Specific Inhibition of Tumor Cell DNA Synthesis In Vitro by Lymphocytes from Peritoneal Exudate of Immunized Syngeneic Guinea Pigs

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Abstract. Tumor immunity to a transplantable diethylnitrosamine-induced hepatoma in inbred guinea pigs has been found to be immunologically specific and cell mediated. We have investigated this cellular immunity using a quantitative, reproducible, and simple assay based on the ability of leukocytes to inhibit the incorporation of tritiated thymidine (TdR^3H) by tumor cells. Tumor cell suspensions were obtained from the ascites form or tissue culture monolayers of the hepatoma. Cells from the peritoneal exudate of immunized guinea pigs inhibited tritiated thymidine uptake by tumor target cells to a significantly greater degree than cells from the peritoneal exudate of unimmunized animals. Immune lymph node, peripheral blood, and spleen leukocytes were not inhibitory. The assay was sufficiently sensitive to detect relatively weak tumor immunity. The in vitro inhibition was correlated directly with the presence of delayed hypersensitivity and/or inhibition of tumor cell growth in local passive transfer studies. Irradiation of peritoneal exudate cells (3000 R) blocked their inhibitory effects on tumor cells. Fractionation of the peritoneal exudate cells by centrifugation in zones of bovine serum albumin of different density also revealed the lymphocytes to be responsible for the specific inhibitory effects whereas macrophages inhibited in an immunologically nonspecific fashion.

The existence of specific cellular immunity in inbred guinea pigs against transplantable hepatomas induced by diethylnitrosamine has been demonstrated. Such guinea pigs, when immunized with tumor cells designated as line 1, develop typical delayed hypersensitivity reactions to line 1 cells and suppress line 1 tumor growth at the site of intra cutaneous tumor cell injection. A variety of assays have failed to detect humoral antitumor immunity in these guinea pigs. These assays have included complement fixation, C_{1a} fixation and transfer, indirect immunofluorescence, and local passive transfer with serum.

Ming et al. have demonstrated inhibition of tumor cell growth by allogeneic lymph node cells from animals immunized with tumor. Using a similar approach we have found that leukocytes from peritoneal exudate (PE) of immunized syngeneic guinea pigs consistently and specifically inhibited the incorporation of tritiated thymidine by suspensions of ascitic or tissue cultured tumor cells in vitro. PE cells from unimmunized guinea pigs were significantly less active in producing such inhibition.
Materials and Methods. Tumors: Administration, in drinking water, of the carcinogen diethylnitrosamine will induce primary hepatomas in inbred strain 2 guinea pigs. The antigenic and biologic properties of the transplantable ascites tumors derived from these primary hepatomas have been described. In the experiments described below, tumor lines 1 and 7 were used. These two transplantable ascites tumors contain different tumor-specific transplantation antigens. Animals immunized to line 1 tumor show delayed cutaneous hypersensitivity reactions to and suppress the growth of line 1 tumor cells but not of line 7 cells. However, animals immunized to line 7 do not show delayed cutaneous hypersensitivity reactions to line 7—occasional weak reactions have been seen—nor line 1 tumor cells. Animals immunized to line 7 suppress the growth of line 7 cells but not of line 1 cells.

Method of immunization: Animals were immunized to line 1 tumor by weekly intradermal injections of $3 \times 10^6$ living ascites tumor cells. Line 1 tumor cells grow and regress spontaneously when injected intradermally and do not need to be excised. Animals received one injection a week for 3 weeks. This was followed by a rest period of 2-3 weeks. A second, and in some experiments a third, series of weekly immunization injections was then given. Animals were selected for experiments on the basis of having delayed skin reactions to line 1 tumor with a radius of at least 25 mm.

Animals were immunized to line 7 tumor by an intradermal injection of $0.75 \times 10^6$ living line 7 ascites tumor cells. Line 7 tumor cells grow progressively when injected intradermally and excision of the tumor about 18 days after injection is required for immunization. These animals then received weekly intradermal injections of living ascites tumor cells. The dose of tumor cells used for immunization was doubled each week until a dose of $3 \times 10^6$ cells was reached. Animals used in these experiments received 6–10 immunizing injections. Animals were selected for experiments on the basis of their ability to suppress an intradermal challenge of $3 \times 10^6$ living line 7 ascites tumor cells. PE cells, peripheral blood leucocytes, lymph node, and spleen cells were collected 7 days after the last immunizing injection.

Control guinea pigs were either unimmunized, immunized with a tumor line other than the one against which their cellular immunity was being tested, or given 0.1 ml complete Freund’s adjuvant (kindly provided by Dr. S. H. Stone, NIH) in each footpad in parallel with the tumor immunized animals.

Preparation of tumor cells for in vitro tests: Ascites-variant forms of antigenically distinct transplantable hepatomas, lines 1 and 7, were harvested using sterile techniques. They were suspended in 12% homologous guinea pig serum and 88% RPMI 1640 medium with 50 units of penicillin and 50 μg streptomycin/ml.

On several occasions line 1 cells, which had been maintained in tissue culture for about 1 month, were the source of the tumor cell suspensions. These tumor cell monolayers were treated with 1/5000 versene which yielded a 95% viable tumor cell suspension as determined by exclusion of 0.4% trypan blue.

Preparation of leucocytes: Peritoneal exudate cells were harvested about 70 hr after intraperitoneal injection of 60 ml of a light mineral oil, Drakeol-6VR (Pennsylvania Refining Company, Butler, Pa.). PE cell viability was always more than 95% when tested with 0.4% trypan blue. In order to have sufficient numbers of cells, the PE cells were usually obtained from two immunized or two control guinea pigs and pooled. Differential counts of immunized and control guinea pigs were similar and usually showed about 65% macrophages, 25% lymphocytes, and 10% granulocytes. In some experiments the PE cells were fractionated by centrifugation in tubes containing zones of albumin of different density. This yielded an upper fraction (I) consisting of 90% macrophages, an intermediate fraction (II) with about 85% macrophages and 15% lymphocytes, and a lower fraction (III) usually with 15% macrophages, 45% lymphocytes, and 40% polymorphonuclear leucocytes.

Spleen, lymph node, and peripheral blood leucocytes were obtained and suspended at the appropriate concentration in the aforementioned culture medium.

Culture conditions: Equal volumes of the sensitized or control lymphoid cells
were mixed at various concentrations, usually ranging from $1 \times 10^4$ to $1 \times 10^6$/ml with tumor cells at concentrations ranging from $10^4$ to $10^6$/ml. Duplicate 1-ml cultures of these mixtures were usually incubated from 44 to 92 hr at 37° C in covered stationary 1-dram vials in an atmosphere of 5% CO₂ and 95% air. Tumor cells and leucocytes were also cultured by themselves. In addition, replicate leucocyte cultures were incubated with 8 $\mu$g phytohemagglutinin (Burroughs Wellcome, Tuckahoe, N.Y.). The mean and standard error of unstimulated control PE cultures was $3495 \pm 648$ cpm. The response of unstimulated immune PE cells was $2922 \pm 500$ cpm.

**Processing of cultures:** All cultures were incubated for the final 12–16 hr with 1 $\mu$Ci tritiated thymidine (TdR³H, sp act. 6.0 Ci/mM, Schwarz BioResearch). They were processed for radioactivity precipitable by trichloroacetic acid as previously described.⁹

**Statistical analysis:** The data was transformed to logarithmic numbers because the degree of variation was observed to be proportional to the value of the counts per minute (cpm). A polynomial regression analysis was done on the logarithmically transformed data using a computer program (prepared by Dr. C. MacLean). This revealed that the standard error of the estimate of cpm ranged from 9 to 34.6% with a mean of 15%. Hence any two points that differ by more than 30% usually exceed the 95% confidence boundaries of the curves and are therefore significantly different from one another.

**Local passive transfer test:** In three experiments in which sensitized PE cells inhibited the in vitro incorporation of TdR³H by line 1 ascites cells, intradermal injections of nonimmune recipients by tumor cells mixed with PE cells sensitized to line 1 produced erythema and induration at 24 hr, and inhibited cutaneous tumor papule growth.⁷ In contrast, control PE cells mixed with line 1 did not produce such delayed hypersensitivity reactions and intracutaneous papules developed at the site of injection.

**Results.** Inhibition of tumor cell DNA synthesis in vitro by peritoneal exudate cells: Line 1 and 7 ascites tumor cells incubated in vitro manifested considerable TdR³H incorporation. The difference in the number of cpm incorporated by duplicate cultures of a given tumor cell line usually varied by less than 15% (in 54 of 55 points shown on Fig. 1). The effect of adding various concentrations of control or sensitized PE cells to cultures of line 1 tumor cells was compared (Fig. 1). Significant inhibition over and above the “nonspecific” inhibition by control PE cells of the incorporation of TdR³H by tumor cells was achieved by $10^4$ and $5 \times 10^4$ sensitized exudate cells. When $10^6$ PE cells were used the degree of nonspecific inhibition by control cells increased to an even greater extent than the inhibitory effect of the sensitized PE cells.

At the optimal concentration of $5 \times 10^6$, the sensitized PE cells inhibited uptake of TdR³H maximally in cultures containing from $10^4$ to $10^6$ line 1 tumor cells. The inhibition of tumor cell growth was not significant when $10^6$ line 1 tumor cells were present. Therefore, the most complete inhibition was obtained at ratios of sensitized PE cells to tumor cells of 50:1, but the number of counts decreased maximally at 10:1. However, significant inhibition was evident even at ratios of 1:1.

In a number of experiments, suspensions of $10^4$–$10^6$ line 1 tumor cells were prepared from tissue culture monolayers. The ability of sensitized PE cells to inhibit the TdR³H uptake by these tumor cells was comparable to their inhibition of ascites tumor cells.

**Kinetics of in vitro tumor cell inhibition:** Significant inhibition of tumor cell incorporation of TdR³H by sensitized PE cells was apparent after only 19 hr of
incubation. The degree of inhibition was greater after 44 and 66 hr of incubation. This increased inhibition of tumor cell DNA synthesis by the sensitized, and to a lesser degree control, PE cells indicated that inhibition occurred throughout the incubation period. This increment in specific inhibition with time occurs despite a concomitant progressively increased tumor cell growth over the same period of time. However, by the seventh day of incubation, the tumor cell growth had exceeded the inhibitory effects of control as well as sensitized PE cells, except at the lower initial tumor cell concentration of $10^3$–$10^4$ ml.

The rapidity with which PE cells cause demonstrable tumor inhibition in vitro suggests that DNA synthesis by lymphocytes contributes little to the TdR$^3$H incorporation by the cultures. In these experiments phytohemagglutinin-stimulated PE cell cultures did not incorporate TdR$^3$H until 66 hr of incubation, whereas PE cells already inhibited tumor cells by 19 and 44 hr. Therefore, it is unlikely that the tumor cell antigens would have stimulated significant lymphocyte proliferation until 66 hr of incubation. Furthermore, our attempts to demonstrate PE lymphocyte proliferation when mixed with irradiated (15,000 R, 200 kV, 15 mA) Westinghouse Quadrocondex x-ray machine) tumor cells and incubated for 3–7 days were unsuccessful. Irradiation blocked 95% or more of the tumor cell TdR$^3$H uptake but failed to reveal any significant increment in DNA synthesis in cultures containing both tumor and sensitized or control PE cells. Therefore, incorporation of TdR$^3$H by lymphocytes presumably did not interfere with our detection of inhibition of tumor cell growth.

**Localization of sensitized cells in vivo:** We compared the efficacy of leucocytes obtained from the peritoneal exudate with those from axillary, inguinal and mesenteric lymph nodes, peripheral blood, and spleens of immunized and control guinea pigs in inhibiting tumor cell DNA synthesis. Only the sensitized PE cells were able to inhibit line 1 cell growth to a greater degree than control cells. There was no detectable specific inhibition by lymph node and spleen cells even when concentrations as high as 1 and $2 \times 10^6$/ml were used. Specific lymph

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**Fig. 1.**—Representative experiment comparing the incorporation of tritiated thymidine by different concentrations of line 1 tumor cells cultured for 90 hr by themselves or in the presence of (A) $1 \times 10^6$, (B) $5 \times 10^6$ or (C) $1 \times 10^7$ sensitized or control guinea pig peritoneal exudate (PE) cells. In each of the figures the points represent the cpm of duplicate cultures. The cpm of unstimulated PE cultures were subtracted from the cpm of cultures containing both PE and tumor cells. The PE cells were obtained from two immunized and two control strain 2 guinea pigs and pooled.
node, peripheral blood, and spleen leucocytes incubated on a shaker (4–6/min) also failed to show any specific inhibitory effects. Furthermore, attempts to elicit specific inhibition by $5 \times 10^9$/ml sensitized spleen and lymph node cells by mixing them with $5 \times 10^4$/ml autologous PE cells were not successful. Thus inadequate "processing" of tumor antigens by macrophages is not likely to be responsible for the failure of lymph node and spleen cells from immunized animals to inhibit tumor cell DNA synthesis. In contrast, in 16 of 18 experiments, the sensitized PE cells were more inhibitory than control PE cells. One of the negative experiments was contaminated with bacteria and in the other the local passive transfer test was also negative. We therefore conclude that the distribution of sensitized cells capable of inhibiting tumor cell growth is uneven and greatest in the PE cell population.

**Effect of various types of peritoneal exudate cells:** PE cells were fractionated by centrifugation in tubes containing zones of albumin of different density (Fig. 2). The macrophage-rich fraction (I) caused considerable nonspecific but
little specific of TdR$^3$H incorporation by the tumor cells. The middle fraction (II) manifested the same degree of nonspecific but less specific inhibition than the unfractionated PE cells. It was in the lymphocyte and neutrophil-rich fraction (III) that the degree of specific inhibition by the sensitized cells was enriched, whereas the degree of nonspecific inhibition was diminished in comparison with the unfractionated PE. These results suggest that the mechanism of inhibition by macrophages is immunologically nonspecific, whereas the PE lymphocytes are responsible for the specific inhibition of tumor cell TdR$^3$H incorporation.

**Effect of irradiation of peritoneal exudate cells:** Irradiation (3000 R) of PE cells increased the degree of nonspecific tumor cell inhibition and ablated the specific tumor cell inhibition of sensitized PE cells (Table 1). Thus the sensitized

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<th>No. of line 1 ascites cells</th>
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<tr>
<td></td>
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*PE cells were irradiated with 3000 R washed $\times$ 3 with phosphate-buffered saline and incubated for 66 hr.

†Mean increment in cpm of duplicate cultures above the cpm of corresponding unmixed PE cell cultures. These consisted of 9240 cpm for control PE cells and decreased to 146 cpm after irradiation. Unmixed immune PE cell cultures incorporated 4852 cpm TdR$^3$H, and only 117 cpm after irradiation.

PE cell suppression of tumor cell growth involves a radiosensitive process. This result also supports the aforementioned observation that lymphocytes rather than macrophages are responsible for the immunologically specific inhibition, since lymphocytes are considerably more radiosensitive than the macrophages. 10

**Specificity of in vitro inhibition:** We compared the inhibitory effect of PE cells sensitized to line 1 or to line 7 on the DNA synthesis of line 1 tumor cells. At a concentration of $5 \times 10^6$ PE cells/ml, the anti-1 PE cells inhibited from $10^3$ to $5 \times 10^6$ line 1 cell growth to a greater degree than anti-7 PE cells. The latter therefore behaved like the control PE cells in the previous experiments.

**Inhibition of line 7 by peritoneal exudate cells:** The sensitivity of the assay was tested by comparing the inhibitory effect on line 7 ascites tumor cells of control versus anti-7 PE cells. This tumor line usually does not evoke delayed cutaneous hypersensitivity reactions. We were able to demonstrate specific inhibition of line 7 tumor cells in vitro by using PE cells from guinea pigs which had received six intracutaneous injections of line 7 tumor cells. Since the presence of delayed hypersensitivity has been correlated with tumor immunity, detection of inhibition of tumor DNA synthesis in its absence indicates that our assay was more sensitive in detecting such cellular immunity than the skin tests.
Discussion. We have presented additional experimental evidence for the existence of cell-mediated tumor immunity in inbred guinea pigs against several diethylnitrosamine-induced tumors. This has been achieved using a relatively simple, quantitative, reproducible, and rapid assay of inhibition of TdRH incorporation by ascites and tissue culture suspensions of tumor cells. Such inhibition was detectable after only 19 hr of incubating mixtures of guinea pig peritoneal exudate cells and tumor cells. This phenomenon was immunologically specific since PE cells from guinea pigs immunized with a particular tumor line consistently produced greater inhibition of that tumor's cell growth than normal PE cells or exudate cells immunized with an antigenically unrelated tumor. In contrast with other studies, we used syngeneic animals to ensure that tumor rather than histocompatibility antigens were responsible for this effect. The assay detects such specific inhibition over a wide range of tumor cell concentrations. Occasionally total inhibition was seen at lower concentrations of less than 10⁴ tumor cells/ml and it became less evident or totally obscured in the presence of more than 10⁶ tumor cells/ml. The specific inhibitory effects could be detected over a wide range of PE to tumor cell ratios of 50:1–1:1. When more than 10⁶ PE cells were used, increased nonspecific inhibition usually resulted in obscuring any immunologically specific effects.

This specific inhibition of tumor cell growth in vitro by sensitized PE cells was directly correlated with positive local passive transfer of delayed cutaneous reactivity. This reactivity in turn has been associated with suppression of in vivo tumor cell growth.7

The in vitro assay was also sufficiently sensitive to detect inhibition of tumor cell growth even in the case of the less-antigen line 7 tumor. Line 7 is inhibited in in vitro passive transfer studies by sensitized PE cells with little or no evidence of associated delayed inflammatory reaction.

However, the in vitro assay failed to detect any immunologically specific inhibition by peripheral blood leucocyte, lymph node, or spleen cell suspensions from guinea pigs with effective sensitized PE cells. Sensitized lymph node and spleen cells have also failed to inhibit tumor growth in passive and systemic transfer experiments.11

Studies with fractionated PE cells showed that cell fractions rich in lymphocytes and neutrophils were responsible for specific inhibitory effects; inhibition caused by macrophage rich fractions was nonspecific. This lymphocyte-rich fraction also exhibited the greatest activity in local and systemic passive transfer experiments. Irradiation of the PE cells blocked the specific immune inhibitory effects which also suggests that the lymphocyte is responsible for the specific response. Irradiated PE cells also failed to suppress tumor growth in the local passive transfer assay.2 Thus, PE cells contain either more potent or greater proportions of lymphocytes specifically able to inhibit tumor cell growth than lymphocytes from other sources. Investigations by Pick et al.12 and Old13 have shown greater efficacy of PE cells in several assays of cellular immunity. This supports the view that these exudates contain very potent lymphocytic mediators of cellular immunity.

The mechanism by which PE lymphocytes inhibit tumor cell growth is not
clear. There is no evidence of participation of antibodies in the immune response of the guinea pigs to the tumors. The process appears to be radiosensitive but also is apparent before any measurable degree of antigenically stimulated in vitro lymphocyte DNA synthesis. The inhibition may in part be reversible but at times is probably caused by irreversible cytotoxic effects since occasionally tumor cell growth was completely inhibited. Whether the inhibition of tumor cell growth requires cell-cell contact14 or may be mediated by lymphotoxin15,16 or a proliferation inhibition factor17 remains to be seen.

This in vitro assay can and should be applied to the study of human tumor immunity. It would be especially appropriate when identical twins or HI-A matched subjects with and without tumors are available. Our findings suggest that testing the influence of lymphocytes on DNA synthesis by tumor cells could be used to monitor varying degrees of tumor immunity in man.

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* Requests for reprints may be addressed to Dr. J. J. Oppenheim, Building 10, Room 322, National Institute of Dental Research, National Institutes of Health, Bethesda, Md. 20014.
2 Zbar, B., unpublished observations.
9 Oppenheim, J. J., R. A. Wolkstencroft, and P. G. H. Gell, Immunology, 12, 89 (1967).
11 Kronman, B. S., and H. T. Wepsic, unpublished observations.