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Role of respiratory-burst products from polymorphonuclear leukocytes in the antitumor activity of *Propionibacterium acnes* vaccine

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Summary. Tumor cells injected into Balb/c mice together with heat-killed 48-h *P. acnes* cells were rendered nontumorigenic as early as 12 h after injection, as determined by the inability of the tumor cells to give rise to tumors when transferred to a new host. Determination of tumor cell antigen levels by ELISA indicated that the tumor antigens had virtually disappeared by 24 h after injection of tumor cells and *P. acnes*. In contrast, in control animals injected with tumor cells only, there was an initial drop in tumor antigen levels at 12 h, after which the level rose steadily and tumors developed in 7–10 days. Since the cellular exudate at 12 h was almost entirely composed of polymorphonuclear leukocytes (PMN), we tested the ability of PMN, stimulated by phagocytosis of 48-h *P. acnes* cells, to produce substances toxic to tumor cells. Results indicated that the supernatant fluid from a phagocytosis mixture of PMN and *P. acnes* contained material toxic to tumor cells and also to Chinese hamster ovary cells. Tests with scavengers and inhibitors of oxygen-derived radicals suggested that the toxic material is either hydrogen peroxide (H_2O_2) or hydroxyl radicals (OH). Suspensions of 12-h *P. acnes*, *P. acnes* cells walls, *P. freudenrichii*, or latex beads were ineffective in preventing tumor growth, and induced little toxicity when phagocytosed. We conclude that in this test system 48-h *P. acnes* prevents tumor growth by stimulating the production of toxic oxygen metabolites during phagocytosis by PMN.

Introduction

The treatment of cancer by injection of bacterial products was first carried out by Coley, who developed the first 'mixed toxins' preparations that were used with some success in the treatment of tumors from 1891 until 1936 [12, 13]. In the 1960s Halpern stimulated renewed interest in the treatment of tumors by bacterial preparations when he reported the regression of an Ehrlich ascites tumor after injection of mice with a killed vaccine made from a strain of *Propionibacterium acnes* (then referred to as *Corynebacterium parvum*) [6]. Woodruff and Boak reported similar results [23], and it was then suggested that the mechanism responsible for the killing of tumor cells by these vaccines was T-cell-mediated [24, 25]. Olivotto and Bomford found that alveolar macrophages, harvested after injection with *P. acnes*, were cytotoxic to tumor cells [14]. Since then, it has been shown that natural killer cells may also play an important role in the killing of tumor cells after injection with *P. acnes* (e.g. Lichtenstein et al. [9]).

However, despite much investigation over the past 20 years the actual mechanism by which these vaccines induce tumor cell destruction in vivo is still by no means clear. To a large extent, the mode of action may depend on the route of administration. Systemic administration (intravenous or intraperitoneal) is likely to produce a general non-specific immunostimulation, while local administration at the tumor site will give a more specific cell-mediated immune response [4, 10, 11]. However, specific antitumor responses are likely to be effective only with tumors known to be immunogenic. In general, the antitumor activity of bacterial vaccines is monitored in vivo by survival rates, or by measuring tumor size or numbers of metastases. These characteristics provide evidence that the tumor mass has been partly or completely destroyed, but give little hint as to mechanisms. In the present communication, we present evidence that when tumor cells are injected intramuscularly together with *P. acnes* vaccine, tumor cell destruction occurs within 12 h, and its probable cause is some metabolite associated with the respiratory burst accompanying phagocytosis of vaccine material by polymorphonuclear leukocytes.

Materials and methods

Bacterial strains. Propionibacterium acnes 0009, *P. freudenreichii* 0407 and *P. avidum* 0589 were from the Culture Collection of the Department of Anaerobic Microbiology, VPI&SU. *P. granulosum* CN 5888 was from Burroughs Wellcome, Beckenham, Kent, UK. Stock cultures were maintained in chopped meat medium [7]. *Micrococcus lysodeikticus* ATCC 4698 was obtained as a spray-dried powder from Miles Laboratories, Elkhart, Indiana.

Preparation of vaccines. Bacterial suspensions were prepared as described previously [2]. A 48-h culture was used as inoculum in all cases, so that flasks to be harvested at 12 h had been initially inoculated with 48-h cells.

Mice. Male Balb/c mice, approximately 6-weeks old weighing 16–18 g, were obtained from Dominion Labora-

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tories, Dublin, Va. They were fed with Purina mouse chow and supplied with tap water ad libitum.

Tumor cell suspensions. The tumor cell line used originated as a methylcholanthrene-induced fibrosarcoma in Balb/c mice produced in the laboratory of Dr. Elgert, VPI&SU. The tumor was maintained by transfer to fresh mice every 12–14 days. To prepare tumor cell suspensions, mice with tumors about 1 cm in diameter were killed by cervical dislocation, the tumor was removed aseptically and the tumor material gently pushed through a 50-gauge wire mesh. The resulting cell suspension was centrifuged in the cold at 400 *g* for 5 min, washed twice with 10 ml cold Hanks balanced salt solution (HBSS), and finally resuspended in 1.5 ml HBSS. Viability was determined by trypan blue exclusion, and the suspension adjusted to 10^6 viable cells/ml: viability was always 90% or higher. For routine transfer of tumors 0.1 ml suspension (10^5 tumor cells) was injected intramuscularly into the right hind leg.

Antitumor activity of bacterial vaccines or latex beads. The concentration of viable tumor cells was adjusted to 2×10^6 cells/ml and mixed with an equal volume of bacterial suspension at 10 mg/ml in HBSS. Mice received an injection of 0.1 ml of this mixture (= 10^5 tumor cells and 500 μg vaccine material) in the right hind leg. Control mice were injected with 10^5 tumor cells alone. Latex beads (Difco, 0.8 μm diameter) were used in the same way (i.e. 500 μg in 0.1 ml) as the bacterial suspension.

*Anti-(*P. acnes*) antiserum.* This was prepared in rabbits by giving i.v. injections of partly disintegrated suspensions of *P. acnes* 0009. Injections were given twice weekly for 3 weeks and the animals bled 1 week after the last injection.

Preparation of antitumor antiserum. Rabbits were injected subcutaneously at the back of the neck with 1 ml emulsion consisting of 1×10^5 tumor cells in 0.5 ml HBSS and 0.5 ml Freunds complete adjuvant (Difco). One month later the animals were given a subcutaneous injection of 1×10^5 tumor cells in HBSS, and were bled out 1 week later. To remove cross-reacting antibodies, sera were absorbed with splenocytes. Spleens from normal Balb/c mice were gently homogenized in buffered saline with a loose-fitting glass grinder, the cells recovered by centrifugation and washed twice in phosphate-buffered saline (PBS). The washed cells were resuspended at 10^6 cells/ml in undiluted antitumor serum and held overnight at 4° C, after which the mixture was centrifuged (400 *g*) to recover the serum. Absorption was performed several times until no cross-reactivity with splenocytes was detected, as measured by enzyme-linked immunosorbent assay.

Fluorescent antibody tests. Indirect fluorescent antibody tests were done as described in Weir [20] using anti-(*P. acnes*) antiserum and goat anti-(rabbit immunoglobulin) antiserum labelled with fluorescein isothiocyanate (Sigma).

Histological preparations. At various times after injection, mice were killed by cervical dislocation; the tissue at the injection site was removed, cut into pieces about 3 mm in diameter and fixed in buffered 5% formalin overnight. The pieces were embedded in one block, and sections were stained with hematoxylin and eosin.

Enzyme-linked immunosorbent assay (ELISA). ELISA tests were done in Falcon flexible microtiter plates, using goat anti-(rabbit immunoglobulin) conjugated with alkaline phosphatase. Preliminary tests established that 1/1000 was a suitable working dilution of the absorbed antitumor serum, and this dilution was used throughout.

Standard absorbance curve. A standard curve relating absorbance to tumor cell numbers was first constructed. A tumor cell suspension, prepared as described above, was diluted in carbonate buffer (pH 9.0) to give 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 tumor cells in 0.2 ml. Wells in the microtiter plate were coated with cells by adding 0.2 ml of the various cell suspensions and incubating overnight at 37° C, using three wells for each cell dilution. All wells were then washed five times with phosphate-buffered saline containing 0.05% Tween 20 (PBS/Tween pH 7.4) and 0.2 ml antitumor serum diluted 1/1000 in PBS/Tween was added to each well. After incubation for 1 h at 37° C, wells were washed again three times with PBS/Tween and 0.2 ml alkaline-phosphatase-conjugated serum, diluted 1/500, was added. After 1 h at 37° C, the wells were washed three times with PBS/Tween, and 0.2 ml alkaline phosphatase substrate (1 mg/ml) in diethanolamine buffer pH 9.6 was added to each well. After a final incubation of 30 min at room temperature, the reaction was stopped by the addition of 0.05 ml 4 M NaOH and absorbances were read at 405 nm in an ELISA automatic reader. Controls consisted of (a) wells to which no tumor cells were added, (b) wells containing 10^5 tumor cells but no antitumor serum, (c) wells containing 10^5 splenocytes, (d) wells containing disaggregated muscle tissue cells from uninjected mice, and (e) wells containing tumor cells and antitumor serum but no phosphatase-conjugated anti-(rabbit Ig) serum. The absorbed serum was only very weakly reactive with cells other than tumor cells, producing absorbance readings of 0.05 with 10^5 splenocytes as against a background absorbance of 0.03 (wells to which no cells were added).

Estimation of tumor cell numbers in lesions. Mice were killed by cervical dislocation; tissue at the injection site was removed, minced coarsely with scissors and digested in 2.5 ml of a mixture containing 1 mg/ml collagenase and 1 mg/ml trypsin in PBS for 30 min at 37° C. All the cells recovered from each lesion were centrifuged, washed in PBS, resuspended in 0.2 ml carbonate buffer, and ELISA tests were performed as described above. Tumor cell recovery was about 95% as judged by ELISA tests in control mice injected with 10^5 tumor cells and then killed immediately. The absorbance readings were converted to presumptive tumor cell numbers using the standard curve. Experimental groups usually consisted of three mice, and cell numbers in the lesions from each mouse were assayed separately.

Lesion transfer assay. In these tests, tumor cells were obtained from lesions at 12, 24, 48 or 96 h after injection and transferred to new hosts to determine whether the tumor cells recovered from the lesions were still viable. At each time of sampling nine mice were killed and the lesion material from three mice was combined and injected into a single new host. The material was thus concentrated to allow smaller numbers of tumor cells to be detected. To per-

form lesion transfer assays, lesions were removed and digested with trypsin and collagenase as described above (Estimation of tumor cells numbers in lesions). Cell suspensions from three mice were combined and layered on a Ficoll step gradient prepared by layering 3 ml Ficoll, relative density 1.077, over 3 ml Ficoll relative density 1.1119. The double gradient with the sample was centrifuged at 200 *g* for 30 min. Under these conditions tumor cells pelleted to the bottom, while host cells formed a layer at the interface. The tumor cell pellets from each group of three mice were removed by pipette, washed in HBSS and resuspended in 0.2 ml and injected into one new host, which was examined at intervals for tumor development. A separate set of samples from lesions of comparable age to those in the experimental groups were examined for *P. acnes* by the fluorescent antibody technique.

Chemiluminescence. Balb/c mice were injected intraperitoneally with 0.5 ml 5% sterile glycogen solution in PBS. After 5 h, the mice were anaesthetized with Methofane and decapitated to obtain blood for serum; polymorphonuclear leukocytes (PMN) were obtained from the same mice by intraperitoneal lavage with ice-cold HBSS (5 ml/mouse). To remove red blood cells the suspension was centrifuged and resuspended in ice-cold 0.22% saline, vortexed briefly, and the isotonicity restored by adding an equal volume of cold 1.54% saline. After counting, the suspension was adjusted to 1×10^6 PMN/ml. Preparations for phagocytosis, consisting of 250 µg/ml bacteria or latex beads in PBS, were opsonized by treatment with an equal volume of fresh mouse serum at 37° C for 30 min. The opsonized suspension was centrifuged and resuspended in HBSS to give 50 µg (= approx. 2×10^8 bacteria) of opsonized cells or beads in 0.4 ml. Without pretreatment with fresh mouse serum no phagocytosis of either bacteria or latex beads could be detected in Wright's-stained slides of phagocytosis mixtures. For detecting chemiluminescence a Beckman LS-250 liquid scintillation counter was used at room temperature with a gain of 275 and a window of 0–500, and with the coincidence circuit switched to the rear photomultiplier tube to provide maximum sensitivity. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was used to enhance chemiluminescence. Readings with PMN alone were made with 1 ml PMN suspension in dark-adapted polypropylene vials. The suspensions were equilibrated for about 5 min until a stable background of about 10000 cpm was reached. Phagocytosis was started by adding 0.4 ml opsonized organisms or latex beads and 0.4 ml luminol solution (20 ng/ml in dimethyl sulfoxide). Final concentrations were 10 µM luminol and 200 organisms or beads per PMN. After addition, the vials were hand-shaken for 10 s, placed back in the counting chamber and counted at 1-min intervals for at least 30 min.

Phagocytosis toxicity assay. Suspensions of PMN were prepared, and vaccines or latex beads opsonized, as described under Chemiluminescence; 1.5 ml of a suspension of opsonized material was then mixed with 3 ml PMN suspension. After incubation for 30 min at 37° C, the mixture was centrifuged at 60 *g* for 10 min and the supernatant fluid removed. Tumor cells were suspended in 1-ml portions of the phagocytosis supernatant to yield 10^5 tumor cells/ml. This was done in triplicate for each supernatant and the mixtures were incubated for 2 h at 37° C. The tumor cell supernatant mixtures were centrifuged, the cells washed once in HBSS, resuspended in 0.1 ml HBSS and injected into the right hind leg of a mouse. Controls included (a) tumor cells only in HBSS, (b) tumor cells incubated with PMN but no bacteria, and (c) tumor cells incubated with *P. acnes* suspension only.

Cytotoxicity tests with chinese hamster ovary cells (CHO cells). CHO cells were grown in minimal essential medium (MEM) + 10% fetal calf serum in 96-well microtiter culture plates to 50% confluency. The volume in each well was 200 µl. For the tests, the phagocytosis supernatants, prepared as described for the phagocytosis toxicity assay, were diluted 1/2, 1/5 and 1/10 in HBSS and 20 µl each dilution added to a well containing growing CHO cells, so that the final dilutions were 1/20, 1/50, and 1/100. The plate was incubated in a CO_2 incubator overnight at 37° C, and then examined under phase contrast to determine cell death, which was shown by a change in cell morphology from spindle-shaped to round. Tests were done in triplicate. Control wells were inoculated with 20 µl supernatants from incubation mixtures containing PMN only, vaccine only, or HBSS only. Some wells remained uninoculated to check the viability of the CHO cell culture.

Filtration of supernatants. To confirm that the toxicity of phagocytosis supernatants was due to low-molecular-mass material, supernatants were filtered through 25-mm Amicon UM05 filters (cut-off of approximately 500 Da) in an Amicon 8MC Ultrafiltration system (Amicon Corp., Danvers, Mass) under 70–103 kPa (10–15 lb/in^2)N_2. Filtration was performed at 4° C. The filters were soaked in several changes of distilled water, and about 20 ml distilled water were passed through the filter before use. Thorough washing was necessary since unwashed filters were found to destroy the toxicity of supernatants.

Treatment of phagocytosis mixtures with enzymes, inhibitors or scavengers. The effects of various potentially inhibitory substances were tested by adding them to the phagocytosis mixture in the following final concentrations: catalase and superoxide dismutase (DDI Pharmaceuticals, Los Angeles, Calif) 75 µg/ml; NaN_3 (Fisher), 1 mM; D-mannitol (Difco), 0.02 M; L-methionine (Sigma), 0.02 M. In most cases, the inhibitory substances or enzymes were added to the phagocytosis mixture at the same time as the bacterial suspension, and the mixture was incubated for 30 min before centrifuging to obtain the supernatant. However, in some experiments inhibitors were not added until after phagocytosis had proceeded for 30 min. In these cases the mixture was incubated for a further 30 min before being centrifuged. As controls, suspensions of PMN and inhibitory substances without bacteria were used to ensure that these substances were not by themselves toxic to tumor cells.

Measurement of oxygen (O_2) consumption. A Clark oxygen electrode was used to measure O_2 consumption by PMN during phagocytosis of bacteria or latex beads. The electrode was calibrated, and O_2 consumption measured by the method of Robinson and Cooper [16] using *N*-methylphenazonium methosulfate and NADH. PMN were obtained by intraperitoneal injection of glycogen as described under Chemiluminescence. The PMN suspension was adjusted to 1×10^6 cells/ml; 1.3 ml (= 1.3×10^6 PMN)

was placed in the Clark cell and aerated, and the endogenous O_2 consumption measured. After 5 min 100 µl (= 2×10^8 organisms) of one of the opsonized vaccine preparations was added to give approximately 1.5×10^2 organisms/phagocyte, and the recorder deflection measured over the next 30 min. From the slope of the curve, a figure for O_2 consumption (µl O_2 ml^{-1} min^{-1}) could be calculated after subtracting the endogenous consumption.

Statistical analysis. Where appropriate, the statistical significance of the differences between means was calculated using the student's *t*-test.

Results

Before describing the results in detail, it may be noted that species of propionibacteria differ greatly in their reticulostimulatory abilities [2]. Moreover, in the case of *P. acnes*, the full reticulostimulatory activity is shown only by suspensions of post-log-phase cells (48 h or more) and suspension of 12-h cells have little activity [3]. Cell wall fragments also have little activity, even if prepared from fully active 48-h bacteria.

In most experiments, therefore, we have tried to cover a range of antitumor activities by using 48-h *P. acnes*, 12-h *P. acnes*, *P. acnes* cell walls, and a strain of *P. freudenreichii*. Strains of *P. freudenreichii* have previously been shown to have very poor reticulostimulatory activity [2, 3]. Latex bead suspensions were tested as representing particulate material not of bacterial origin.

The comparative antitumor effects of these various materials are shown in Table 1.

Fate of injected tumor cells

In the development of tumors in control animals, palpable tumors were not detectable before 6–7 days, and it was of interest to determine at what point in this early period the tumor cells were killed in vaccine-treated mice. This problem was attacked in two ways, first by transfer of lesions to new hosts, and secondly by assaying for tumor antigen using an ELISA test.

Lesion transfer experiments

The results of a typical experiment is illustrated in Fig. 1, which shows the effect of transferring into untreated recipient mice tumor cells obtained from animals previously injected with tumor cells with or without *P. acnes*. In the experiment shown, the transfers were done 12 h after the original injections. Essentially identical results were obtained when transfers were made after 24, 48 and 96 h, except that the older the lesion transferred to a second host the more rapidly did tumors develop, presumably because more tumor cells were being transferred in the older lesions. If the original animals were injected with tumor cells alone, the mice receiving the transfers always developed tumors, even if transfers into these recipient mice were made as early as 12 h after injection of original mice. This shows that a number of viable tumor cells sufficient to cause a tumor in a new host was present 12 h after injection of tumor cells into the original mice. However, if the original mice were injected with tumor cells and *P. acnes*, animals receiving the transfers of tumor cells did not develop tumors, even if transfers were made as early as 12 h after injection of original mice. This indicates that tumor cells injected together with *P. acnes* were rendered nonviable as early as 12 h after injection.

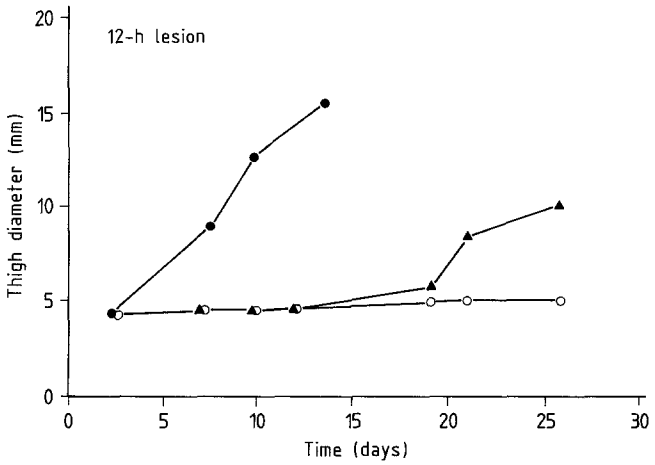

Fig. 1. Tumor development in fresh Balb/c mice injected with tumor cells obtained from 12-h-old lesions of mice previously injected with either tumor cells only or tumor cells + *P. acnes*. Control mice received 10^5 tumor cells obtained from a disaggregated 13-day tumor. Data points represent the average of five mice.
● 10^5 tumor cells control;
▲ Tumor cells alone;
○ Tumor cells + *P. acnes*

Table 1. Antitumor effect of various materials

Material injected	Thigh diameter measurements (mm)[a]			
	6	10	14	20 days
10^5 tumor cells	5.0	6.3	9.2	13.8
+ 500 µg 48-h *P. acnes*	6.0	5.4	5.6	5.0[b]
+ 500 µg 12-h *P. acnes*	5.1	6.0	8.9	13.2
+ 500 µg *P. acnes* cell walls	5.7	6.5	8.4	11.2
+ 500 µg *P. freudenreichii* (0407)	5.1	6.5	9.6	14.3
+ 500 µg latex beads	4.8	5.4	8.7	13.8

[a] Mean of five mice
[b] No tumors developed up to 90 days

The material transferred to the recipient mice was routinely examined by fluorescent antibody to *P. acnes* to insure that it was free of bacterial cells (see Materials and methods). Separation of tumor cells from *P. acnes* prior to transfer was made simple by the fact that at 12 h almost all of the bacteria had been already phagocytosed by PMN. Successful separation of these leukocytes from tumor cells was then easily accomplished by the step gradient system used.

Enzyme-linked immunosorbant assay

The second method used to monitor the fate of tumor cells in lesions was to assess the amount of tumor antigen present using an ELISA test. This method is obviously indirect since it measures tumor antigen rather than actual numbers of tumor cells, but in practice it gives results which appear closely to parallel those of the lesion transfer experiments.

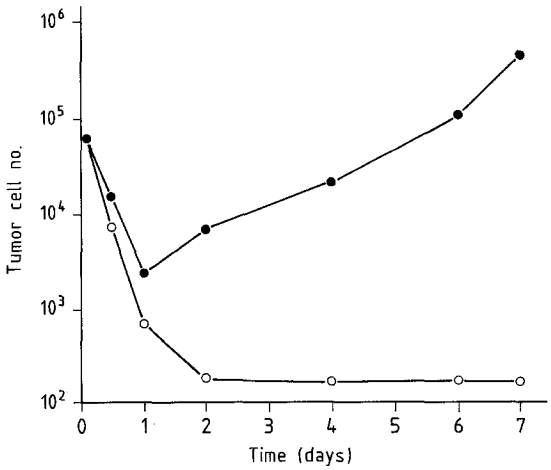

Fig. 2. Tumor cell number present in lesions versus time at which lesions were removed from Balb/c mice and subjected to analysis by ELISA. The curves represent results with mice injected with tumor cells only, or with tumor cells + *P. acnes*. Data points represent tumor cell numbers obtained from the ELISA standard curve using the average of three absorbance readings for each lesion.
● 10^5 tumor cells;
○ 10^5 tumor cells + 500 μg *P. acnes*

A standard curve, relating absorbance to tumor cell numbers, was constructed and there appeared to be a satisfactory relationship between absorbance and cell numbers over the range between 10^3 and 10^5 tumor cells. On the other hand 10^5 splenocytes gave an absorbance barely above background. This standard curve was used to estimate tumor cell numbers in lesions as shown in Fig. 2.

In control mice given 10^5 tumor cells, there is a sharp drop in antigen content of the lesion over the first 24 h, but then the amount steadily rises. In animals given 10^5 tumor cells and 500 μg *P. acnes* vaccine, however, antigen levels fall precipitously in the first day, have virtually disappeared by day 2, and do not rise thereafter.

Histological examination of lesions

Evidence from tumor transfer experiments and estimation of tumor cell numbers by ELISA tests indicated that *P. acnes* vaccines caused destruction of tumor cells at an early stage. In particular, the tumor transfer experiments suggested that the presence of vaccine material rendered the tumor inoculum nonviable as early as 12 h after inoculation. Histological examination of lesions at different times showed that while tumor cells alone elicited almost no cellular reaction in the host (Fig. 3), the injection of 500 μg *P. acnes* vaccine (with or without tumor cells) produced a well-marked polymorphonuclear response, which was particularly intense between 8 h and 14 h (Fig. 4 and Fig. 5). However, a similar PMN response was elicited by latex beads (Fig. 6), which did not give any substantial protection against the development of tumors (see Table 1). The PMN response elicited by *P. acnes* seemed, therefore, to be unusually toxic or destructive, and this point was investigated by incubating tumor cells in phagocytosis supernatants and then reinjecting them into another animal. The degree of chemiluminescence produced and the oxygen consumption of PMN when stimulated by phagocytosis of various different vaccines was also examined.

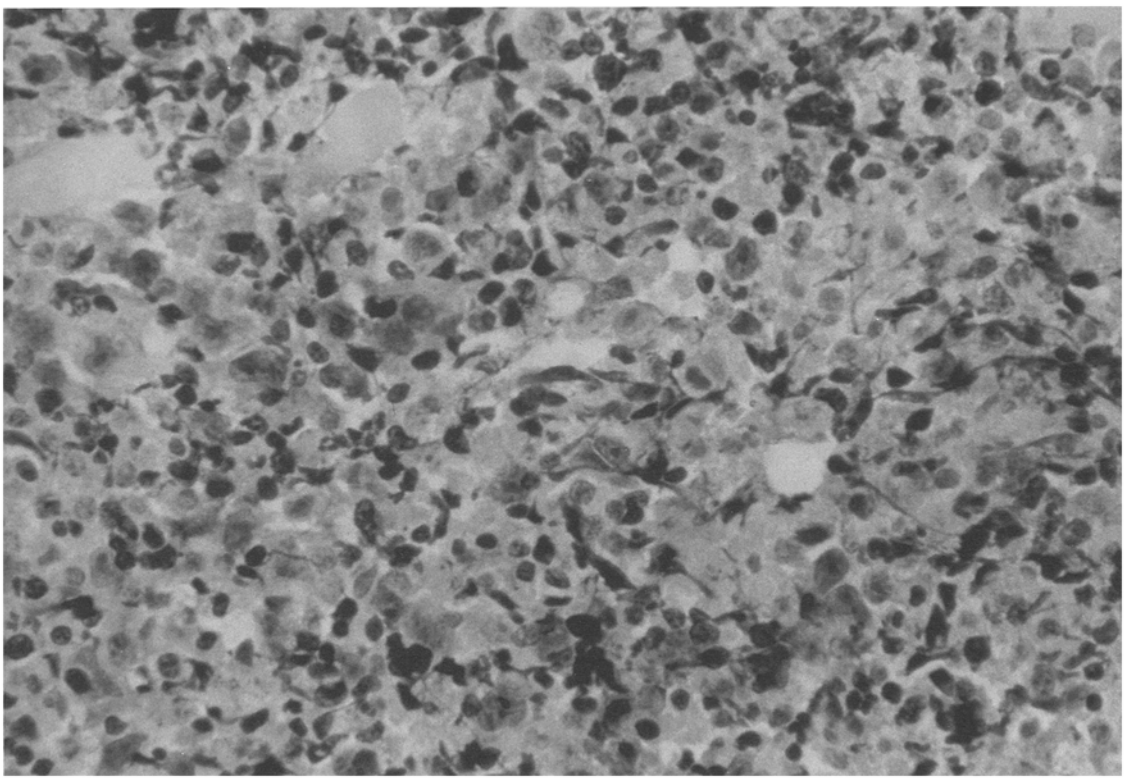

Fig. 3. Hematoxylin and eosin staining of muscle tissue section from Balb/c mouse leg, 8 h after intramuscular injection with tumor cells

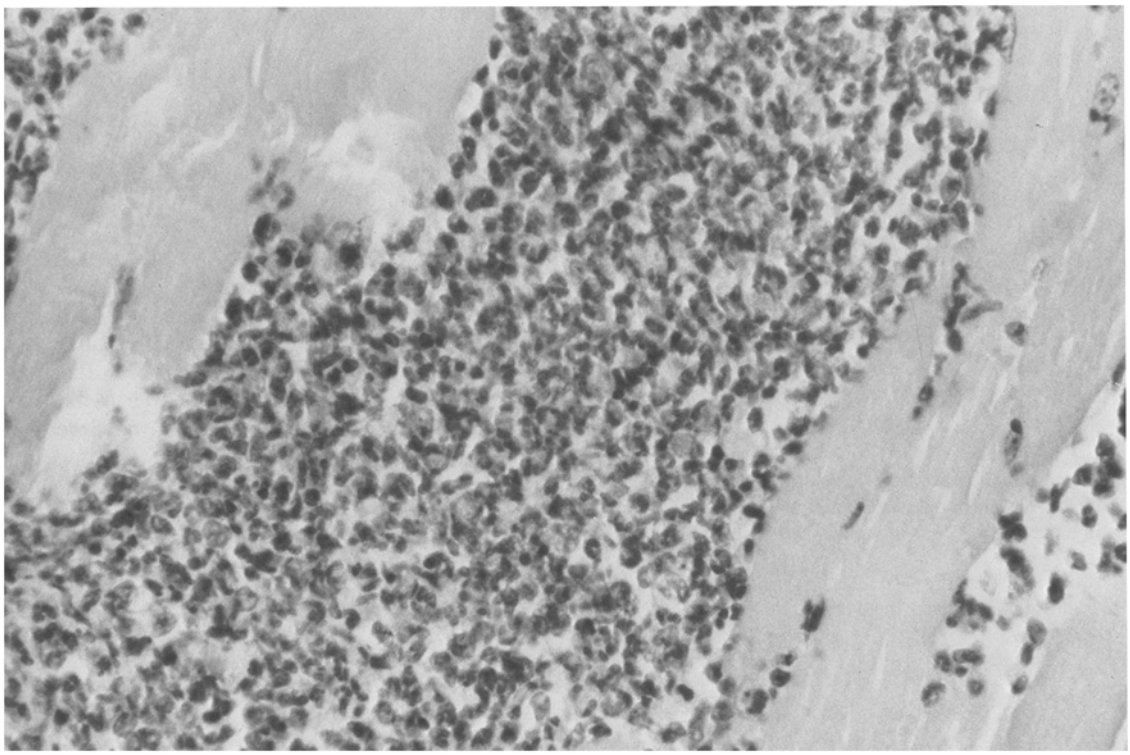

Fig. 4. Hematoxylin and eosin staining of muscle tissue section from Balb/c mouse leg, 8 h after intramuscular injection with 48-h *P. acnes* + tumor cells

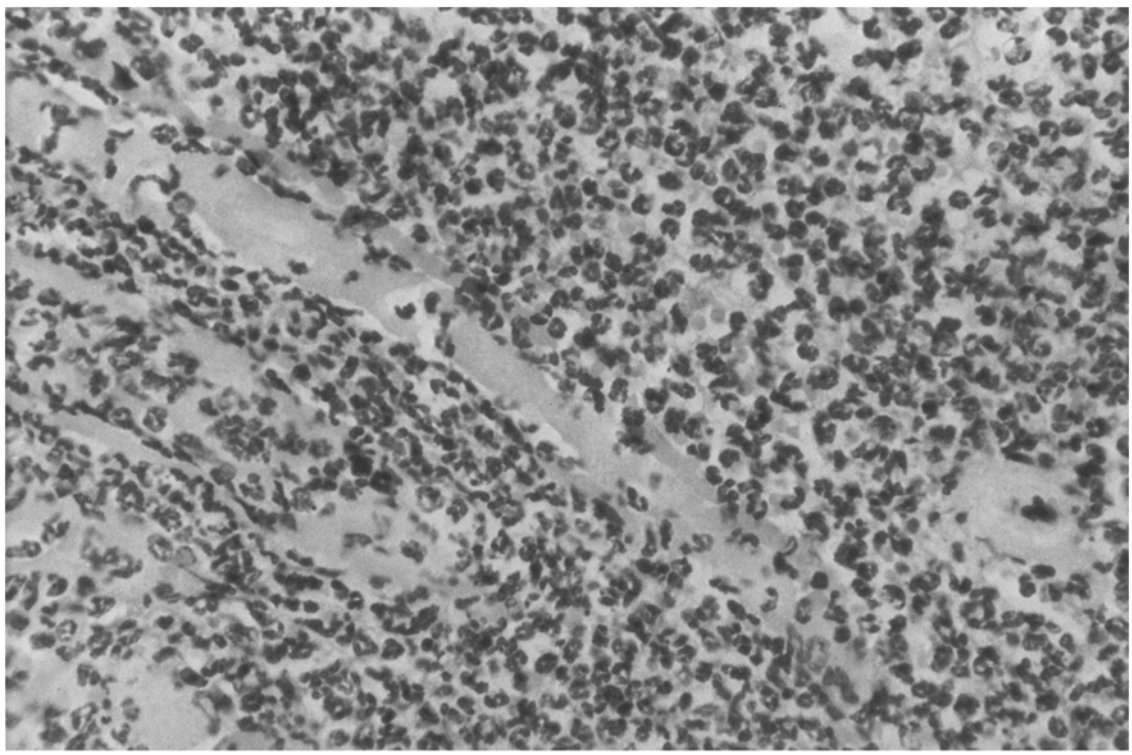

Fig. 5. Hematoxylin and eosin staining of muscle tissue section from Balb/c mouse leg, 8 h after intramuscular injection with 48-h *P. acnes* vaccine

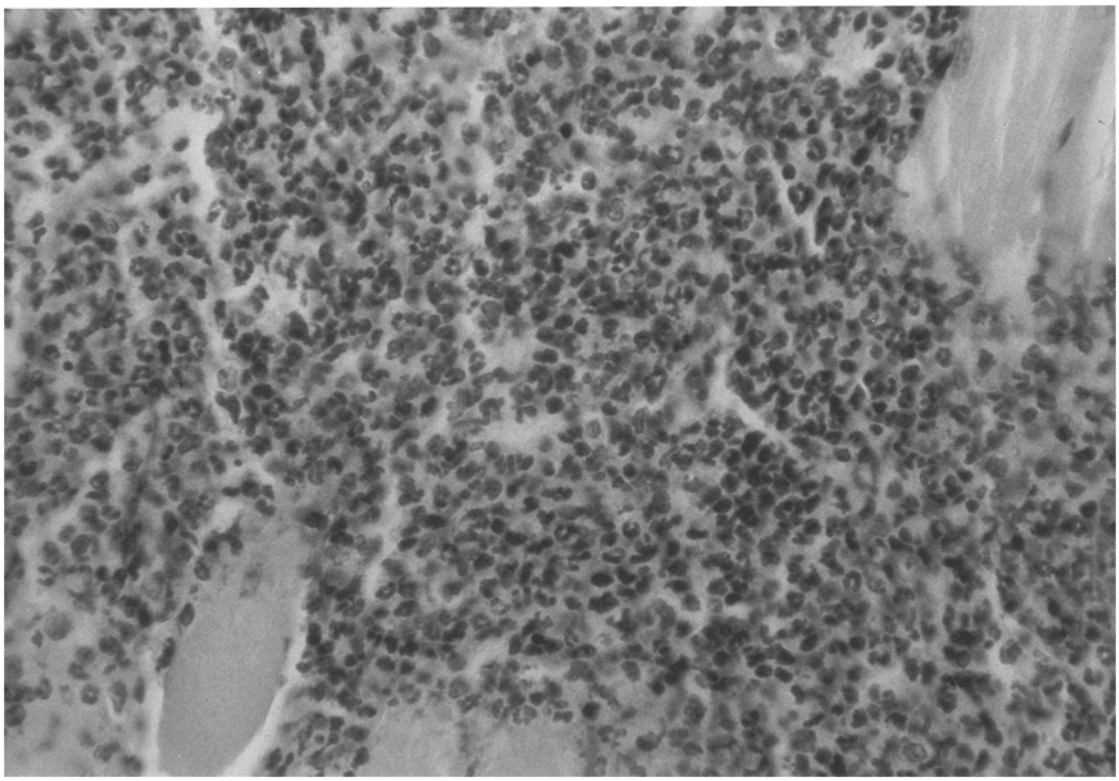

Fig. 6. Hematoxylin and eosin staining of muscle tissue section from Balb/c mouse leg, 14 h after intramuscular injection with latex beads

Toxicity of supernatants from phagocytosis mixtures

Lesion transfer experiments and ELISA tests indicated that tumor cells, given together with active vaccine at the same site, were killed soon after injection at a time when the inflammatory exudate was composed almost entirely of PMN. These findings strongly suggested that PMN were the cells primarily responsible for the death of tumor cells. Suspensions of tumor cells were therefore incubated in the supernatants from phagocytosis mixtures in which mouse polymorphs were allowed to phagocytose various suspensions. The results are shown in Table 2, and it is clear that after tumor cells have been incubated in supernatant fluid from a mixture of 48-h *P. acnes* and mouse polymorphs, they can no longer give rise to tumors, while this is not the case with the other phagocytosis mixtures. It may be noted also that incubation with bacterial suspension alone, or with unstimulated PMN alone, was not toxic to tumor cells.

Tests for the toxicity of phagocytosis supernatants using Chinese hamster ovary cells (CHO cells) showed a very similar result (Table 3). With CHO cells, all supernatants were toxic to some extent, but the supernatants from polymorphs and *P. acnes* 48-h cells were still fully toxic when

Table 2. Tumor development after incubation of tumor cells with supernatants from various phagocytosis mixtures

Material injected	Thigh diameter measurements (mm)[a]	
	17 days	21 days
HBSS	5.0	5.1 (0)[b]
10^5 tumor cells incubated with supernatants from mixture containing:		
10^6 PMNc + 48-h *P. acnes*	5.1	5.0 (0)
10^6 PMN + 12-h *P. acnes*	15.5	17.1 (12)
10^6 PMN + *P. acnes* cell walls	14.3	16.5 (12)
10^6 PMN + *P. freudenreichii*	15.1	17.0 (12)
10^6 PMN + latex beads	14.5	15.8 (12)
10^6 PMN only	14.9	16.5 (12)
48-h *P. acnes* only	15.1	16.3 (12)

[a] Mean of 12 mice
[b] Number in parenthesis represents the actual number of mice that developed tumors
[c] PMN, polymorphonuclear leukocytes

Table 3. Effect of supernatant from various phagocytosis mixtures on chinese hamster ovary (CHO) cells

Incubation mixture with CHO cells	CHO cell death[a] with supernatant dilutions of			
	Undiluted	1/2	1/5	1/10
Control no supernatant added	−	−	−	−
HBSS	−	−	−	−
PMN only	−	−	−	−
PMN + 48-h 0009	+	+	+	+
PMN + 0407	+	−	−	−
PMN + latex beads	+	−	−	−

[a] Death is measured by morphological change of all cells from spindle to round shape, as they become detached from the surface of the microtiter plate when dead (+). No change in morphology, as compared to control (−)

Table 4. Effect of radical scavengers and inhibitors on PMN-mediated cytotoxicity for tumor cells[a]

Material injected	No. of mice	Mean thigh diameter (mm) at 21 days	No. of mice with tumors	Significance, *P* (comparison with 21-day results for 10^6 PMN + 500 μg *P. acnes*
HBSS-only	3	5.1	0	
10^5 tumor cells treated with supernatant from:				
10^6 PMN only	21	15.1	21	<0.001
10^6 PMN + 500 μg *P. acnes*	21	5.2	0	
+ azide	15	15.0	15	<0.001
+ catalase	15	14.1	15	<0.001
+ superoxide dismutase	6	16.4	6	<0.001
+ mannitol	9	11.3	9[b]	<0.001
+ methionine	9	6.2	1[c]	NS[d]

[a] This table is compiled from the results of several experiments. In all cases the scavengers or inhibitors were added at the same time as the bacterial suspension, i.e. at the start of phagocytosis. For details, see Materials and methods
[b] Tumor development delayed but all animals developed tumors by 21 days
[c] Tumor developed after 17 days in one animal
[d] NS, not significant at 5% level

diluted 1/10 whereas the others were only toxic when used undiluted.

If a toxic phagocytosis supernatant was passed through a washed UM05 filter (cut-off at 500 Da), the filtrate was as toxic as the original while the retentate had no toxicity, showing that the toxic material is of low molecular mass. This would appear to rule out a protein, such as a lymphokine of some kind or an enzyme such as neuraminidase, as the basis for the toxicity.

Effect of radical scavengers and inhibitors on PMN-mediated cytotoxicity

The effects of various known scavengers of oxidative radicals were tested, with the results shown in Table 4. As might be expected, the addition of sodium azide completely inhibits the toxic effect on tumor cells, thus showing how closely the effect is connected with the respiratory oxidative burst produced by phagocytosis. When added to the phagocytosis mixture at the start, both catalase and superoxide dismutase abolished the toxic effect on tumor cells as effectively as azide. If mannitol (a scavenger of hydroxyl radicals) was added to the phagocytosis supernatant there was an obvious reversal of toxicity, although the mean thigh diameter was significantly smaller at 20 days than with azide, catalase or superoxide dismutase (e.g. 15.0 mm for azide against 11.3 mm for mannitol). However, all the animals in the mannitol experiment developed tumors, and it seems quite probable that a higher concentration of mannitol would have completely abolished toxicity. Methionine, a scavenger of hypochlorous acid (HOCl) was ineffective in reversing the antitumor effect of *P. acnes*, although one animal in nine did develop a tumor.

Similar effects of scavengers on the toxicity of phagocytosis supernates were shown in the CHO cell model (Fig. 7). In this model the dilution at which the supernate showed toxicity (i.e. produced death of CHO cells) was taken as a measure of activity. In experiments using CHO cells we compared the effect of adding the potential inhibitors at the start of phagocytosis, or after 30 min. If added after 30 min, the reaction mixture was incubated for a further 30 min before centrifugation to remove polymorphs and bacteria. Both catalase and superoxide dismutase were

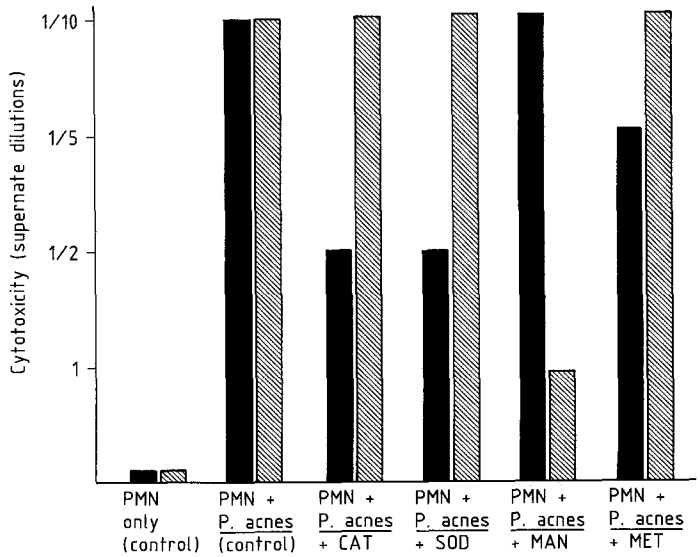

Fig. 7. Effect of time of addition of various radical scavengers/inhibitors on toxicity of supernatant from phagocytosis mixture containing PMN and 48-h *P. acnes* cells. Solid bars indicate addition of scavenger/inhibitor at the start of phagocytosis and hatched bars indicate addition 30 min after the start of phagocytosis. Scavenger/inhibitors used are catalase (CAT), superoxide dismutase (SOD), D-mannitol (MAN), and methionine (MET). Controls consist of PMN only or PMN + *P. acnes* cells to which no scavenger/inhibitor was added

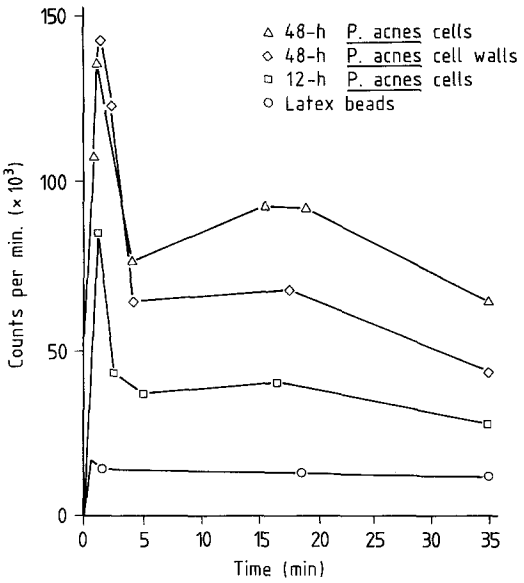

Fig. 8. Effect of various *P. acnes* cell preparations on chemiluminescence emission of polymorphonuclear leukocytes (PMN) during phagocytosis

quite effective in destroying toxicity if added at the start of phagocytosis, but ineffective if added 30 min *after* the start. Methionine showed little effect in either case.

Chemiluminescence and oxygen consumption of PMN

Because the results indicated that *P. acnes* 48-h vaccine was far more active in phagocytosis mixtures than the other preparations tested, the chemiluminescence and O_2 consumption accompanying phagocytosis of the different preparations were measured. All preparations except latex beads induced a marked burst of chemiluminescence within 2–3 min of adding the material to the suspension of PMN. (Fig. 8) *P. acnes* suspensions and *P. acnes* cell wall fragments produced almost equal peaks of about 140000 cpm. In addition, *P. acnes* 48-h cells produced a second rather flat peak of chemiluminescence at about 15–20 min. In terms of O_2 consumption, there was little difference between various bacterial preparations (Table 5), but latex beads stimulated almost no consumption of oxygen above the endogenous baseline.

Discussion

The results from the lesion transfer experiments indicate clearly that the killing of tumor cells in the model used is an event that occurs within 12 h of injection. These results were reinforced by the ELISA determinations, in which tumor antigen levels in lesions from mice injected with 10^5 tumor cells and 500 μg *P. acnes* dropped to background levels after 12 h. Since ELISA is based on antigen-antibody interactions it is not certain whether the decrease detected by this assay represents a drop in the actual numbers of tumor cells, or whether it simply indicates a change in the cells' antigenic configuration. However, whether tumor cells are killed directly or are first rendered non-oncogenic by loss or change of antigens and then killed, it is clear from our results that the tumor cells are no longer able to produce tumors as early as 12 h after injection with *P. acnes*.

Table 5. Oxygen consumption of PMN during phagocytosis of various vaccine materials

Vaccine used	$[O_2]$ consumed (μl ml^{-1} min^{-1})[a]
P. acnes (48-h 0009)	40.1
P. granulosum (5888)	40.4
P. avidum (0589)	38.9
P. freudenreichii (0407)	38.1
M. lysodeikticus	37.7
Latex beads	0.0

[a] Data represent the adjusted values of O_2 consumed after stimulation minus O_2 consumed before stimulation (endogenous)

The histological studies indicate that at 12 h the majority of the inflammatory cells present in the lesion are PMN. Weiss and Slivka [21] as well as other investigators have found that stimulated PMN can produce various oxygen-derived radicals capable of killing tumor cells [1, 17]. Although the primary role of PMN is that of phagocytosis, secretion of these radicals can occur with the potential for damage to adjacent cells. Through the phagocytosis toxicity and CHO cytotoxicity studies, it appears very likely that the mechanism responsible for the killing of tumor cells following intramuscular injection of mixtures with *P. acnes* involves a substance produced during phagocytosis of the *P. acnes* by PMN, since the supernatant obtained from phagocytosis mixtures in vitro was toxic to tumor cells. This substance was shown to be an oxygen-derived radical by the ability of azide and catalase to prevent completely the killing of tumor cells when added to the PMN/*P. acnes* mixture at the start of phagocytosis. The fact that catalase was ineffective, if added 30 m after phagocytosis had started, suggests that the hydrogen peroxide produced in the initial stages of the reaction behaves as intermediate and, if not destroyed, leads to the production of other toxic agents. Mannitol, a scavenger of hydroxyl radicals, was more effective when added at 30 min, suggesting that hydroxyl radicals may be largely responsible for the toxicity. These radicals are known to be produced by the interaction of superoxide anions and hydrogen peroxide through the Haber-Weiss reaction [19].

It is not clear why 48-h *P. acnes* cells should be so much more effective than the other materials in causing the production of toxic substances, especially in view of the fact that all the vaccines (but not latex beads) stimulated about the same amount of O_2 consumption during phagocytosis. It is known that 48-h cells of *P. acnes* are more effective than either 12-h cells or cell walls in longer-term stimulatory effects, e.g. the production of hepatosplenomegaly in mice [3]. This may be due to the ability of 48-h cells to withstand destruction after phagocytosis [15] and perhaps suggests that after the initial primary respiratory burst, triggered equally by all vaccines, the details of secondary radical production may depend on differences in cell surface composition in various organisms.

In recent years the possible tumoricidal role of PMN has received attention especially in view of the fact that they are particularly active in producing oxidative radicals when stimulated by phagocytosis (e.g. [8]) and that some of these oxidants may be long-lived [18, 21, 22]. It has also been shown that human granulocytes stimulated by phorbol myristate acetate destroy T-lymphoblast target cells and render them non-oncogenic [21]. Glaves [5], in a dif-

ferent system, showed that PMN were primarily responsible for the accelerated pulmonary clearance of melanoma cells in mice treated with trypan blue.

Although the evidence is admittedly indirect, our own results reported here appear to point clearly to the polymorphonuclear leukocyte as the cell responsible for tumor cell destruction. In the first place, tumor cells were destroyed in vivo at a time when the inflammatory exudate is almost entirely composed of PMN. Secondly, the tumoricidal activity induced by various vaccine materials in phagocytosis mixtures containing PMN in vitro parallels the antitumor activity of the same vaccines in vivo.

The mechanisms by which bacterial preparations prevent the development of tumors have not been fully elucidated, but they are generally thought to act by enhancing cell-mediated immune responses, for example by 'activating' macrophages, natural killer cells or T cells to become cytotoxic to tumor cells. However, it appears likely that, on intramuscular injection in conjunction with tumor cells, the tumoricidal effect of *P. acnes* is not due to its function as an immunostimulant but to its action as a trigger for the release of toxic oxidative metabolites from PMN.

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