

CYTOSTATIC ELIMINATION OF SYNGENEIC RAT TUMOR CELLS  
IN VITRO BY NONSPECIFICALLY ACTIVATED  
MACROPHAGES\*

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Evidence for the presence of tumor-specific antigens in human and animal tumors (1-7) and for the important role of specific cellular immunity in the process of tumor cell elimination (8-11) continues to accumulate. This considerable body of information has, however, failed to account for the often observed capacity of the host to reject homografts while unable to overcome a tumor. The fact that tumors generally grow unchecked and metastasize has been variously ascribed to the elaboration of specific antibody or other mechanisms capable of blocking cellular immune reactions (12-16); however, confidence in such explanations is diminished by well-established situations in which prompt initial tumor growth can no longer be overcome by the delayed immune response, or by the paradox of the "sneaking through" of a few tumor cells despite the obvious existence of a substantial specific immune response.

There is now a large body of information showing that the host resistance to infection can be modified and strikingly increased by nonspecific means (17-20). Recent investigations have indicated that resistance to tumors may involve nonspecific factors as well as acquired specific immunity (21, 22). For example, the growth of both allogeneic and syngeneic tumors has been shown to be markedly inhibited in rats and mice infected with *Nippostrongylus brasiliensis* (23, 24), *Schistosoma mansoni* (25), *Toxoplasma* or *Besnoitia* (26), or in animals injected with BCG (22, 24, 27-29), complete Freund's adjuvant (30), *Corynebacterium parvum* (22), poly I/poly C<sup>1</sup> (24, 31), endotoxin (22, 24), peptone (24, 32), and still other affirmed stimulants of the reticulo-endothelial system.

In showing that virus-induced or carcinogen-induced syngeneic tumor cells may be effectively killed in vitro by activated normal rat peritoneal cells, the present work extends this body of evidence; these findings are consistent with the thesis that the nonspecific mechanisms heretofore associated with resistance to infection can contribute significantly to the host's defense against tumors.

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<sup>1</sup> Abbreviations used in this paper: DMBA, dimethylbenzanthracene; [<sup>3</sup>H]TdR, tritiated thymidine; MCA, methylcholanthrene; PMN, polymorphonuclear leukocytes; Poly I/Poly C, polyinosinic polycytidylic acid.

### *Materials and Methods*

*Animals.*—Inbred DA rats were used throughout this study. They were kept on a standard diet and water *ad libitum*.

*Induction of Syngeneic Tumors.*—Three different types of tumors were used:

*Polyoma tumors:* Polyoma virus (wild type; kindly donated by Dr. R. Weil, Dept. de Biologie Moléculaire, Genève) was suspended to contain  $5 \times 10^8$  plaque-forming units/ml. Polyoma tumors were obtained after subcutaneous (s.c.) injection of 0.06 ml of the virus suspension into the neck of 2-day old, neonatally thymectomized DA rats. The subcutaneous tumors were maintained in vivo either by serial s.c. implants or by s.c. injection of tumor cell suspensions into syngeneic recipients. As a source of target cells for in vitro tests, tissue culture lines were prepared from the transplanted polyoma tumors, grown in a medium described by Schindler et al. (33) and containing 10% normal rat serum (heat-inactivated for 60 min at 56°C), and maintained by in vitro serial passage. Cells were subcultured every 3rd day and  $10^6$  cells were seeded per 250 ml Falcon culture flask (Falcon Plastics, Division of BioQuest, Oxnard, Calif.).

*Dimethylbenzanthracene tumors:* Dimethylbenzanthracene tumors were obtained after a single subcutaneous injection of 3.3 ml of 7.12 dimethylbenz(a)anthracene ([DMBA] Fluka AG, Buchs, Switzerland; dissolved in olive oil) into inbred DA rats weighing 150–200 g. The tumors were maintained in vivo through serial passage by implanting pieces of tumor subcutaneously into rats weighing 80–120 g. Tissue culture lines of DMBA tumors were developed as for polyoma tumors and grown in the same medium supplemented with heat-inactivated 20% fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.). Cells were subcultured every 2–3rd day and  $10^6$  cells were seeded per 250 ml culture flask.

*Methylcholanthrene tumors:* Methylcholanthrene tumors were obtained after instillation into the stomach of 20 ml of methylcholanthrene ([MCA] Fluka AG) on each of 6 consecutive days into female DA rats that had been splenectomized 1 or 2 days previously. Mammary tumors were passaged in vivo as described for DMBA tumors. Tissue culture lines were developed as described for polyoma tumors and grown in the same medium supplemented with 10% normal, heat-inactivated rat serum. Cells were subcultured every 4th day and  $10^6$  cells were seeded per 250 ml culture flask.

These tumor cell cultures were regularly tested for the presence of mycoplasma organisms after incubation for 2–4 days at 37°C on mycoplasma agar. No mycoplasma contamination was ever detected in any of the cultures.

*Preparation of Macrophage Monolayers.*—Peritoneal cells were obtained either from untreated donor DA rats, or 3 days after intraperitoneal (i.p.) injection of 10 ml of 10% proteose peptone (Fluka AG [32]) by washing out the cavity with culture medium. Cultures were prepared by seeding approximately  $2 \times 10^6$  macrophages into 35 × 10 mm Falcon plastic Petri dishes. After 15 min the medium was decanted and 2 ml of medium supplemented with 10% heat-inactivated normal rat or fetal bovine serum was added. After 2 h of incubation, the non-adhering cells were removed by intensive washing of the monolayers with jets of tissue culture fluid. After this procedure, lymphocytes constituted less than 3% and granulocytes less than 0.2% of the culture. Tumor cells ( $2 \times 10^5$  cells/dish) were immediately added to the monolayer or after a further 5 h of culturing.

*Preparation of Polymorphonuclear Leukocytes (PMN's).*—Peritoneal cells were harvested from inbred DA rats 4 h after injection of 10 ml of 0.1% glycogen i.p. (Fluka AG [34]) and cultured for 2 h in plastic Petri dishes as described before to remove macrophages. The nonadhering cells consisting of at least 95% PMN's were used as a source of effector cells.

*Assessment of the Effects of Interacting Target Cells with Effector Cells.*—Besides the assessment of the morphology (by phase-contrast microscopy or after fixation with methanol and staining with Giemsa) and of the viability (with 0.2% trypan blue) of effector and target cells, two more objective methods were used to follow the possible consequences of effector/target cell interaction.

*Cytotoxicity Tests.*—Suspensions of tumor cells were first incubated for 30 min at 37°C with  $^{51}\text{Cr}$  (sodium chromate; Eidgen. Institut für Reaktorforschung, Würenlingen, Switzerland; 50  $\mu\text{Ci } ^{51}\text{Cr}$  per  $10^8$  cells). Tumor cells were washed three times, then added to the macrophage monolayers ( $2 \times 10^5$  tumor cells/35 mm Falcon plastic dish) and cultured at 37°C for 15 h in a moist atmosphere containing 5%  $\text{CO}_2$ . Afterwards, the medium was decanted and the nonadhering cells were removed by intensive washing of the dishes. After centrifugation, radioactivity was measured in sediments, supernatants, and the liquid obtained by solubilization of the adhering cells through the addition of 2 ml of 0.5 N NaOH. Cultures containing labeled tumor cells alone were incubated and sampled simultaneously with the experimental cultures to determine the amount of label leaching spontaneously from the cells. Labeled tumor cells were killed by heating (60°C for 20 min) to determine the total releasable label. Using these controls, cell death was expressed in the form:

$$\frac{\text{Experimental release} - \text{control release}}{\text{Total releasable label} - \text{control release}} \times 100.$$

*Assessment of Residual Tumor Cell Proliferation.*—Tumor cells ( $2 \times 10^5$  cells/dish) were cultivated either alone or together with syngeneic peritoneal cells in  $35 \times 10$  mm Falcon plastic tissue culture dishes for 2, 4, 6, 8, 10, 12, or 14 h at 37°C. Afterwards, the cells were exposed for 60 min at 37°C to 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine/dish ([ $^3\text{H}$ ]TdR; methyl- $^3\text{H}$ ; 5,000 mCi/mmol; The Radiochemical Centre, Amersham, Buckinghamshire, England) and washed three times with cold medium. To ensure that the radioactive precursor was incorporated into DNA, nucleic acid radioactivity was measured in a liquid scintillation counter after it had been solubilized with hot perchloric acid (35). In some experiments, effector cells were first irradiated in vitro with 5,000 R and then immediately cultured together with target cells.

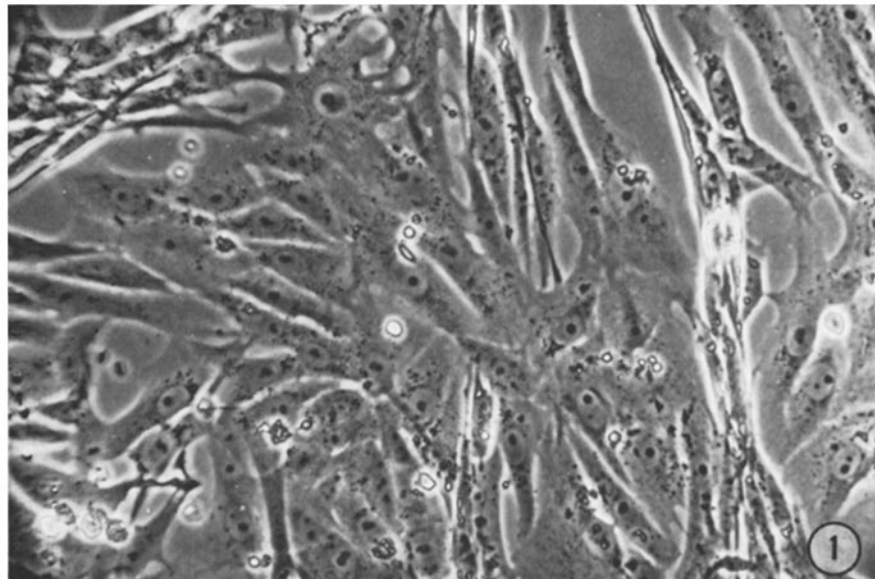


FIG. 1. Morphology of polyoma-induced rat tumor cells. Tumor cells were plated at  $2 \times 10^5$  cells per 35 mm dish in medium supplemented with 10% heat-inactivated normal rat serum and grown for 15 h. Living cells, phase-contrast microscopy.  $\times 125$ .

To assess the effect of adherent peritoneal cells on RNA synthesis by tumor cells,  $2 \times 10^5$  tumor cells cultivated for 14 h with peptone-induced macrophages were exposed for 60 min at  $37^\circ\text{C}$  to  $1 \mu\text{Ci}$  [ $^3\text{H}$ ]uridine/dish ([ $5\text{-}^3\text{H}$ ]uridine; 20,000 mCi/mmol; The Radiochemical Centre), processed, and radioactivity measured as described for DNA incorporation.

#### RESULTS

*Morphological Changes after the Interaction In Vitro of Syngeneic Tumor Cells and Activated Peritoneal Macrophages.*—Exploratory experiments revealed that the procedure used in the Walker carcinosarcoma model system to assess effector/target cell interactions, i.e. enumerating the tumor cells that could be washed off from the cultures after incubation, was not strictly quantitative since the syngeneic tumor cell lines also adhered firmly to the culture vessel. However, a marked difference was observed consistently when tumor cells were grown for 15 h; cultured in the absence of effector cells, the tumor cells covered part of the surface of the dish and were well spread (Fig. 1). In preparations where tumor cells had been cultured for 15 h together with peptone-induced macrophages, tumor cell number was greatly decreased, the cells were shrunken, and most of these cells were covered by adhering macrophages. Despite the obvious decrease in tumor cell number, no increase in cell debris was ever found in these preparations (Fig. 2). When normal, nonstimulated peritoneal cells were

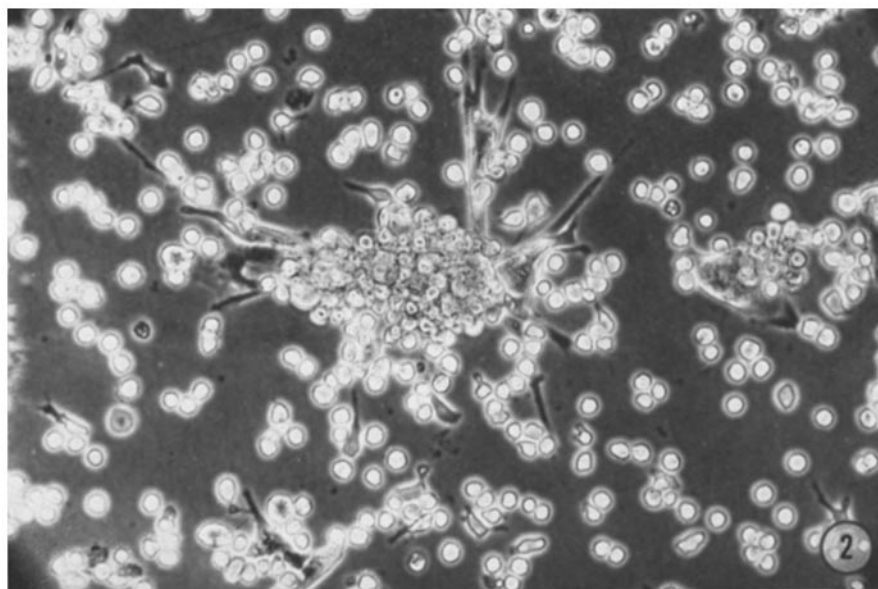


FIG. 2. Morphology of polyoma-induced rat tumor cells grown under identical conditions as indicated in Fig. 1 but in the presence of  $2 \times 10^6$  activated, nonimmune rat peritoneal macrophages. Reduction in tumor cell number: tumor cells were shrunken and covered by adhering macrophages. Living cells, phase-contrast microscopy.  $\times 125$ .

cultured with tumor cells after the nonadhering cells had been washed off, a less marked effect was seen: tumor cells grown rather densely showed partly normal appearance and were well spread, partly shrunken, and were often covered by a large number of adhering mononuclear cells (Fig. 3). A somewhat similar picture was seen when tumor cells were cultured with glycogen-induced PMN's (Figs. 4 and 5).

*The Nature of the Cytocidal Process.*—Once morphological observations had indicated that both activated macrophages and exudative PMN's could rapidly

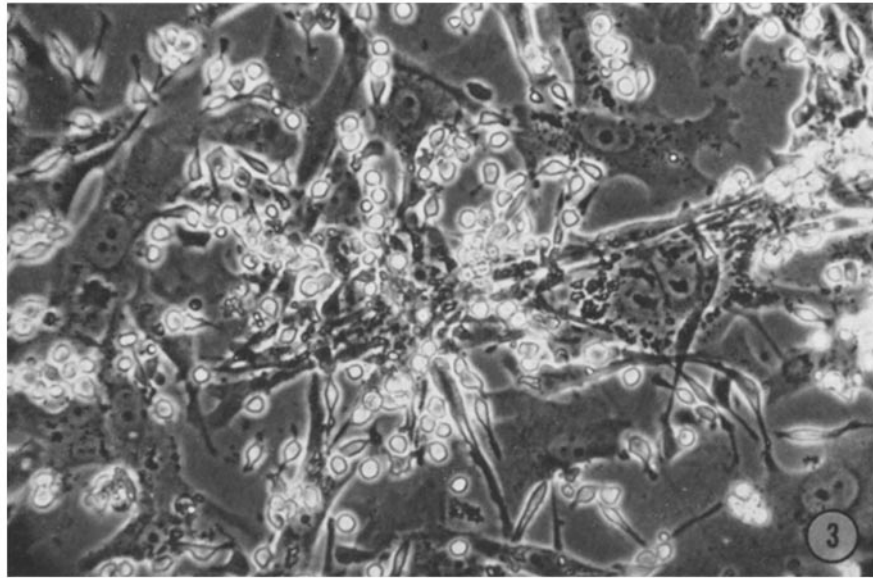


FIG. 3. Morphology of polyoma-induced rat tumor cells grown under identical conditions as indicated in Fig. 1 but in the presence of  $2 \times 10^6$  adherent, normal peritoneal cells. Liver cells, phase-contrast microscopy.  $\times 125$ .

and effectively eliminate syngeneic tumor cells *in vitro*, more objective means of assessing this process were investigated.

*Cytotoxicity.*—When  $^{51}\text{Cr}$ -labeled polyoma- or carcinogen-induced tumor cells were cultured alone, the amount of label leaching spontaneously from the cells was approximately 2%/h. Although up to 30% of the label was thus released over 15 h, the cells retained viability (>97%) as indicated by dye exclusion tests (Table I). Culturing of  $^{51}\text{Cr}$ -labeled polyoma- or carcinogen-induced tumor cells with activated peritoneal macrophages at a ratio of approximately 1:10 for 15 h consistently yielded a low but significant release of the label from target cells (Table I); and under these conditions the viability of effector and target cells, as assessed by trypan blue exclusion, was regularly slightly decreased.

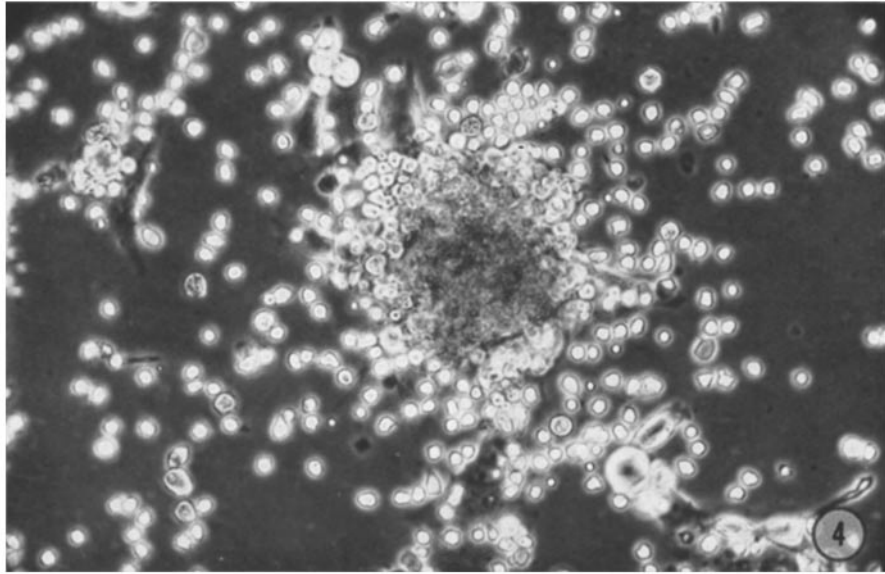


FIG. 4. Morphology of polyoma-induced rat tumor cells grown under identical conditions as indicated in Fig. 1 but in the presence of  $2 \times 10^6$  glycogen-induced syngeneic polymorphonuclear leukocytes. Living cells, phase-contrast microscopy.  $\times 125$ .

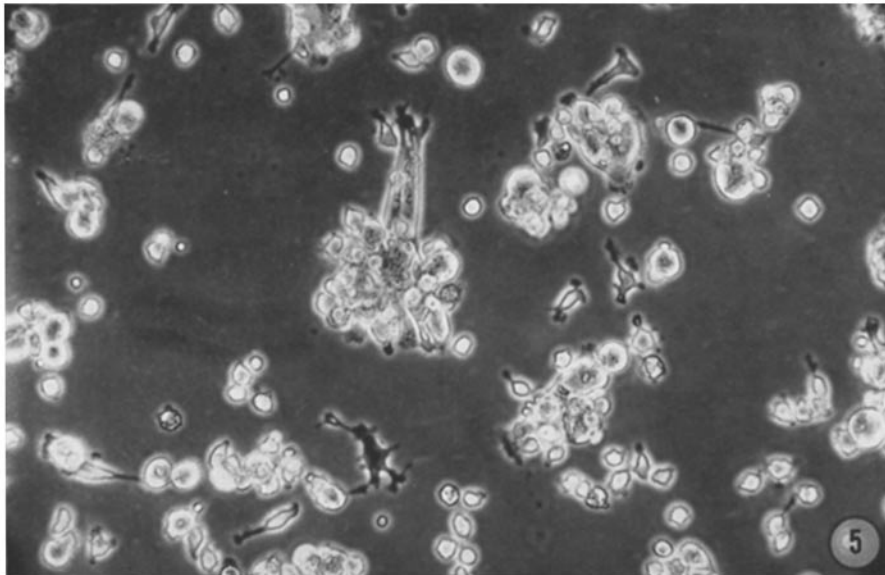


FIG. 5. Morphology of polyoma-induced rat tumor cells grown under identical conditions as indicated in Fig. 1 but in the presence of  $2 \times 10^6$  glycogen-induced syngeneic polymorphonuclear leukocytes. Note accumulation of effector cells on tumor cells. Living cells, phase-contrast microscopy.  $\times 125$ .

In contrast, culturing exudative PMN's with the various target cell lines produced no cytotoxic effects; the release of  $^{51}\text{Cr}$  and the ability to exclude the vital dye were within the same range as the controls (Table I).

In following the time-course of macrophage cytotoxicity, the main increase in  $^{51}\text{Cr}$ -release was found to occur within the first 6 h of interaction with targets; afterwards levels of  $^{51}\text{Cr}$  in the supernatants remained more or less constant (not shown). A similar time-course was evident when viability of target cells, as assessed by trypan blue exclusion tests, was taken as a measure of cytotoxicity. Provided that the effector to target cell ratio was within the same range (10:1) as in the preceding experiments, adherent peritoneal cells from normal, untreated DA rats, cultured for 6 h before the addition of tumor cells, displayed a degree of cytotoxicity similar to peptone-induced, adherent peritoneal cells.

*Proliferation of Tumor Cells.*—The loss by tumor cells of the capacity to incorporate DNA precursors, such as [ $^3\text{H}$ ]TdR, was marked when they were exposed to nonimmune, activated macrophages or exudative PMN's (Table II). In contrast, tumor cell proliferation remained unaffected when tumor cells were cultured alone at a similar density (initial tumor cell number  $2 \times 10^6$ ). Control experiments confirmed that macrophages or PMN's alone did not incorporate

TABLE I  
*Comparative Cytotoxicity for Target Cells Exerted by Activated Macrophages and by Exudative PMN's*

Cells present in culture*	$^{51}\text{Cr}$ release from target cells	Target cell death (trypan blue)	Effector cell death (trypan blue)
	%	%	%
Tumor cells‡	0§	2.1 ( $\pm 0.4$ )	—
Activated macrophages	—	—	7.5 ( $\pm 1.2$ )
Activated macrophages + tumor cells (10:1)	13.2 ( $\pm 4.1$ )	12.5 ( $\pm 3.6$ )	14.6 ( $\pm 4.3$ )
Exudative PMN's	—	—	4.6 ( $\pm 0.8$ )
Exudative PMN's + tumor cells (10:1)	0	2.4 ( $\pm 0.6$ )	5.6 ( $\pm 1.1$ )

Each value represents the mean of 20 determinations.

\* Incubation was for 15 h.

‡ Polyoma-induced tumor cells were used in the present experiments.

§ Spontaneous release from targets was taken as 0.

TABLE II  
*Residual Tumor Cell Proliferation after Culturing for 14 h with Effector Cells (Controls = 100%)*

Cell mixture	Mean % of [ $^3\text{H}$ ]TdR incorporation at 14 h
Macrophage/tumor cell (10:1)	8.6 ( $\pm 4.4$ )*
PMN/tumor cell (10:1)	45.3 ( $\pm 6.2$ )*

\* Each value represents the mean of 30 determinations.

measurable amounts of the radioactive precursor (36, 37). This system thus reflects the residual proliferative capacity of target cells. Since the potential for growth of the tumor cells was markedly affected by the presence of macrophages or PMN's, this procedure was used to evaluate the elimination process.

The capacity of tumor cells ( $2 \times 10^5$ /dish) to incorporate the RNA precursor [ $^3\text{H}$ ]uridine was greatly decreased after interaction for 14 h with  $2 \times 10^6$  activated macrophages ( $11 \pm 4.7\%$  of controls). Since macrophages alone incorporated a much smaller percentage ( $3 \pm 1.9\%$ ) of the label incorporated by tumor cell controls, this decrease reflects the residual RNA synthesis of tumor cells.

*Cytkinetics of the Cytostatic Process.*—The time-course of the elimination process was followed using populations of activated peritoneal macrophages or glycogen-induced PMN's. Incorporation of [ $^3\text{H}$ ]TdR was measured after effector cells plus tumor cells or tumor cells alone had been cultured for periods of time ranging from 2 to 14 h. Results such as those shown in Fig. 6 demonstrate that in the presence of nonimmune, activated macrophages, the ability of

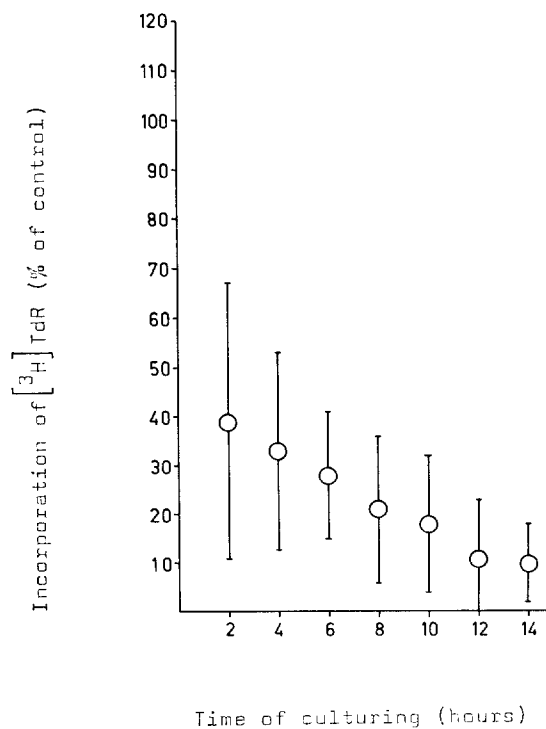


FIG. 6. Time-course of ability to incorporate [ $^3\text{H}$ ]TdR by polyoma-induced tumor cells in the presence of activated, nonimmune macrophage (effector to target cell ratio approximately 10:1). Incorporation by target cells alone = 100%. Each value represents the mean of 24 determinations.



polyoma-induced syngeneic tumor cells to incorporate [ $^3\text{H}$ ]TdR into acid-precipitable material is progressively lost as incubation proceeds. In this experiment where the ratio between macrophages ( $2 \times 10^6$ ) and tumor cells ( $2 \times 10^5$ ) was approximately 10:1, a marked decrease was detectable within 2 h, and by 14 h proliferation of tumor cells had virtually ceased. Similar results were obtained when macrophages were cultured with carcinogen-induced tumor cell lines. Moreover, a similar decrease in proliferative activity of the various syngeneic tumor cell lines was seen when tumor cells were allowed to grow for 4–8 h and peptone-induced, adherent peritoneal cells were then added to the cultures.

Glycogen-induced peritoneal cells consisting mainly of PMN's cultured with target cells at a ratio of approximately 10:1, yielded a somewhat similar picture (Fig. 7). However, as compared with macrophages, PMN's were clearly less effective in any of the experimental conditions examined; moreover, decrease in target cell proliferation occurred more slowly. Nonetheless the data show convincingly that in this system, PMN's as well as macrophages do have a distinct effect on target cell proliferation.

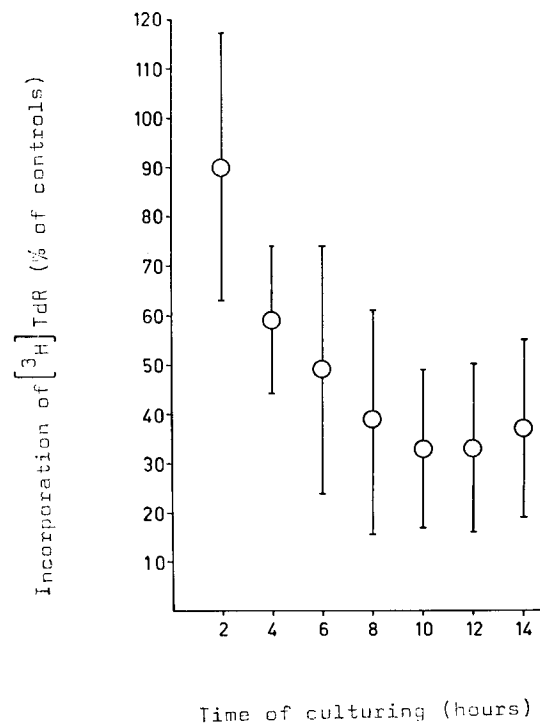


FIG. 7. Time-course of ability to incorporate [ $^3\text{H}$ ]TdR by polyoma-induced tumor cells in the presence of exudative polymorphonuclear leukocytes (effector to target cell ratio approximately 10:1). Incorporation by target cells alone = 100%. Each value represents the mean of 20 determinations.

*The Effector to Tumor Cell Ratio as Determinant of the Cytostatic Effect.*—The preceding experiments provide evidence that under the in vitro conditions of this investigation a rather small proportion of macrophages or PMN's suffice to effectively diminish proliferation of tumor cells. It was, however, essential to obtain further information on the extent to which the process depends on the ratio of effector cells to target cells. Accordingly, incorporation of [ $^3\text{H}$ ]TdR by  $2 \times 10^5$  tumor cells was measured 14 h after culturing these cells with varying increments of activated macrophages or glycogen-induced PMN's. The results with polyoma-induced tumor cells represented in Fig. 8 show that the macrophage killer effect is strikingly dependent on the effector cell to target cell ratio. When macrophages were in a majority of 5- to 25-fold (not shown), a dramatic effect was observed consistently. Even when the effector to target cell ratio was

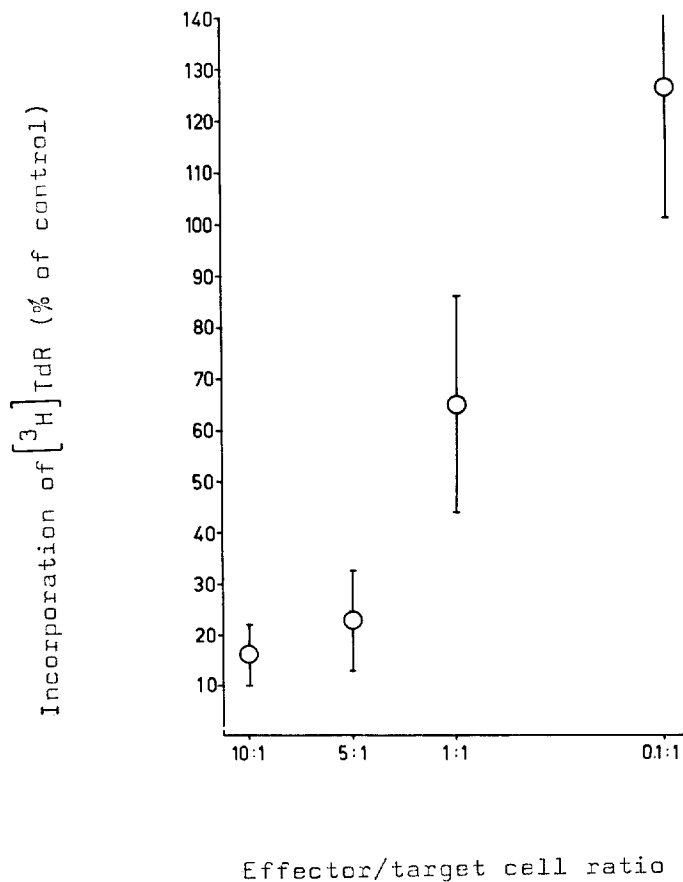


FIG. 8. Dependence of ability of polyoma-induced tumor cells to incorporate [ $^3\text{H}$ ]TdR on the ratio of activated, nonimmune macrophages in the incubate. Tumor cells alone = 100%. All systems were cultured for 14 h in medium containing 10% heat-inactivated rat serum. Each value represents the mean of 24 determinations.

reduced to 1:1, a significant reduction in tumor cell proliferation was still detectable, but a cell ratio of 0.1:1 often resulted in an increase in target cell proliferation. Similar results were obtained with carcinogen-induced tumor cell lines.

To ascertain whether the observed marked decrease in the growth potential of tumor cells achieved in the presence of macrophages was not merely a consequence of increased cell density in the culture,  $10^4$  tumor cells were interacted for 14 h with  $10^4$  activated macrophages, and incorporation of [ $^3\text{H}$ ]TdR then assessed. Under these conditions, the reduction in tumor cell proliferation was proportionately similar ( $50 \pm 6.8\%$ ) to that obtained with 20-fold greater numbers of both effector and target cells (ratio 1:1; Fig. 7).

The dependence of residual proliferative ability of polyoma-induced tumor cells on the actual ratio of PMN's present in the incubate is shown in Fig. 9.

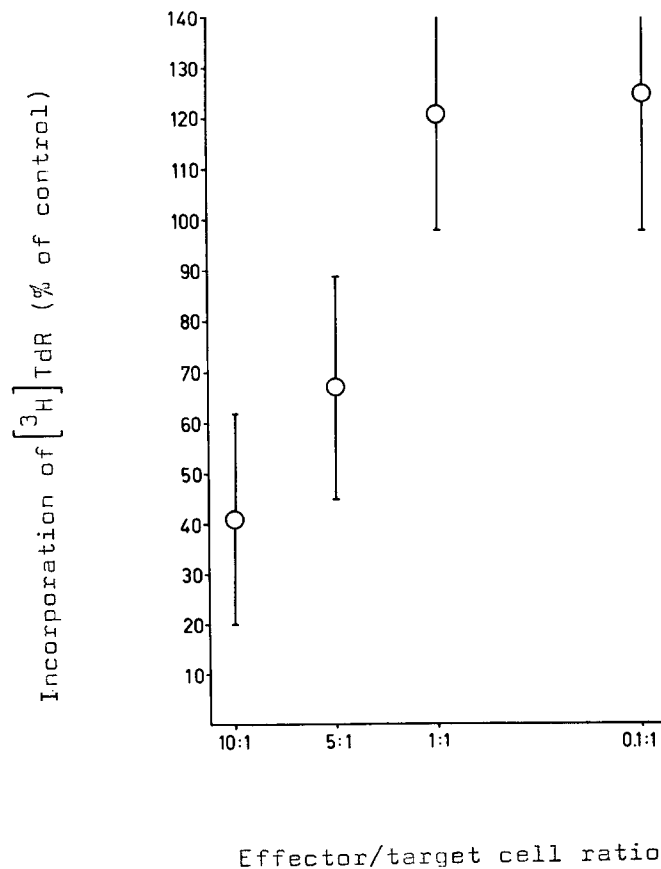


FIG. 9. Dependence of ability of polyoma-induced tumor cells to incorporate [ $^3\text{H}$ ]TdR on the ratio of exudative polymorphonuclear leukocytes in the incubate. Tumor cells alone = 100%. All systems were cultured for 14 h in medium containing 10% heat-inactivated rat serum. Each value represents the mean of 18 determinations.

Comparison of these results with those obtained using macrophages as effector cells reveal that the macrophages are far more effective in limiting tumor cell proliferation than are PMN's, and that less than 1:1 effector to target cell ratios enhance rather than inhibit target cell proliferation.

Results of further experiments assessing the influence of irradiated (5,000 R) effector cells on target cell proliferation showed that the elimination of target cells is not limited by X-irradiation (Table III).

*The Relative Significance of Cytostatic vs. Cytotoxic Effects in the Elimination of Syngeneic Tumor Cells.*—Once it had become evident that activated, nonimmune macrophages could modulate tumor cell proliferation by either cytotoxic or cytostatic effects (whereas exudative PMN's are producing only an inhibition of tumor cell proliferation), it was important further to distinguish the sig-

TABLE III  
*Effect of Untreated or Irradiated (5,000 R) Effector Cells on Proliferation of Polyoma-Induced Tumor Cells (Incorporation of [<sup>3</sup>H]TdR; Percent of Control ±SD)*

Pretreatment of effector cells	Macrophage/target cell ratio			
	10:1	5:1	1:1	0.1:1
None	15.7 (±6.1)	23.6 (±9.6)	65.1 (±21.5)	126.1 (±24.9)
Irradiation	19.1 (±7.1)	19.3 (±6.6)	74.0 (±11.4)	116.0 (±13.7)
	PMN/target cell ratio			
	10:1	5:1	1:1	0.1:1
None	41.0 (±21.1)	66.9 (±22.6)	121.0 (±23.5)	125.1 (±27.3)
Irradiation	31.5 (±7.8)	51.0 (±22.5)	61.5 (±24.4)	86.7 (±20.1)

Each value represents the mean of 12 determinations.

nificance of these two mechanisms. To this end, both macrophage-induced cytotoxicity and inhibition of tumor cell proliferation were followed in cultures of activated macrophages plus targets in the presence of silica (100–200 µg/ml), an agent known to be selectively toxic for macrophages (38, 39). The results summarized in Table IV show that in the presence of silica, tumor cells proliferate essentially as they would in the absence of effector cells, despite the generation of a low but consistent degree of cytotoxicity. These data show clearly that cytotoxic effects do not contribute appreciably to the elimination process involved in the present in vitro model system. Thus, all available data are consistent with the view that activated, nonimmune macrophages and exudative PMN's kill tumor cells primarily by affecting their proliferative ability.

*The Relative Significance of Cell-to-Cell Contact vs. Humoral Effects in the Elimination Process.*—To determine whether the general mechanism of anti-tumor cell action in the present in vitro system involved merely some change in the medium unfavorable for tumor cell growth, as opposed to direct cell-to-cell contact, the effect of supernatant medium from macrophage cultures and of macrophage lysates on tumor cell proliferation was investigated. The data repre-

sented in Table V demonstrate that in presence of supernatants or lysates tumor cell proliferation continued unaffected. Similar experiments were performed to evaluate the possibility that the increased tumor cell growth observed at an effector to tumor cell ratio of 1:1 might reflect some favorable "condition-

TABLE IV  
*Utilization of Silica Destruction of Macrophages to Discriminate between Cytostatic and Cytotoxic Mechanisms for Tumor Cell Elimination*

Incubation mixture*	<sup>51</sup> Cr release	Tumor cell proliferation
	%	%
Tumor cells‡	0§	100 (±9.6)
Tumor cells + silica	0.5 (±2.1)	92.6 (±8.7)
Activated macrophages + tumor cells (10:1)	7.0 (±1.8)	14.7 (±5.6)
Activated macrophages + tumor cells (10:1) + silica	4.8 (±2.6)	83.5 (±10.2)

Each value represents the mean of 12 determinations.

\* Incubation was for 15 h.

‡ Polyoma-induced tumor cells were used for the present experiments.

§ Spontaneous release from targets was taken as 0.

TABLE V  
*Evidence for Lack of Significant Cytostatic Action on Tumor Cells by Macrophage Supernatants and Lysates*

Quantity of macrophage supernatants or lysates* added to tumor cells	Tumor growth‡ (incorporation of [ <sup>3</sup> H]TdR)
Supernatants from 10 <sup>4</sup> macrophages	112 (±14.5)
Supernatants from 10 <sup>5</sup> macrophages	101 (±7.5)
Supernatants from 10 <sup>6</sup> macrophages	84 (±11.6)
Supernatants from cultures of macrophages + tumor cells§ (ratio 1:1)	115 (±12.4)
Supernatants from cultures of macrophages + tumor cells§ (ratio 5:1)	70 (±18.5)
Supernatants from cultures of macrophages + tumor cells§ (ratio 10:1)	72 (±19.6)
Supernatants from cultures of macrophages + tumor cells§ (ratio 1:10)	63 (±21.2)
Supernatants from 2 × 10 <sup>5</sup> tumor cells	102 (±6.8)
Lysates from 10 <sup>4</sup> macrophages	100 (±9.7)
Lysates from 10 <sup>5</sup> macrophages	99 (±11.5)
Lysates from 10 <sup>6</sup> macrophages	95 (±9.3)
Lysates from 10 <sup>7</sup> macrophages	81 (±17.4)

Each value represents the mean of 12-16 determinations.

\* These were "activated" rat peritoneal macrophages taken 3 days after intraperitoneal injection of peptone.

‡ 2 × 10<sup>5</sup> polyoma-induced tumor cells cultured in 2 ml of "conditioned" medium for 14 h at 37°C (2 × 10<sup>5</sup> tumor cells in fresh medium = 100%).

§ 2 × 10<sup>5</sup> polyoma-induced tumor cells were cultured with macrophages for 14 h at 37°C.

ing" of the medium. The results given in Table V show that tumor cell proliferation was not affected either by supernatants from macrophage cultures or from macrophage/tumor cell cultures.

#### DISCUSSION

Observations made many years ago and summarized by Gorer (40) drew attention to the fact that, besides the generally accepted killer properties of lymphoid cells in specific immune response against neoplasias, macrophage reactions are prominent in the rejection of tumor cells, especially during extensive vascularization. There has since accumulated much more evidence that macrophages from immunized hosts can eliminate tumor cells either by phagocytosis (41-43) or by killing them through cell contact (44-47).

Recent work has shown that normal macrophages incubated with hyperimmune spleen cells or supernatants of cultures of immune lymphocytes and the specific antigen are toxic for specific target cells; moreover, contact with the specific antigen also renders them capable of killing antigenically unrelated target cells (48). Under these conditions, the killing of tumor cells by macrophages involves an immunologically specific interaction succeeded by a nonspecific lethal reaction. This mechanism is analogous to the well-known antimicrobial activity of macrophages in animals infected with living microorganisms (18). More recent studies have demonstrated that cross-protection among intracellular microorganisms extends beyond phylogenetic lines and is effective in the absence of the specific antigen (19, 20). The present experiments confirm and extend some earlier findings (23, 24, 26, 31, 32, 49) indicating that intensive nonspecific stimulation as achieved by persistent infection or by nonspecific stimulants of the reticuloendothelial system enables phagocytic cell systems to kill tumor cells in a nonspecific manner.

The present *in vitro* studies using various syngeneic rat tumor cell lines as targets confirm earlier morphological observations that normal activated macrophages interfere in a striking manner with allogeneic or syngeneic tumor targets (24, 26, 32, 49; Figs. 1-3); moreover, the present work verifies this interaction by more objective means. These data convincingly demonstrate that tumor cell proliferation, as assessed by their ability to incorporate [<sup>3</sup>H]TdR, is quickly and markedly inhibited in the presence of a rather modest majority of activated macrophages (effector to target cell ratio approximately 10:1) and virtually ceases after 14 h of contact (Fig. 6). Under the same experimental conditions, RNA synthesis by tumor cells, assessed by their ability to incorporate [<sup>3</sup>H]uridine, was found to be similarly depressed. It is noteworthy that tumor cell proliferation is significantly inhibited even at a macrophage to tumor cell ratio of 1:1, but is often favored by a still lower proportion of macrophages in the cultures (Fig. 8). Whether stimulation of target cell proliferation by relatively few macrophages reflects artificial conditions of cell culture or is rather more representative of *in vivo* conditions needs further clarification. The

present data nonetheless show that compared with so-called killer lymphocytes, where ratios of several hundred lymphocytes per target cell are often required, the present system appears remarkably efficient in coping with tumor cells.

It is generally agreed that growth of cells in culture depends greatly on the cell density. It therefore seemed important to evaluate whether the marked decrease in the growth potential achieved by the presence of nonimmune, activated macrophages was a consequence either solely or in part of increased cell density in the culture. The observations that (a) tumor cells cultivated with macrophages at a ratio of 1:1 showed a similar decrease in their proliferative capacity irrespective of whether the total cell number was  $2 \times 10^4$  or  $4 \times 10^5$  and (b) that tumor cell proliferation remained unaffected when tumor cells were cultured alone at a similar density as with macrophages ( $2 \times 10^6$  cells/dish) are taken as indicating that the inhibition of tumor cell growth by effector cells is real and not a reflection of high cell density. Another important issue was whether in the present in vitro system the general mechanism involved in anti-tumor cell action was a reflection of the medium becoming unfavorable for tumor cell growth or, the completely opposite alternative, a consequence of cell-to-cell contact. Various observations, such as (a) the strong parallelism in the appearance of cell aggregation and inhibition of tumor cell proliferation, (b) the failure of macrophages in presence of silica to affect tumor cell growth, and (c) the failure of macrophage culture supernatants or lysates to inhibit tumor cell growth, all point towards cell-to-cell contact phenomena between functional effectors and targets as the necessary precondition for attaining tumor cell damage.

Signs of cytotoxicity for target cells, such as release of  $^{51}\text{Cr}$  and uptake of trypan blue, are already evident at an early stage of the interaction but are less pronounced than the inhibition of proliferative capacity (Table I). Up to now, cytotoxicity has been held largely responsible for tumor cell killing by macrophages from immune and nonimmune hosts (26, 31, 48). Silica, an agent established as specifically toxic for macrophages (38, 39), was utilized to discriminate between cytotoxic and cytostatic mechanisms. In the presence of silica in the cultures, tumor cell proliferation proceeds essentially as though macrophages were absent. Cytotoxic activity, on the other hand, is generated to a degree similar to the silica-free controls, a finding that argues for a cytostatic mechanism having a predominant role in accounting for the macrophage effects seen in the present in vitro system (Table IV).

The present work has shown that exudative PMN's can also kill targets, apparently by a mechanism similar to that of activated macrophages (Figs. 4, 5, 7, and 9). If such mechanisms are in fact operative in vivo, PMN's could also participate in the nonspecific elimination of tumor cells, especially under circumstances where the migration time and the number of effector cells available in a given region are limited. The conclusion that target cell damage by macrophages and PMN's is achieved by a similar mechanism is also supported by the failure

to detect cytotoxic effects by PMN's even at high effector to target cell ratios (50:1), an observation which differs from that made recently in another rat tumor (50). This discrepancy possibly involves the fact that in these studies, syngeneic ascites tumor cells were grown *in vivo* before being cultured *in vitro* together with PMN's and may thus have specific cell-bound antibody on their surface (50). Our finding that heavy irradiation of effector cells does not diminish their killer properties argues against an involvement of lymphoid cells in this process (51). On the other hand, it is entirely conceivable that lymphokines play an important part in effector cell activation.

Many important questions remain unanswered and some others newly arise from the present work. As this and earlier studies (52) indicate, "activation" of normal, nonimmune macrophages is a central and indispensable precondition for their achieving the capacity to eliminate tumor targets. Present knowledge does not permit the definition of which functional and biochemical activities are enhanced in the course of macrophage activation and which are decisive for the effector cell to recognize and to kill tumor targets. From the present demonstration that in principle mononuclear phagocytes as well as polymorphonuclear leukocytes are able to eliminate tumor cells it may be argued that the killer mechanism elicited by the two types of effector cells may be similar or even identical. All experimental work thus far suggests that both types of effector cells achieve tumor cell killing only via close contact, but the actual mechanism involved remains obscure.

Any meaningful analysis of the role of macrophage activation is greatly complicated by the now established finding that normal, unstimulated peritoneal cells cultured *in vitro* become progressively adherent through increasing contact with the surface of the culture vessel; a few hours of such culture conditions can lead to activation that parallels that attained *in vivo*. Such *in vitro* adherent cells achieve in all measurable respects the capacity to eliminate tumor cells just as macrophages taken from stimulated animals. Since adherence is the characteristic feature of macrophages utilized for separating this cell from all other cell types, it becomes difficult, indeed presently impractical, to differentiate between normal and activated macrophages. However, despite this bar to fully controlled experiments there are various findings suggesting that activation of macrophages is indeed a necessary condition for eliminating tumor targets (32, 52). Other basic issues such as the involvement of the tumor cell itself in the performance of macrophages, the recognition mechanism involved, and the specificity of the macrophage elimination process for tumor targets are the subject of continuing investigations (Keller, R., and T. Bächli, data in preparation).

The majority of experimental studies on tumor immunity, both natural and acquired, have understandably focused attention on specific immune phenomena, notably on thymic lymphocytes as the host cell responsive to the neoantigens that characterize autochthonous tumors and their acquisition of killer



properties. However, as this and other studies (24, 26, 31, 32, 49, 50) show, non-immune phagocytes by a separate mechanism are effective in killing tumor cells. The study of these cells and their mode of action on tumor targets therefore provide new opportunities for understanding the cellular basis of tumor resistance. Indeed, the elimination of tumor cells by activated, "nonimmune" phagocytes represents a form of host surveillance far more primitive and basic than that ascribed to T lymphocytes.

#### SUMMARY

Syngeneic tumor cell lines induced in inbred DA rats by polyoma virus, dimethylbenzanthracene, or methylcholanthrene were interacted in vitro with syngeneic effector cells. Glycogen-induced peritoneal exudate cells, predominantly polymorphonuclear leukocytes, and proteose peptone-induced peritoneal cells, principally macrophages, were the effector cells employed. Activated, non-immune macrophages or exudative polymorphonuclear leukocytes produced pronounced morphological changes in syngeneic tumor cells as evidenced by a substantial reduction in tumor cell numbers and appearance of shrunken cells, even though there was no increase in cell debris. Polymorphonuclear leukocytes exerted a generally similar but quantitatively much diminished effect. These effector cells constantly produced a decrease in the incorporation by tumor cells of DNA precursors such as [<sup>3</sup>H]thymidine and of RNA precursors such as [<sup>3</sup>H]uridine. In this regard, the effector cells were quite refractory to high doses of X-irradiation.

Interaction of target cells with activated, nonimmune macrophages yielded low but consistent signs of cytotoxicity, whereas polymorphonuclear leukocytes gave no such effects. Elimination of functional macrophages by silica, an agent specifically toxic for macrophages, resulted in unrestricted tumor cell proliferation despite continued generation of cytotoxicity. Accordingly, cytostatic mechanisms appear to play a predominant role in the elimination of tumor cells by nonimmune phagocytes. Evidence from a variety of experimental approaches suggest that the cytostatic effect is dependent on cell-to-cell contact.

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