# THE REQUIREMENT OF AN ADHERENT CELL SUBSTRATUM FOR THE GROWTH OF DEVELOPING PLASMACYTOMA CELLS IN VIVO

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The intraperitoneal (i.p.) injection of mineral oil or the pure alkane, pristane (2,6,10,14-tetramethylpentadecane), induces the formation of a granulomatous tissue on the mesentery, diaphragm, omentum, and other peritoneal connective tissues that is composed predominantly of phagocytic cells which have ingested oil and adhered to these surfaces. The granuloma is vascularized and also contains lymphocytes, granulocytes, and plasma cells (1).

In BALB/c mice, the i.p. injection of mineral oil or pristane induces a high incidence of plasmacytomas (PCT)[1] that arise i.p. in close association with the granuloma evoked by the oil. Most primary PCT cells fail to grow when transplanted to the peritoneal cavity of normal, syngeneic mice at doses of $10^5$ cells or less (2). Successful transplantation of all primary PCTs may be obtained, however, by conditioning the recipient mice with an i.p. injection of pristane 30–60 days before tumor cell inoculation (2). This finding, in addition to the close association of primary PCTs with the oil granuloma, suggests that the primary PCT is dependent upon microenvironmental factors provided by the granuloma during early stages of tumor development. The dependence of primary PCTs on the pristane-conditioned environment is transient, and most tumors acquire the ability to grow in the normal peritoneum after several transplant generations. Since the granuloma is composed largely of macrophages and polymorphonuclear leukocytes (PMNL) which have ingested oil, the possibility that these cells facilitate the growth of primary PCTs must be considered.

Other independent studies indicate that adherent cells influence the growth of PCT cells. Namba and Hanaoka (3) found that the adaptation of the PCT MOPC 104E to long term tissue culture requires the presence of adherent cells or adherent cell products. In further studies they isolated a factor from a normal phagocytic cell line that overcomes a block in the $G_1$ stage of the cell cycle of MOPC 104E cells grown in vitro (4). Metcalf (5) described a serum factor that promotes soft agar colony formation by PCT cells obtained directly from established in vivo cell lines. The i.p. injection of mineral oil increased this activity in the serum. Thus, findings to date support the notion that adherent cells are a source of factors which promote PCT growth in vitro. The relation of these effects to the environment produced by the pristane-induced granuloma in vivo is less clear. Accordingly, we have extended our previous study in order to clarify the role of the adherent

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[1] *Abbreviations used in this paper:* APC, adherent peritoneal cell; BSS, balanced salt solution; FCS, fetal calf serum; HC, hydrocortisone; MEM, minimum essential medium; PCT, plasmacytomas; PMNL, polymorphonuclear leukocytes; TGM, thioglycolate medium.

peritoneal cell (APC) population and the APC granuloma in promoting primary PCT growth in vivo.

Takakura et al. (6) observed that hydrocortisone (HC) administration reduced both granuloma formation and PCT development in mineral oil-treated mice. Thompson and VanFurth (7) demonstrated that HC prevented the migration of mononuclear cells into the inflamed peritoneal cavity, but did not reduce the existing population of peritoneal macrophages. Studies with HC and paramethasone indicate that these drugs have similar effects upon the PMNL component of inflammatory exudates (8, 9), although the elevation of the numbers of these cells in the circulation was not prevented. These results suggest that if the pristane-induced APC are critical for the establishment of a conditioned environment, then HC treatment should abrogate the conditioning effect. Consequently, we have studied the effects of HC on the pristane-conditioning effect. Additionally, we have examined the conditioning capabilities of thioglycolate medium (TGM), an agent that produces an elevation of the free APC population, but does not result in granuloma formation. We have also tested the ability of passively transferred, TGM-induced APC to restore conditioned environment production in mice treated with pristane and HC simultaneously.

## Materials and Methods

*Mice.* BALB/c AnN mice, 6–8 wk of age, weighing approximately 20 g, were used in all studies. Mice were obtained from the National Institutes of Health Breeding Colony, Bethesda, Md.

*Drugs and Chemicals.* Pristane (2,6,10,14-tetramethylpentadecane) was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., and injected i.p. using 25G × ½ inch needles. HC (Cortef acetate, Upjohn Co., Kalamazoo, Mich.) was injected subcutaneously at a dosage of 0.5 mg per mouse in the regimens indicated. Sterile liquid TGM (Difco Laboratories, Detroit, Mich., 0.29% in water) was injected i.p. at a dosage of 0.5 ml in the regimens indicated. Eagle's minimum essential medium (MEM), Hanks' balanced salt solution (BBS), fetal calf serum (FCS), and trypan blue dye were obtained from Grand Island Biological Company, Grand Island, N.Y. Extra high amino acids medium (EHAAM) (10) was obtained from the National Institutes of Health Media Unit, Bethesda, Md. 2-Mercaptoethanol was obtained from Eastman Organic Chemicals, Div., Eastman Kodak Co., Rochester, N. Y. [$^3$H]thymidine in sterile aqueous solution, at 1 mCi/ml, was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

*Primary Ascitic Tumor Cells for In Vivo Transplantation.* PCTs were induced in BALB/c mice through multiple injections of pristane as previously described (11). Ascitic fluid was drained from the primary host using a 16G × 1½ inch hypodermic needle and maintained in an ice bath. Viable cell counts were performed using the exclusion of an 0.4% solution of trypan blue dye as the criterion for viability. The ascites were diluted with BSS to produce a density of $2 \times 10^5$ or $2 \times 10^4$ viable cells per ml, and 0.5 ml of this suspension was administered to recipient mice.

*Fixed Tissue Sections.* Tissues were removed from animals and fixed for 24 h in Fekete's modification of Tellesznicky's solution. The sections were then cut and stored in 70% ethanol before embedding, sectioning, and staining with hematoxylin-eosin.

*Scoring of Tumor Growth.* Mice were screened at 5-day intervals for successful PCT transplantation by the examination of a Wright's stained smear of peritoneal fluid. The presence of numerous large, hyperchromatic PCT cells, characteristic of PCTs, was the criterion for a positive smear. This method afforded an accurate method for the early detection of progressively growing tumors.

*Peritoneal Cell Counts.* Mice were sacrificed by cervical dislocation. The peritoneum was opened and the contents everted in 30 ml of calcium-magnesium-free BSS. The resulting suspension was centrifuged for 5 min at 3,000 *g*, and the pellet was resuspended in 5 ml of MEM made 10% with FCS. Total cell counts were immediately performed in a hemacytometer. Smears for differential cell counts were made using one drop of the cell suspension and one drop of FCS to facilitate

smear preparation. These smears were air dried and later stained with Wright's stain and counted under 430 or 1,000 × magnification. Percentages were calculated from counts of at least 200 cells. The remaining suspension was transferred to 60-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air for 1 h. After gentle agitation, the nonadherent cells were decanted and counted. The adherent cell count was calculated as the total cell count less the nonadherent count. In some experiments, the adherent cells remaining in the tissue culture dishes were also stained and differential counts were performed.

*TGM-Induced APC for Cell Transfer Experiments.* Donor mice were treated i.p. with 0.5 ml of TGM. After 5–7 days, the mice were sacrificed by cervical dislocation, and the peritoneal wall was exposed. A 5-ml aliquot of BSS was injected i.p. and then withdrawn with a 16G × 1½ inch needle. The resulting cell suspension was delivered to a 50-ml centrifuge tube and maintained on ice. The washings of 10 mice were pooled and centrifuged in the cold at 1,800 *g* for 10 min. The pellet was resuspended in 25 ml of MEM made 10% with FCS, placed in 100-mm plastic tissue culture dishes (Falcon Plastics), and incubated for 1 h. The nonadherent cells were withdrawn after gentle agitation. The adherent cells were scraped from the dish with a sterile rubber policeman and resuspended in 5 ml of BSS. The density was adjusted to $4 \times 10^6$ viable cells per ml, and 0.5 ml was injected i.p. in recipient mice.

## Results

*Rapid Conditioning of the Peritoneal Environment.* In previously reported studies (2), conditioning was established by injecting 0.5 ml of pristane 30–60 days before the transplantation of primary PCT cells. In the present experiments, pristane was administered 3, 14, and 30 days before transplantation. The two PCTs tested failed to grow in untreated control groups by day 100, but developed rapidly in all three of the pristane-treated groups (Table 1). The 3-day conditioning period appeared more efficient when the latent periods of these groups were compared by a Mann-Whitney U test, but additional experiments would be required to establish this as a general rule. These findings indicated the feasibility of a 3-day conditioning period and facilitated experiments on the effects of HC.

*Changes in the Free Peritoneal Population After Pristane Conditioning and HC Treatment.* Total cell counts were performed as described in three groups: (*a*) pristane-treated mice, (*b*) mice given 0.5 mg of HC daily beginning simultaneously with pristane injection, and (*c*) mice given 0.5 mg of HC daily beginning 3 days after pristane injection (HC-lag group). The proportion of adherent to nonadherent cells was established by determining the number of cells which adhered to a plastic tissue culture dish after 1 h of incubation.

Within 3 days after pristane injection, the free peritoneal cell population rose from a normal value of $1.55 \times 10^6$ to $4.10 \times 10^6$. After 7 days, an additional increase to a level of $5.28 \times 10^6$ had occurred. Thereafter, the total cell population remained relatively constant until day 50 (Table II, Fig. 1). The adherent, mononuclear cell population was the primary source of this increase, as the nonadherent cell counts did not deviate significantly from the normal value during this period, and the proportion of mononuclear cells in the peritoneal population rose from 35 to greater than 50% (Table II). Although the relative proportion of PMNL decreased over this period, the actual numbers increased. Moreover, these cells contributed noticeably to the adherent cell population in pristane-treated mice (Table III). Generally, the adherent cell population appeared to reflect the combined mononuclear and PMNL components of the peritoneal exudate population.

TABLE I

*Transplantation of Primary Plasmacytoma Cells in Mice Treated with Pristane at Varying Intervals Before Tumor Inoculation*

| Tumor | Days of pristane exposure before inoculation | Day PCT cells first detected | Proportion dead at 100 days postinoculation | Average day of death and range |
|---|---|---|---|---|
| FLOPC 4 | 0 | – | 0/10 | – |
| | 3 | 25 | 10/10 | 42 (35–50) |
| | 14 | 40 | 10/10 | 58 (50–66) |
| | 30 | 40 | 10/10 | 60 (50–70) |
| MUTIV no. 20 | 0 | – | 0/10 | – |
| | 3 | 30 | 10/10 | 47 (40–60) |
| | 14 | 25 | 10/10 | 66 (52–70) |
| | 30 | 25 | 10/10 | 64 (54–78) |

Mice were injected i.p. with 0.5 ml of pristane 3, 14, or 30 days before receiving $10^5$ cells of the indicated primary plasmacytoma. Mice were observed for tumor growth as described in the text. Control mice received tumor cells only.

TABLE II

*Changes in the Free Peritoneal Cell Population After Pristane and HC Treatment*

| Day | Pristane | | | | | Hydrocortisone | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total cells × $10^6$ | Nonadherent × $10^6$ | Differential counts | | | Total cells × $10^6$ | Nonadherent × $10^6$ | Differential counts | | |
| | | | PMNL | Monocytes | Lymphocytes | | | PMNL | Monocytes | Lymphocytes |
| | | | % | % | % | | | % | % | % |
| 0 | 1.55 ± 0.06[a] | 1.12 ± 0.06[a] | 36 ± 1.2 | 35 ± 0.6 | 29 ± 0.6 | | | | | |
| 3 | 4.10 ± 0.12[b] | 1.15 ± 0.08[a] | 24 ± 1.5 | 49 ± 1.5 | 23 ± 2.0 | 1.77 ± 0.15 | 1.05 ± 0.05 | 30 ± 1.2 | 24 ± 1.2 | 46 ± 2.3 |
| 7 | 5.28 ± 0.08[c] | 1.23 ± 0.06[a] | 22 ± 1.5 | 54 ± 1.2 | 24 ± 1.8 | 1.73 ± 0.06 | 1.05 ± 0.09 | 36 ± 4.3 | 14 ± 6.2 | 50 ± 2.5 |
| 10 | 5.10 ± 0.06[c] | 1.24 ± 0.05[a] | 24 ± 1.5 | 54 ± 1.2 | 22 ± 2.1 | 1.85 ± 0.13 | 1.13 ± 0.03 | 24 ± 1.8 | 14 ± 2.0 | 62 ± 3.8 |
| 20 | 5.20 ± 0.06[c] | 1.27 ± 0.13[a] | 25 ± 1.2 | 52 ± 2.2 | 22 ± 2.6 | 1.70 ± 0.06 | 1.05 ± 0.05 | 29 ± 2.1 | 17 ± 2.1 | 54 ± 1.2 |
| 30 | 5.10 ± 0.10[c] | 1.40 ± 0.06[a] | 27 ± 0.6 | 53 ± 4.5 | 19 ± 0.9 | 1.72 ± 0.09 | 1.13 ± 0.07 | 19 ± 4.6 | 18 ± 2.3 | 63 ± 2.9 |
| 40 | 4.93 ± 0.06[c] | 2.87 ± 0.09[b] | 38 ± 0.9 | 35 ± 1.0 | 26 ± 1.9 | 1.72 ± 0.09 | 1.05 ± 0.08 | 17 ± 3.5 | 19 ± 1.8 | 64 ± 3.7 |
| 50 | 5.82 ± 0.42[d] | 3.20 ± 0.06[b] | 26 ± 0.9 | 34 ± 1.9 | 39 ± 1.5 | 1.82 ± 0.04 | 1.05 ± 0.05 | 25 ± 3.1 | 15 ± 2.3 | 60 ± 5.0 |

Cell counts were performed as described in the text on three groups: (*a*) mice treated with pristane alone; (*b*) mice treated with pristane and concomitantly placed upon a regimen of 0.5 mg HC daily. Each entry is mean ± SE of three mice. One-way analysis of variance was performed on each set of counts and, where significant differences were found at the 5% level, Student Neuman-Kuels tests were performed. Superscripts indicate the groups delineated by this test. Differential cell counts were performed on Wright's stained smears prepared from peritoneal cell samples. The percentages are calculated from the mean counts of three mice. Each count consisted of at least 200 cells.

Continuous HC treatment beginning simultaneously with pristane injection prevented any change in the total free peritoneal cell population, although there was a depression in the proportion of mononuclear cells and an increase in the proportion of lymphocytes (Table II, Fig. 1). In the HC-lag group, the normal and 3-day values agreed well with those of the pristane-treated group. Further, the total peritoneal cell count did not decrease after the onset of HC treatment in this group, but remained substantially higher than the normal value through day 30. As in the pristane-treated group, the adherent cells were the predominant source of this increase (Fig. 2).

Interestingly, by day 50 there was an additional increase in the total peritoneal cell population of pristane-conditioned mice which appeared to be attributable to the nonadherent cell population (Table II, Fig. 1).

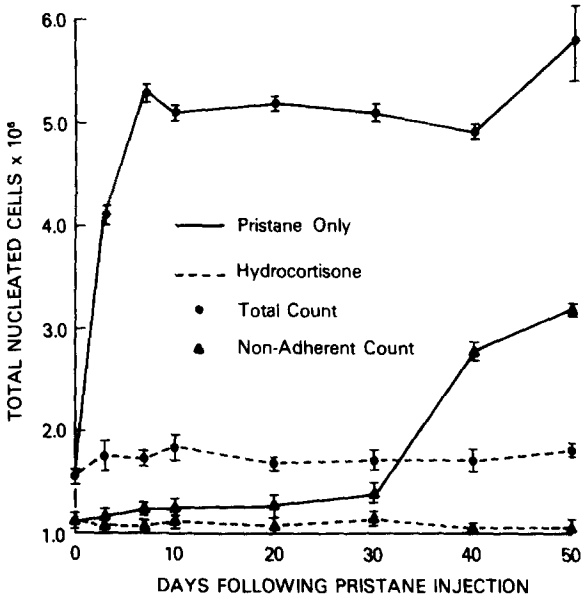


FIG. 1. Mice were sacrificed at intervals after either pristane or pristane-HC treatment, and the peritoneal contents were washed in 30 ml of calcium-magnesium-free Hanks' BSS. The resulting suspension was centrifuged at 3,000 *g* for 5 min and the pellet resuspended in Eagle's MEM. Total cell counts were performed immediately, and the remaining cell suspension transferred to 60-mm plastic tissue culture dishes and incubated 1 h. The nonadherent cells were decanted and counted. Each point is the mean ± SE of counts on three mice.

TABLE III

*Differential Adherent Peritoneal Cell Counts in Pristane- and TGM-Treated Mice*

| Days after irritant injection | Pristane | | | Repeated thioglycolate | | |
|---|---|---|---|---|---|---|
| | PMNL | Monocytes | Lymphocytes | PMNL | Monocytes | Lymphocytes |
| | % | % | % | % | % | % |
| 0 | 47 | 48 | 5 | | | |
| 3 | 40 | 59 | 1 | 45 | 48 | 8 |
| 7 | 36 | 63 | 1 | 35 | 60 | 6 |
| 10 | 46 | 47 | 7 | 21 | 75 | 4 |
| 30 | 49 | 49 | 2 | – | – | – |

Differential cell counts were performed on plates of adherent peritoneal exudate cells that were directly Wright's stained. The percentages are calculated from the mean counts of three plates. Each count consisted of at least 200 cells.

*Granuloma Formation in Pristane- and HC-Treated Mice.* Granuloma formation was followed in groups of mice treated identically as those in the cell count experiments by the examination of fixed tissue sections of the peritoneal mesentery. In pristane-treated mice, the onset of granuloma formation was apparent as early as 3 days after pristane injection (Fig. 3). The granuloma became progressively larger through day 30, and contained numerous droplets of entrapped oil (Fig. 3). In mice that received HC treatment beginning simulta-

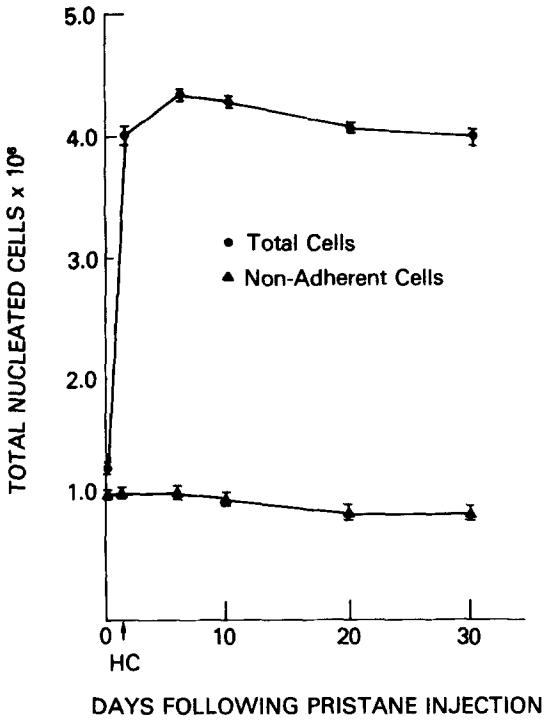


FIG. 2. Cell counts were performed as described in Fig. 1 on groups of mice treated with pristane and HC beginning 3 days later.

neously with pristane injection, however, minimal granuloma formation was observed during this period. In the HC-lag group, however, granuloma formation closely paralleled that of the group given pristane alone.

*The Effect of HC Treatment on Pristane Conditioning.* The effect of HC on pristane conditioning was examined with eight different primary PCTs (Table IV). Either $10^4$ or $10^5$ primary PCT cells were injected i.p. in four groups of 10 mice each. Control mice (Group I) received no previous treatment; Group II received pristane 3 days before tumor inoculation; Group III received pristane 3 days before tumor inoculation and were simultaneously placed upon daily HC treatment; and Group IV was conditioned with pristane 3 days before transplantation and placed upon HC treatment on the day of transplantation (HC-lag).

All of the primary PCTs were dependent upon the pristane-conditioned environment for growth. Only one of the 120 inoculated mice developed a tumor, whereas all of the pristane-conditioned mice developed progressively growing tumors. In contrast, only 26 of the 120 mice treated with HC beginning simultaneously with pristane injection developed tumors. Groups that were exposed to pristane for 3 days before beginning HC treatment, however, closely paralleled the groups receiving pristane alone, indicating that the lack of tumor growth in Group III was not due to toxic effects of HC. All primary PCTs assayed to date have produced similar results (Table IV).

*The Inability of TGM to Produce a Conditioned Environment.* A single i.p. injection of TGM produced a brief elevation of the free peritoneal cell population

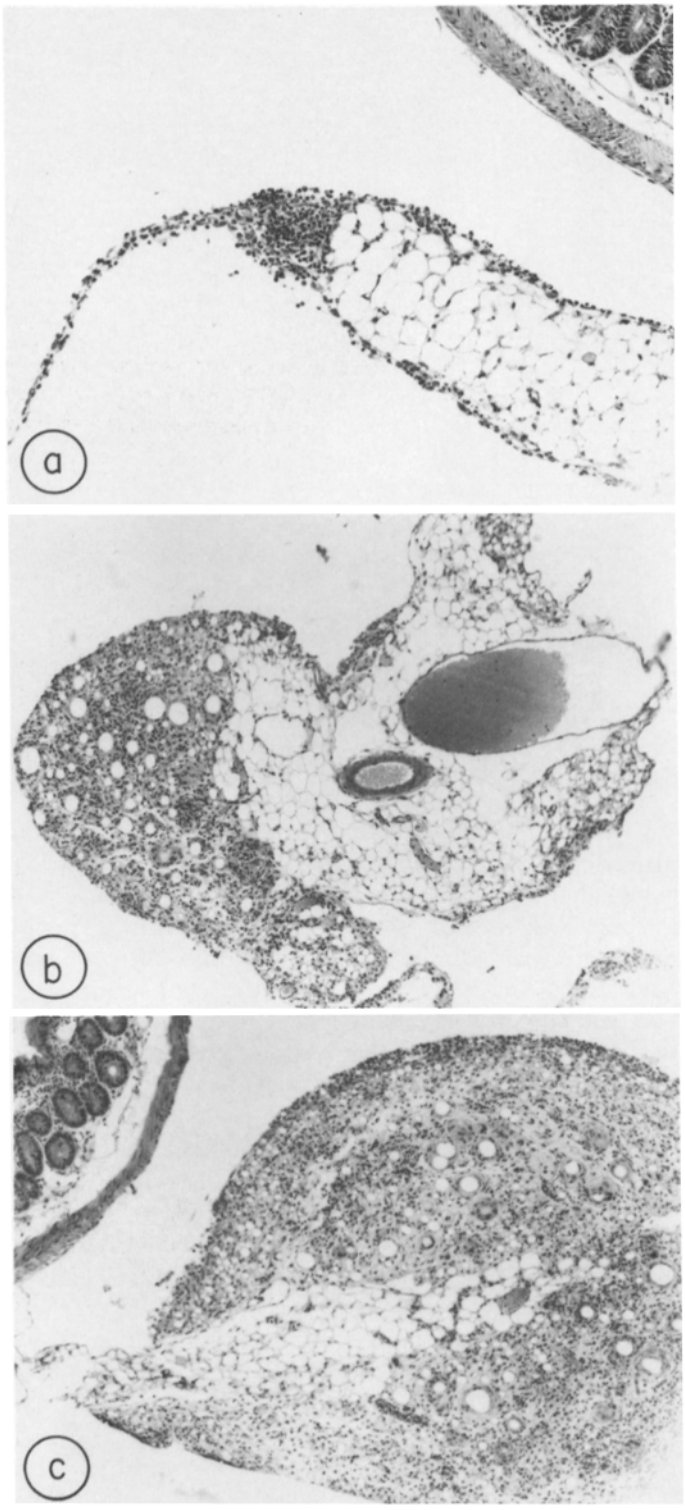
a
b
c

TABLE IV

*Summary of Tumors Tested in Pristane-Conditioned and Hydrocortisone-Treated Mice*

| Tumor | Cells inoc. log 10 | Number of mice in group dead at 100 days | | | | Average day of death and range | | | | Average day of positive smear and range | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CON I | PRIS II | HC III | LAG IV | CON I | PRIS II | HC III | LAG IV | CON I | PRIS II | HC III | LAG IV |
| TEPC604 | 5 | 0 | 10 | 4 | 10 | – | 55 (50-70) | 70 (65-80) | 60 (55-70) | – | 45 (35-50) | 60 (50-70) | 50 (45-55) |
| TEPC604 | 4 | 0 | 10 | 3 | 10 | – | 60 (55-75) | 80 (75-90) | 62 (50-70) | – | 50 (40-60) | 75 (65-90) | 55 (50-65) |
| MOPC702 | 5 | 0 | 10 | 2 | – | – | 50 (40-65) | 80 (70-90) | – | – | 40 (35-50) | 65 (60-70) | – |
| MOPC702 | 4 | 0 | 10 | 2 | – | – | 52 (45-60) | 85 (70-100) | – | – | 40 (35-50) | 70 (65-75) | – |
| PC55 | 5 | 0 | 10 | 2 | 10 | – | 50 (40-65) | 72 (65-80) | 60 (40-75) | – | 50 (40-60) | 55 (55-60) | 45 (40-55) |
| PC55 | 4 | 0 | 10 | 2 | 10 | – | 75 (70-85) | 70 (65-75) | 80 (70-95) | – | 65 (55-70) | 60 (60) | 65 (50-90) |
| PC8 | 5 | 0 | 10 | 2 | 10 | – | 55 (45-65) | 75 (70-80) | 60 (45-70) | – | 45 (35-50) | 62 (50-70) | 42 (35-50) |
| PC8 | 4 | 0 | 10 | 1 | 10 | – | 70 (60-85) | 92 (85-100) | 80 (70-90) | – | 65 (55-70) | 80 (70-85) | 62 (55-80) |
| NIPC21 | 5 | 1 | 10 | 2 | 10 | 80 | 40 (35-40) | 50 (45-55) | 45 (40-60) | 65 | 30 (25-35) | 32 (25-40) | 35 (25-45) |
| NIPC22 | 5 | 0 | 10 | 2 | – | – | 45 (35-40) | 60 (55-65) | – | – | 35 (30-40) | 45 (40-55) | – |
| DMBA16 | 5 | 0 | 10 | 1 | 10 | – | 55 (45-60) | 70 | 65 (55-70) | – | 40 (30-45) | 62 | 50 (45-60) |
| TEPC828 | 5 | 0 | 10 | 3 | 10 | – | 63 (49-72) | 77 (70-84) | 68 (52-80) | – | 39 (31-52) | 47 (46-53) | 45 (38-56) |

Primary tumor cells were inoculated i.p. to four groups of recipient mice: I. Control mice receiving cells only; II. pristane-treated mice received 0.5 ml of pristane 3 days before tumor inoculation; III. HC-treated mice received 0.5 ml of pristane and were placed upon 0.5 mg HC daily 3 days before tumor inoculation; and IV. HC-lag received 0.5 ml of pristane 3 days before tumor inoculation and were placed upon HC treatment concomitant with tumor inoculation.

which reached a maximum value of $3.5 \times 10^6$ 7 days after injection (Fig. 4). When TGM was given repeatedly at 4-day intervals, a prolonged elevation of the free peritoneal cell population similar to that obtained with pristane was seen. The total peritoneal cell population reached a maximum value of $6.55 \times 10^6$ on day 10. By day 20, this number had decreased to a value of $4.38 \times 10^6$ and remained at this level through day 30 (Fig. 4). Again, the adherent cell population was the primary source of this increase. The initial proportion of monocytic cells to PMNL in the TGM-induced APC population paralleled that obtained with pristane (Table III). By day 10, however, there was a greater proportion of monocytic cells in the TGM-treated mice. In contrast to pristane treatment, however, no granuloma formation was found on the peritoneal connective tissues by day 30. To determine whether granuloma formation was necessary for the conditioning effect, the ability of TGM treatment to produce a conditioned environment was examined with six primary PCTs. Cells were injected i.p. in four groups of 10 mice each. Control mice received no prior treatment; TGM-treated mice received 0.5 ml of TGM 3 days before tumor inoculation. Repeated TGM-treated mice received 0.5 ml of TGM i.p. every four days beginning 3 days before tumor inoculation. A group of pristane-treated mice was included as a positive control.

The control and pristane-treated groups indicated that the PCTs used were dependent upon a conditioned environment for growth. In the TGM-treated groups, no PCT growth was observed (Table V). This finding suggested that the elevation of the free APC population without granuloma formation was not sufficient to produce a conditioned environment.

*Reconstitution of the Conditioning Effect in Pristane-HC-Treated Mice with*

FIG. 3. Stages of developing granuloma in BALB/c mice after pristane treatment. (*a*) Mesentery 3 days after pristane injection, 140 ×. (*b*) Mesentery 20 days after pristane injection. Note oil droplets entrapped in the forming granuloma, 88 ×. (*C*) Mesentery 30 days after pristane injection, 88 ×. All sections were stained with hematoxylin-eosin.

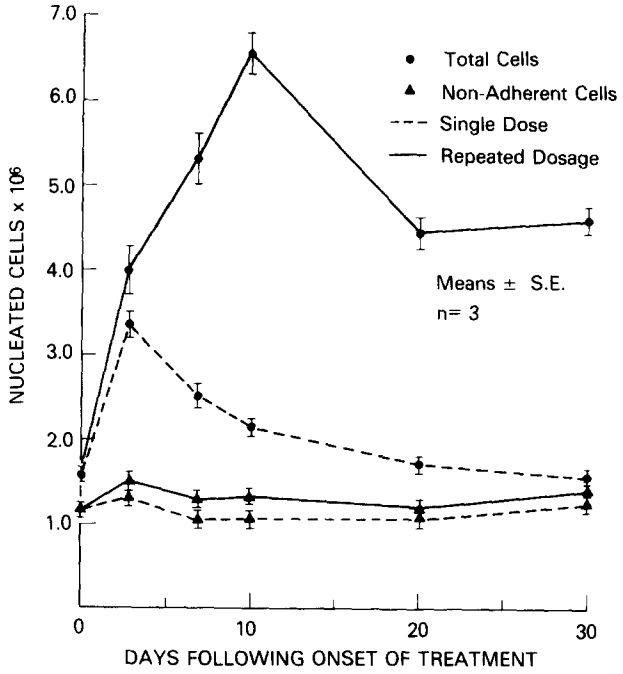


FIG. 4. Cell counts were performed as described in Fig. 1 on mice treated with either a single injection of 0.5 ml of TGM i.p., or with 0.5 ml of TGM i.p. repeatedly at 4-day intervals.

*Passively Transferred TGM-Induced APC.* In three experiments of this type, three additional treatment groups were included. These consisted of a pristane-HC-treated group, an HC-lag group (as previously described), and a group that received pristane, simultaneous HC treatment, and four doses of $2 \times 10^6$ syngeneic, TGM-induced adherent cells i.p. at 4-day intervals beginning 3 days before tumor inoculation. The purpose of these groups was twofold. First, they examined whether TGM-induced APC could provide a conditioned environment when stimulated to form a granuloma by pristane. Second, they provided a means of determining whether passively acquired APC would reconstitute the production of a conditioned environment in mice whose APC response to pristane was suppressed.

In the groups receiving TGM-induced APC, 29 of the 30 mice tested exhibited successful transplantation (Table V). Further, sections of peritoneal mesentery revealed that extensive granuloma formation had taken place. In all other groups, the results were consistent with previous experiments.

## Discussion

An environment conducive to primary PCT growth is produced as few as 3 days after the i.p. injection of pristane. This result extends previous observations that 30–60 days of pristane exposure elicits this effect. Further, a conditioned environment is established simultaneously with the rapid influx and deposition of large numbers of adherent cells in the peritoneal cavity, and suppression of this influx with HC abrogates conditioning. Since pristane is a

TABLE V

*Summary of Transplantation Experiments with TGM-Treated Recipients and Passively Transferred TGM-Induced APC*

| | Number of mice in group of 10 developing progressively growing tumors by day 100 | | | | | | |
|---|---|---|---|---|---|---|---|
| Tumor | Untreated | Pristane | TGM | Repeated TGM | Pristane HC | HC Lag | Pristane HC TGM cells |
| CBPC 116 | 0 | 10 | 0 | 0 | – | – | – |
| TEPC 827 | 0 | 10 | 0 | 0 | – | – | – |
| TEPC 829 | 0 | 10 | 0 | 0 | – | – | – |
| TEPC 831 | 0 | 10 | 0 | 0 | 1 | 10 | 9 |
| TEPC 828 | 0 | 10 | 0 | 0 | 3 | 10 | 10 |
| CBPC 118 | 0 | 10 | 0 | 0 | 2 | 10 | 10 |

Primary tumor cells were inoculated i.p. to each group. The untreated, pristane, pristane HC, and HC lag groups were identical to those in Table IV. TGM-treated mice received TGM 3 days before tumor inoculation. Repeated TGM-treated mice received TGM at 4-day intervals beginning 3 days before tumor inoculation. Reconstituted mice received pristane, simultaneous HC treatment, and four doses of $2 \times 10^6$ TGM-induced APC at 4-day intervals beginning 3 days before tumor inoculation.

nonmetabolizable substance and is not cleared from the peritoneal cavity, these results indicate that pristane in the absence of an adherent cell population does not produce a conditioning effect. This finding strongly implicates the APC population in the conditioning process. Moreover, the transplantation data obtained using delayed HC treatment shows that the adherent cell influx during the initial 3 days of pristane exposure is sufficient to establish a conditioned environment. These results, in conjunction with the peritoneal cell counts in this group, indicate that the pristane-induced APC population may undergo a substantial amount of division in the peritoneum. These findings are consistent with those of Ryan and Spector (12, 13) who showed that granulomata induced by paraffin oil, incomplete Freund's adjuvant, and *Bordetella pertussis* vaccine exhibit high rates of [$^3$H]thymidine incorporation compared to those induced with carrageenan. They attributed this differential stimulation of macrophage mitogenesis to the relative toxicity of the irritant employed and the availability of newly recruited mononuclear cells.

Growth-stimulating or viability-enhancing properties have been attributed to similar situations and cell populations both in vivo and in vitro. Namba and Hanaoka (3, 4) described a soluble, nondialyzable factor elaborated by cultured adherent cell populations which facilitates the long term in vitro cultivation of established PCT cell lines. Metcalf (5) reported that a factor present in the serum of BALB/c mice after mineral oil injection stimulated colony formation by cells obtained from established in vivo cell lines. VanDenBrenk et al. (14) observed increased lung colony-forming efficiency of the tumors Y-P388 and W256 when they were grown in an acutely inflamed environment.

The results obtained herein using TGM-treated recipients, however, show that elevation of the free APC population alone is not sufficient to produce a conditioning effect, and that the establishment of a resident population of these cells is a necessary event in the conditioning process. This interpretation is supported by the results of the passively transferred APC experiments, where

TGM-induced APC are capable of conditioning if stimulated to form a granuloma by pristane. Additionally, these results confirm the role of APC in the conditioning effect in vivo.

The requirement for granuloma formation in vivo may indicate that attached macrophages provide a substratum suitable for the adherence of primary PCT cells. The close association of primary PCTs with the granuloma in vivo (1) supports this possibility. Selective physical interactions between immunoglobulin-bearing lymphocytes and macrophages are well established (15–17), and it would thus not be surprising if PCT cells also behaved in this fashion.

The overall mechanism through which the APC granuloma influences primary PCT growth in vivo might be rather complex. In addition to providing a suitable substrate for cellular adherence and proliferation, soluble growth-enhancing factors similar to those described in vitro (3–5) might also be elaborated. Systems to investigate this possibility are currently being developed in this laboratory. Preliminary results indicate that a soluble material produced by pristane-induced APC enhances the in vitro immunoglobulin production and microcolony formation by primary PCT cells. If soluble APC products are involved in the conditioning process in vivo, however, the singular ability of granuloma-inducing materials to establish a conditioned environment would suggest that such materials are secondary to the adherence phenomena. The close association of the PCT cells with the granuloma would, of course, insure the exposure of the developing tumor to any stimulatory substances produced by the APC. Further, the observations that macrophages stimulated with nonmetabolizable materials exhibit comparatively long term production of collagenase (18) and plasminogen activator (19) imply that the pristane granuloma may be a rich source of soluble, macrophage-elaborated products.

Further, insoluble materials deposited by macrophages may be instrumental in the conditioning process. Culp et al. (20–22) report that substrate-attached materials produced by fibroblasts in vitro provide a substratum for subsequent cellular attachment and proliferation.

Although the bulk of the free peritoneal cell increase after pristane injection is due to macrophages, the PMNL also increase in number and are present in the granuloma. Further, PMNL may comprise as much as 49% of the APC population obtained from pristane-treated mice. Therefore, the possibility that PMNL may play a role in the conditioning process cannot be disregarded. Evidence exists that certain functional characteristics are shared by macrophages and PMNL. These include the production of neutral proteases (23), the presence of surface receptors for C3 and immunoglobulin (24), and the enhancement of T-lymphocyte mitogenic responses to phytohemagglutinin (25). The possible participation of PMNL in the conditioning effect thus warrants further investigation.

The APCs might also provide an immune protection mechanism by altering or masking PCT cell surface antigens or by altering the host's immune system either generally or locally. Pristane conditioning apparently abrogates established immunity to the PCT AdjPC 5, permitting tumor growth (26). Recently, Ptak and Gershon (27) have shown that the in vitro plaque-forming response to sheep erythrocytes is suppressed by peritoneal macrophages or macrophage membranes when they are added during the initial stimulation of the lympho-

cyte cultures. Further, Broder et al. (28) reported that the mononuclear cells of myeloma patients actively suppress immunoglobulin synthesis by B lymphocytes from normal individuals.

It is likely that the pristane-induced APC granuloma exerts its effects through a combination of activities which ultimately result in the production of a unique environment. This environment is a necessary prerequisite for successful primary PCT growth and is probably a critical factor in the emergence of primary neoplastic plasma cell clones.

The exclusive or greatly enhanced growth of developing neoplastic PCT clones in a particular microenvironment suggests the emerging clone is dependent upon extrinsic factors for its growth. This microenvironmental growth dependence closely resembles hormone dependent growth observed in other systems (29). Although primary PCT cells are aberrant with respect to proliferative control, conditioning dependence implies that their response to certain regulatory constraints is still operational. Based upon these considerations, it is tempting to speculate on the implications of the pristane-conditioning effect regarding both plasmacytomagenesis and the regulation of normal plasma cell proliferation.

Completely autonomous cellular proliferation requires that the normal regulatory mechanisms influencing division be lost or rendered ineffective. This is only partially true of primary PCTs, suggesting that at least two regulatory constraints are normally active in this system. The appearance of a primary PCT might reflect the loss of responsiveness to only one of these constraints. Such a preneoplastic clone, however, would not proliferate except when provided with the necessary secondary stimulus such as the physical interaction with a suitable substratum. The pristane-induced oil granuloma would thus provide a selective microenvironment for the preneoplastic clones by providing a continual source of this stimulation.

## Summary

The intraperitoneal injection of pristane (2,6,10,14-tetramethylpentadecane) produces an environment conducive to primary plasmacytoma growth in as few as 3 days. After pristane injection, the total free peritoneal cell population increases from a normal value of $1.55 \times 10^6$ to $5.28 \times 10^6$ and remains at this elevated level for at least 50 days. The adherent peritoneal cell population, composed of both mononuclear cells and polymorphonuclear leukocytes, is the primary source of this increase. In the pristane-conditioned peritoneum, these cells rapidly form a chronic granuloma on the peritoneal connective tissues. Daily subcutaneous treatment of mice with 0.5 mg of hydrocortisone beginning simultaneously with pristane injection prevents the increase in the peritoneal cell population, granuloma formation, and the production of a conditioned environment. In mice treated with hydrocortisone beginning 3 days after pristane injection, however, neither the peritoneal cell increase nor the production of a conditioned environment is prevented. The intraperitoneal injection of thioglycolate medium at 4-day intervals produces an elevation of the free adherent peritoneal cell population similar to pristane, but does not produce a granuloma or a conditioned environment. The intraperitoneal transfer of thio-

glycolate-induced adherent peritoneal cells to mice treated with pristane and hydrocortisone simultaneously restores the production of a conditioned environment. These findings indicate that the adherent peritoneal cell population is responsible for the conditioning effect, and that the establishment of a resident population of these cells is necessary to produce conditioning.

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