\(^-\) cells and 88% PNA\(^*\) cells; the PNA\(^-\) population, about 83% PNA\(^-\) cells and 17% PNA\(^*\) cells; and the LNC, about 49% PNA\(^-\) and 51% PNA\(^*\) cells.](image-url)
ratio tested. The ratio of cells added to the MLC to achieve 50% suppression was 0.3:1 for the PNA- fraction, 0.5:1 for LNC, and an extrapolated ratio of about 20:1 for the PNA+ fraction. These numbers also approximated the differential content of PNA- T-cells in each fraction before TPA treatment (Table 5). The LNC contained about 50% PNA- cells, the PNA- fraction contained about 85% PNA- cells; and the PNA+ fraction contained about 10% PNA+ cells, in good agreement with the amount of induced suppressor activity observed. Furthermore, the 10% PNA+ cells in the PNA+-enriched fraction can account for the suppressor activity observed in that fraction.

Measurement of TPA-induced Soluble Suppressor Factor Activity. In an attempt to further characterize the mechanism of suppression, TPA-treated cells from the LNC, PNA-, and PNA+ populations were tested for release of soluble suppressor factor(s) as described in "Materials and Methods." The results of four experiments indicated that there was a release of suppressor factor(s) by the TPA-treated LNC (Fig. 5). The supernatant was inhibitory at 10% (v/v) and continued to show increased inhibition to about 40% of the culture medium. The activity in medium from the PNA- cells closely followed that of the LNC. The LNC supernatants had between two and four half-maximal suppressor units; the PNA- supernatants had about three to six units. Each unit was defined as the inverse of the dilution of the supernatant required to give a 50% suppression. In contrast, supernatants from PNA+ cells had no appreciable suppressive activity, and even appeared to give help in three of the four assays. This finding with PNA+ cells also supported the evidence that the suppressor activity was not due to TPA carried over with the cells (11, 13). We had also previously found that TPA did not cause release of cold thymidine or block isotope transport (12).

In another approach in which suppressor factor was assayed after direct supernatant transfer, the results were similar to those observed with the supernatant dilution experiments. The LNC supernatant showed 15 ± 8%, 28 ± 10%, and 45 ± 6% suppression for 0.5, 1.0, and 2.0 × 10^7 TPA-treated cells/ml in the initial cultures, respectively. PNA- cell supernatants gave 17 ± 3, 31 ± 13, and 53 ± 17% suppression for 0.5, 1.0 and 2.0 × 10^7 cells/ml, respectively. In a total of nine determinations the PNA+ supernatant often appeared to give help, but the results were not consistent. Taken together, these experiments indicated a relationship between cell number, cell phenotype, and amount of suppressor material produced.

DISCUSSION

It has been previously reported that TPA blocked the proliferation of lymphocytes in response to various lectins as well as to allogeneic cells (10, 14). One possible explanation was the development of suppressor lymphocytes by TPA (11). In this present study, we confirmed that suppressor cells were induced, and examined the subpopulations required both for the induction and expression of this activity.

The suppressive activity resulted from an interaction of TPA with the lymphocytes in the culture. Macrophages were not required. Their removal or addition had little effect on the generation of suppressor cell activity. In contrast, suppressor activity was enhanced in purified T-cells. Specifically, the effector cells were PNA positive T-cells. The precursors of these were PNA negative T-cells. Supporting this conclusion was the observation that about half of the PNA negative cells acquired a PNA positive phenotype after TPA treatment. This phenotype change correlated with the development of suppressor activity in the cultures.

The suppressor cell activity appeared to act through a soluble mediator. Supernatants from TPA-treated, irradiated cells had enough activity to account for essentially all of the inhibition observed with the suppressor cells, on a cell number basis. However at this time this soluble material has not been further characterized.

The PNA marker was originally chosen for analysis of the suppressor cell subpopulations on the basis of previous studies. First, it had been reported that the major subpopulation of bovine peripheral blood T-cells, but few B-cells were PNA positive (22-24). Secondly, murine cells induced in vitro acquire a PNA positive phenotype along with increased suppressor activity (25). Finally, in cattle the PNA positive T-cell fraction had been reported to contain the nonspecific suppressor activity induced in vitro (17, 26) by ConA. Thus, our finding that the effector cell of TPA induced suppression was PNA positive was consistent with these observations.

When the PNA marker was compared with other bovine T-cell rosetting markers, it was determined that the PNA positive T-cells were predominantly Ea- En+ (75-80%) but contained a minor population of Ea+ En- (20-25%). The PNA negative fraction contained 95% Ea+ En- cells. Earlier work demonstrated that the Ea+ En+ subpopulation of bovine T-cells differentiated into Ea+ En- cells when held in culture for several days (19). The Ea- En- subpopulation of LNC was more responsive to mitogens and alloantigens than the Ea+ En+ cells. For comparison, the PNA positive fraction, predominantly Ea+ En-, gave three to five times greater response to mitogenic lectins and alloantigens than the PNA negative (Ea+ En-) fraction (Hurley and Mastro, unpublished). Together these data suggested that TPA differentiated a less mature T-cell population (PNA-, Ea+ En+) into a more mature population (PNA+, Ea- En-) with suppressor function in vitro.

Nevertheless, it is clear that the PNA phenotype alone is not indicative of suppressor activity in cattle. First, both the precursors and effectors of ConA induced suppressors are PNA positive T-cells. Second, about 50% of bovine LNC (Table 5) and 60-70% of bovine peripheral blood lymphocytes are PNA positive.
positive (23), too large a percentage to represent only suppressor cells in untreated animals. Finally, in this study, cells bearing the PNA* marker are not precursors of TPA induced suppressor cells in spite of the fact that the effector cells are PNA*. The system described may be useful in defining the necessary and sufficient conditions to establish a specific phenotype of nonspecific suppressor cells. Moreover, PNA remains a useful tool for the dissection of mechanism of action of the TPA induction of these cells.

Others have observed phenotypic changes in T-cells after treatment with the tumor-promoting phorbol esters consistent with the induction of suppressor cell function. For example, Ryffel et al. (27) reported the ablation of T4 and T6 markers with concomitant increase in T3 and T8 markers on a human T-cell leukemia line treated with TPA or PDBu. Jagielski et al. (28) found a loss of the T4 marker associated with the inhibition of T-cell helper activity in B-cell immunoglobulin secretion after treatment of human peripheral blood lymphocytes with TPA. However, these studies dealt primarily with changes in phenotype rather than with the mechanism of suppressor function.

In summary based on this study we conclude that at least some of the inhibitory activity of TPA on lymphocyte proliferation is due to the development of cells with suppressor activity. Not all of the inhibition can be accounted for by these cells for the following reasons: PNA positive cells treated with TPA failed to undergo an MLR, but did not suppress the MLR of other cells. In addition, the amount of suppression with addition of the irradiated suppressor cells never reached 100%, while direct addition of TPA to an MLR usually inhibited it by greater than 99%. The direct inhibitory effects of TPA on proliferation involve other activities such as modulation of surface marker expression (27–29), interference with cell-cell interaction (30, 31), and the inappropriate timing of regulatory signals such as the activation or down regulation of protein kinase C (32), or expression of growth factor receptors (33).

In light of the recently described immunobiology of the skin (34), and of suppressor cells in UV-induced skin carcinogenesis (35), a role for suppressor cells in skin tumor promotion is conceivable. However, whether this effect of TPA in vitro has an in vivo corollary remains to be tested.

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REFERENCES

Differentiation of Peanut Lectin Positive Suppressor T-Cells from Peanut Lectin Negative Precursors in Bovine Cells by 12-O-Tetradecanoylphorbol-13-acetate

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