Further Observations on the Effect of Cyclophosphamide on Intratumor and Peripheral Leukocyte Levels

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The injection of cyclophosphamide (CY) into C57BL/6J mice bearing intramuscularly transplanted, MCA-induced tumors (MCA/76-9, 76-64, 76-45, and 77-23) resulted in a reproducible sequence of events involving tumor regression that was followed by recurrence in all but a few cases. Regression itself occurred whether tumors were immunogenic or not and whether tumors were growing in immunocompetent or immunosuppressed mice. Permanent CY-induced regressions, however, were rare under any conditions. The drug induced well-defined changes in the number of bone marrow and blood leukocytes. By 8 days after drug injection, control or tumor-bearing mice showed normal bone marrow counts but elevated peripheral blood leukocyte counts. The latter involved only monocytes and granulocytes, lymphocytes requiring more than 21 days to return to control values. Tumor bearers not injected with CY also showed elevated blood monocyte and granulocyte numbers. After CY injection of mice, tumors showed distinctive histologic changes that were common for all four sarcomas. Within 3 days of injection, neoplastic cells showed evidence of damage, and mononuclear cells became prominent throughout the tumor mass. By 7 days, there were many giant cells and polykaryons, which progressively decreased, so that by Days 10–14 few were discernible. At this time, the bulk of the tumor mass consisted of mononuclear cells having the morphologic characteristics of macrophages. However, between days 7 and 10 well-defined hyperchromatic areas could be seen peripherally in the capsule or the muscle into which the tumor cells were originally implanted. These areas consisted of relatively undifferentiated cell types, with which cells of the granulocyte series were often associated. By Day 14 these regions were packed with granulocytes. Regression usually stopped between Days 14 and 21, at which time the residual tumor mass consisted of birefringent, fibrous tissue containing relatively few cellular elements. Between 21 and 28 days, tumor recurrence was evident in most cases. The histologic changes were quantified by disaggregating the tumors with enzymes before and after CY injection. A good correlation was obtained between histologic appearance and the numbers and types of cells obtained at defined times. Both tumor-associated macrophage and granulocyte counts showed an increase over the first 10 days after CY injection. Frequently, both cell types declined numerically over the first 3 days in parallel with blood monocyte and granulocyte counts and with bone marrow cell counts. The decline depended to a large extent on the total number of cells associated with the tumor mass and the overall effect exerted by CY on these cells. Intratumor macrophage numbers reached a peak by Day 8, whereas granulocytes did not reach maximum values until Day 10. Subsequently, both cell types decreased in number until recurrent tumor growth became apparent. These findings are discussed in terms of the possible relationship between peripheral and tumor-associated leukocytes and in terms of the functions of the latter during CY-induced tumor regression. (Am J Pathol 1980, 99:667–684)

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THE PRECISE RELATIONSHIP between peripheral blood leukocytes and macrophages (monocytes), granulocytes, and lymphocytes accumulating at the site of progressive tumor growth is obscure.[1] One assumes that, following implantation of tumor material or during spontaneous tumor induction, tumor-associated leukocytes (TALs) are derived initially at least from the circulation, but whether there is a continuous replenishment as progression occurs and to what extent the TALs multiply *in situ* is far from clear. Manipulation of the experimental animal, for example, by preirradiation [2,3] or pretreatment with drugs,[4] suggested that the peripheral blood leukocyte levels, which in turn depend on the activity of the bone marrow, did influence the level of TALs. However, to date, there is little information on the rate of accumulation, the longevity, or the functional activity of TALs or how, under defined conditons involving drug treatment, peripheral leukocyte levels influence those in the tumor mass.

In a recent report [5] we demonstrated striking changes in the cellular composition of a murine sarcoma following the injection of tumor bearers with cyclophosphamide (CY). An increase in both tumor-associated macrophages and granulocytes was seen together with a concomitant decrease in the total number of identifiable neoplastic cells. The distribution of macrophages in the regressing tumor mass was different from that of granulocytes, in that the former were spread throughout the tumor mass, and the latter were confined to the capsule or muscle in which the tumor cells had been implanted. Histologic evaluation indicated that the granulocytes were associated with hyperchromatic focal areas of relatively undifferentiated cell types, among which the occasional mitotic figure was seen. In addition, it was apparent that intratumor leukocyte changes paralleled those occurring in the blood and bone marrow.

This report confirms these data and extends the observations to three other tumor systems. It shows that identical changes occurred in other syngeneic, but immunologically unrelated, sarcomas after CY treatment, thereby indicating that such changes are of general interest in terms of trying to understand the mechanisms of the antitumor action of CY. Moreover, the results confirm the previous observation [6] that neither established immunity nor high tumor immunogenicity seems to be a prerequisite for the induction of CY-induced tumor regression.

Materials and Methods

Mice

C57BL/6J female mice, 7–8 weeks of age, were used throughout the experiments.

Tumors

Tumors were induced in the mice by Dr. Liisa Prehn, using 3-methylcholanthrene. The tumors were designated MCA/76-9, 76-64, 76-45, and 77-23 and classified as rhabdomyosarcomas. MCA/76-9, 76-64, and 76-45 sarcomas were strongly immunogenic, as measured by the ability of immunized mice to reject about 10^6 cells. The threshold dose for a 50% take (TD_{50}) in control mice was 10^2–10^3 cells. The MCA/77-23 sarcoma was nonimmunogenic by the rejection criterion, in that the TD_{50}, 10^3 cells, was not rejected by presensitized mice. However, in terms of growth rates, tumors developing from 10^3 cells grew slightly more slowly in presensitized mice than in controls, suggesting that the tumor was weakly immunogenic. Tumors were passed in syngeneic recipients every 2–3 weeks by intramuscular implantation of 10^4 or 10^5 cells. Tumor size was assessed by measuring two diameters at right angles to each other with calipers and expressed as the mean tumor diameter in millimeters. Experimental groups consisted of basic units of five mice per box.

Tumor Disaggregation

This technique has been fully described elsewhere.[2,5] Briefly, tumors contained in the gastrocnemius muscle were excised, cut into fragments with sharp fine scissors, and suspended in a mixture of papain (250 μg/ml, Sigma Type IV), collagenase (250 μg/ml, Sigma Type I), and DNase (1 μg/ml, Sigma). The fragments were stirred by a magnetic stirrer at 37 C for 20 minutes, after which the cell suspension was removed and replaced with fresh enzymes. In the present studies, no tumors took longer than 45 minutes to disaggregate totally into a single-cell suspension. The cells were washed twice in Dulbecco's phosphate-buffered saline or RPMI 1640 by centrifugation for 6 minutes at 500 g and finally resuspended at a concentration of 1–2 × 10^6 cells per ml of medium. Cells were counted in a hemocytometer using phase contrast optics. Viability (usually in excess of 90%) was assessed by the appearance of cells under phase contrast or by exclusion of trypan blue.

Identification of Cells

Tumor-associated cells were identified by the criteria fully described previously.[2,5] Briefly, macrophages were identified by their ability to rosette and phagocytose antibody-coated sheep erythrocytes (EA), and by their positive staining for nonspecific esterase in cytocentrifuge preparations. Granulocytes were assessed on the basis of nuclear morphologic features and positive staining for specific chloroacetate esterase. Neoplastic cells were identified on the basis of morphologic and staining characteristics. Cytocentrifuge preparations were made on a Perkin-Elmer Cytospin, air-dried, fixed in methanol, and stained with Wright's stain. Data on cell populations were expressed in the text either as a percentage or as mean number of cells per tumor mass. As discussed elsewhere,[5] the use of enzymes to disaggregate the tumor fragments did not affect the morphologic characteristics of the released cells; nor were the identifying criteria rendered invalid.

Blood and Bone Marrow

Blood was obtained from the retro-orbital sinus and bone marrow from the tibia by flushing through the bone with 2 ml of RPMI 1640 medium containing 1 μg/ml DNase.[5] Both blood and bone marrow leukocytes were counted in a Coulter Counter, Model ZBI. Blood smears were prepared with the use of the Cytospin and stained with Wright's stain. Differential counts were performed routinely. Blood and bone marrow leukocyte counts were expressed as the number of cells per milliliter or per tibia, respectively.

Cyclophosphamide (CY)

Cytoxan (Mead Johnson, Evansville, Ind) was dissolved in distilled water at a concentration of 60 mg/ml. In all experiments, 0.1 ml (6 mg) was injected intraperitoneally into

each mouse. This dose was equivalent to about 300 mg/kg. The LD_{50} for C57BL/6J female mice was about 425 mg/kg.

Thymectomy

Thymectomy was performed on 4–5-week-old mice. These mice were rested for at least 2 weeks before being used in experiments. The absence of a thymus was checked at the end of all experiments. Sham thymectomy was carried out at the same time.

Irradiation

Mice were exposed to whole body γ-irradiation (WBI) at a rate of 234 R per minute from a Shepard Mark I Cesium irradiator. Thymectomized mice received 450 R WBI 24 hours before the intramuscular injection of 10^6 tumor cells. The LD_{50} for 8-week-old mice was between 800 and 900 R, 1000 R being lethal for 100% of the mice.

Histologic Studies

Excised tumors were fixed and processed by conventional techniques. Sections were cut at three levels to obtain an in-depth impression of intratumoral changes. The stains used were hematoxylin and eosin.

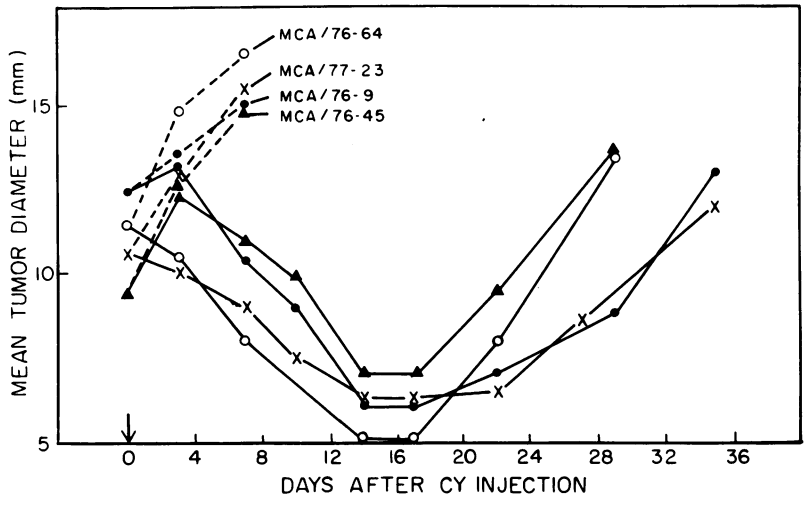
Statistical Analysis

The results were analyzed by the Mann–Whitney U test, by which a 2-tailed *P* value of 0.05 or less was considered significant.

Results

The Effect of CY on the Growth of C57BL/6J Sarcomas

Text-figure 1 summarizes representative data showing the effect of injecting 6 mg CY intraperitoneally into C57BL/6J mice bearing the MCA/76-9, 76-64, 76-45, and 77-23 sarcomas. Basic groups contained 5 mice, but each point is the mean of several experiments. Regression in terms of decrease in tumor size usually became apparent between Days 7 and 10, up to which time it was not unusual to see a slight increase in tumor size. However, after about 10 days after CY injection, tumor size diminished rapidly. Between Days 21 and 28 after CY injection tumors usually began to regrow. From the time recurring tumors became palpable, the rate of increase in tumor size was very rapid. Permanent regressions were rare (taking the overall data, involving at least 500 mice, less than 5% in any tumor system showed permanent eradication). Ranges of variation are not shown for simplicity. These were, in fact, narrow; that is, most tumors responded in a reproducible and predictable manner. Table 1 summarizes data that illustrate the range in variation when comparing diameters of tumors from control mice and those given injections of CY. A single time point, 7 days after giving tumor bearers CY, is given to show the narrow ranges obtained.



TEXT-FIGURE 1.—The effect of CY injection on the growth of four C57BL/6J sarcomas. The broken lines represent tumors growing in control, untreated mice. The basic units consisted of 5 mice per group.

The extent of regression under present conditions was more or less dependent on tumor size: for example, when CY was injected into mice bearing tumors larger than 13 mm in diameter, the tumors either stopped increasing in size for a few days only and then recommended their growth or showed no visible response to the drug. In a few instances only did large tumors respond as shown in Text-figure 1.

When tumors regrew in CY-treated mice, they were, in general, either refractory to further CY treatment or showed only a weak response. This did not appear to be due to the induction of CY-resistant lines, since transplantation of the tumors into syngeneic recipients followed by CY injection resulted in the same pattern of events described above. An attempt to induce CY-resistant lines by serial transplantation of tumors recurring after CY-induced regression has so far failed.

Table 1—A Comparison of Sarcoma Size, Together With Range of Variation, After the Injection of Cyclopospham ide (CY) into Tumor-Bearing C57BL/6J Mice

Tumor	Mean tumor diameter (mm)*	
	Control mice	CY-treated
MCA-76-9 sarcoma	15.0 (13.5–16.0)	10.5 (7.5–11.5)
MCA-76-45 sarcoma	15.0 (13.0–16.5)	11.0 (8.0–11.5)
MCA-76-64 sarcoma	16.5 (15.0–17.5)	7.5 (6.0–8.5)
MCA-77-23 sarcoma	16.0 (13.5–17.0)	9.0 (7.0–10.0)

* The data refer to tumors measured 7 days after the injection of 6 mg CY intraperitoneally.

Histologic Evaluation

A summary of the changes induced by CY injection of mice bearing MCA/76-9, 76-64, 76-45, and 77-23 sarcomas is given in Table 2. The changes apply to each tumor in every detail.

During the first 3 days after CY injection, the nuclei of the neoplastic cells were often swollen and vacuolated. Mononuclear cells began to be seen between the neoplastic cells in greater frequency than found in control tumor sections (Figure 1) taken at any stage of tumor growth. (Control MCA76-9 tumors showed an accumulation of mononuclear cells in the capsule region of the tumor, whereas the other three tumors showed a more generalized distribution throughout the tumor mass.) By Day 7, there were many giant cells and polykaryons (Figure 2). Mononuclear cells were especially prominent and were evidently increasing in density. At this time, granulocytes were not abundant, although they were seen in areas of hemorrhage and necrosis. By Day 10, most of the giant cells and polykaryons had either disappeared or were decreasing in density. At this time two distinct populations were discernible. *a*) The bulk of the tumor mass consisted of small mononuclear cells whose nuclear appearance suggested they were monocytes or macrophages. Large pigment-containing cells were present. These were probably large macrophages containing breakdown products of erythrocytes. *b*) Small dense areas of relatively undifferentiated cells became prominent in both the muscle and the capsule

Table 2—Summary of Some of the Histological Changes Seen During Regression of C57BL/6J Sarcomas* Following the Intraperitoneal Injection of Cyclophosphamide (CY)

Days after injection	Observed changes
1–3	Neoplastic cells show swelling of nuclei and vacuolation. Spaces appear between cells, and mononuclear cells become prominent.
5–7	Formation of giant cells and polykaryons. Increase in the density of mononuclear cells within the tumor mass.
8–10	Formation of hyperchromatic areas of undifferentiated cells in the capsule and muscle. Granulocytes show a localized accumulation, frequently associated with undifferentiated cells. Reduction in neoplastic cell numbers, and the bulk of the tumor mass replaced by mononuclear cells.
10–14	Accumulation of granulocytes in capsule and muscle prominent. Areas of fibrous, birefringent material formed.
14–18	Rapid decrease in the cellularity of the tumor mass. Few neoplastic cells discernible.

* The data refer to MCA-76-9, 76-64, 76-45 and 77-23 sarcomas. CY (6 mg) was injected intraperitoneally on Day 9 or Day 10 after the implantation of 10^6 tumor cells.

(Figure 3). Mitotic figures were also seen among these cells (Figure 4). Granulocytes in varying stages of maturation, from cells with an intact annular nucleus to fully lobulated polymorphonuclear cells, were invariably associated with these areas.

The MCA/76-9 and 76-64 sarcomas showed more or less identical reactions, in that Days 9 and 10 were the days when obvious accumulation of granulocytes began, whereas accumulation was seen as early as Day 7 or Day 8 in the case of MCA/76-45 and MCA/77-23 sarcomas. Between Days 10 and 14 large areas of the capsule consisted entirely of granulocytes (Figure 5). During this time, the tumor mass underwent reduction in size. As regression occurred, the cellularity of the tumor mass diminished until only a fibrous mass with a few cells was seen (Figure 6). Tumor regression itself was associated with deposition of birefringent material to replace cellular elements, although fibroblasts were often seen in these areas. The large pigmented cells became localized in the capsule of the residual tumor mass. At the time tumors were smallest, it was evident that the mass consisted of some mononuclear cells, a few granulocytes, a fibroblastic capsule, and often no detectable neoplastic cells. However, when recurrence occurred, neoplastic cells became clearly visible, and growing tumors soon resembled the original untreated tumor controls. The capsule often showed considerable thickening, compared with that in the original untreated tumors.

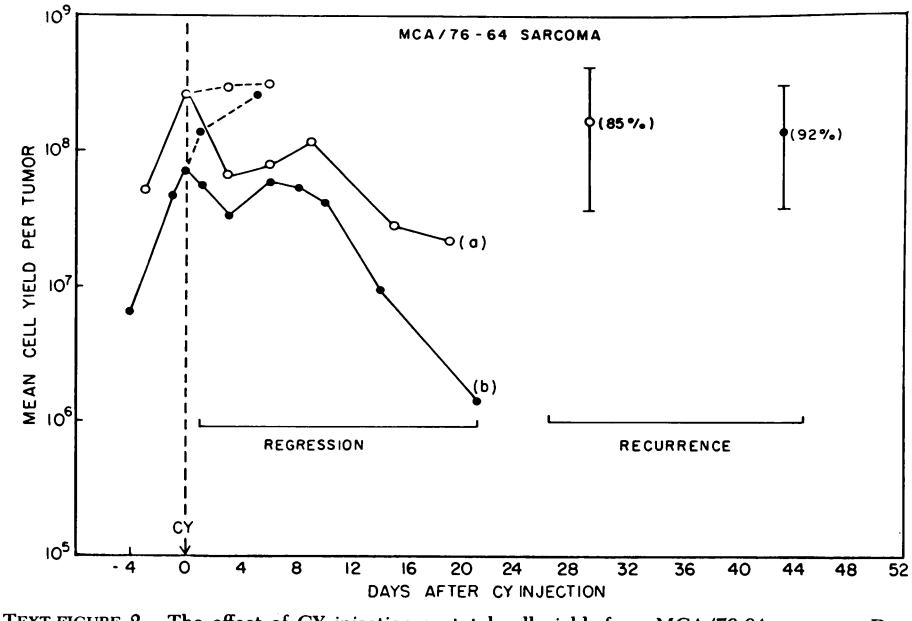
When mice with large tumors were treated with CY, similar distinctive changes occurred, but relatively fewer neoplastic cells were involved. That is to say, macrophages and granulocytes still accumulated in the solid tumor mass or in the capsule, respectively, and giant cells and polykaryons appeared, but the reactions involved less of the overall tumor mass than described above.

Intratumor Cellular Changes in Relation to Peripheral Blood and Bone Marrow Leukocyte Levels

The events described histologically were also monitored by estimating total cell yields and characterizing cells derived from enzymically disaggregated tumors. At the same time, bone marrow and blood differential counts were made. Comprehensive data from the MCA/76-9 and 76-64 sarcoma systems and rather less from the MCA/76-45 and 77-23 sarcoma systems have so far been obtained. For convenience, and to avoid confusion, bone marrow and peripheral blood leukocyte data from control, non-tumor-bearing, or tumor-bearing mice are not described fully, since the detailed description of the MCA/76-9 sarcoma system presented elsewhere [5] was shown to apply also to the other three sarcoma systems. That is, bone marrow counts of normal control and tumor-bearing mice re-

mained essentially stable in all experiments (10.3 ± 1.2 × 10^6 cells per tibia). After the injection of CY into control or tumor-bearing mice, the numbers fell to a minimal value (< 10% of controls) by Day 3. Recovery was rapid, and by Day 8 cell counts had returned to normal levels. Total blood leukocyte counts followed the same pattern in both control animals and tumor bearers after CY injection. Recovery occurred soon after Day 3, resulting in an "overshoot" by Day 8. This involved monocytes and granulocytes, the lymphocyte counts not returning to control values for at least 21 days after CY injection. However, total leukocyte counts in normal non-tumor-bearing, CY-treated mice returned to normal values by Day 14. In the tumor bearers, granulocyte and monocyte counts remained elevated. An elevation of monocytes and granulocytes was also seen from about Day 14 in tumor bearers not given CY.

The effect of CY injection on the total tumor cell yield is summarized in Text-figure 2, which shows the data from two experiments involving the MCA/76-64 sarcoma. The intention was to demonstrate the relationship between the tumor burden (total number of cells) and the extent of the antitumor action of CY. In Text-figure 2, the tumors were of different sizes at the time of CY injection. However, the same pattern of response was seen in both cases. The major differences between the two situations were

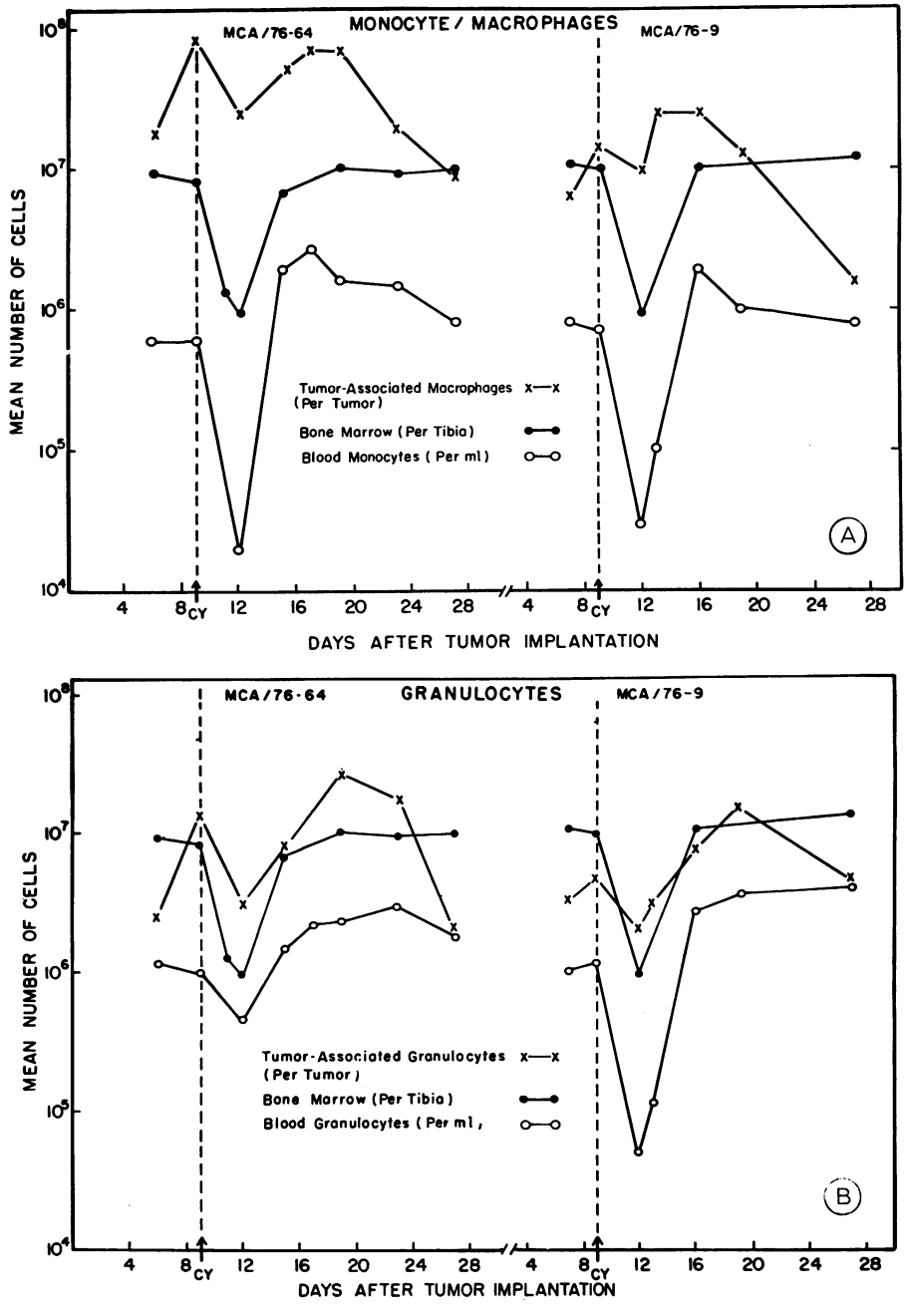


TEXT-FIGURE 2.—The effect of CY injection on total cell yields from MCA/76-64 sarcomas. Data from two experiments (*a* and *b*) are shown. Broken lines represent control tumors. Ranges are given only for yields during recurrence of tumor growth.

1) that regression resulted in nonpalpable masses when the initial burden was not excessive in relation to the dose of CY injected, as in Experiment *b* and 2) that tumor recurrence occurred sooner in the case of *a*, presumably because the number of cells was not reduced to as low a level as in *b*. It is seen that 85% of all mice (a total of 100 in Experiment *a*) showed large recurring tumors by 29 days after CY injection, compared with 92% of the mice in experiment *b* (a total of 65 mice), showing tumors on Day 43. Few residual mice failed to develop tumors.

Text-figures 3A and 3B summarize the data comparing the numbers of bone marrow cells, peripheral blood monocytes, or granulocytes with tumor-associated macrophages and granulocytes after CY injection of MCA/76-9 and 76-64 sarcoma-bearing mice. It is seen that tumor-associated macrophage (Text-figure 3A) and granulocyte (Text-figure 3B) counts paralleled the initial decline and subsequent increase in bone marrow cells and peripheral monocytes or granulocytes. The difference between tumor-associated macrophage counts on Day 3 and Days 7 or 8 after CY injection was highly significant at the 0.05 level ($2P = 0.001$ and 0.008 for MCA/76-64 and 76-9 tumors, respectively). The difference between granulocyte counts on Day 3 and Day 10 was also highly significant ($2P = < 0.001$). In accordance with the histologic evaluation, macrophage numbers increased before granulocytes, the numbers peaking around Days 4–8 after CY injection. At this time, macrophages accounted for up to 70% of the total cell yields, compared with 30% and 35% in control MCA/76-9 and 76-64 sarcomas, respectively. Granulocyte numbers reached a maximum by Day 10 after the injection of CY, constituting up to 35% of the total population, compared with less than 10% in control tumors. Thereafter, there was a rapid fall in both macrophage and granulocyte numbers. This fall occurred concurrently with a decrease in tumor size. The extent of this reduction in cell counts was directly related to the extent of tumor regression. When tumors regressed to small or nonpalpable masses, the cell yields were very low. When tumor regression was of relatively short duration, the level of macrophages and granulocytes appeared to keep pace with the extent of neoplastic cell proliferation.

The effects seen in Text-figure 3 were reproducible under optimal conditions. When the MCA/76-64 sarcoma was used, tumor masses consisting of up to 250×10^6 cells responded in the manner describe above, whereas the MCA/76-9 tumors showed less marked changes if the tumor mass consisted of more than 100×10^6 cells. When larger tumor masses of either type were used, the initial decrease in macrophages and granulocytes was not always seen. However, the overall temporal changes were similar. Although CY-induced regression was minimal when tumors were large (>12



TEXT-FIGURE 3A—A comparison of MCA/76-64 and 76-9 tumor-associated macrophage, bone marrow leukocyte, and peripheral blood monocyte levels after an intraperitoneal injection of CY (6 mg). B—A comparison of MCA/76-64 and 76-9 tumor-associated granulocyte, bone marrow leukocyte, and peripheral blood granulocyte levels after an intraperitoneal injection of CY (6 mg).

mm in diameter) at the outset, increases in tumor-associated macrophage and granulocyte numbers were observed. These increases were reflected in an increased percentage of both types. This percentage was not as high as recorded in the above experiments because of the larger number of neoplastic cells that were unaffected by drug treatment.

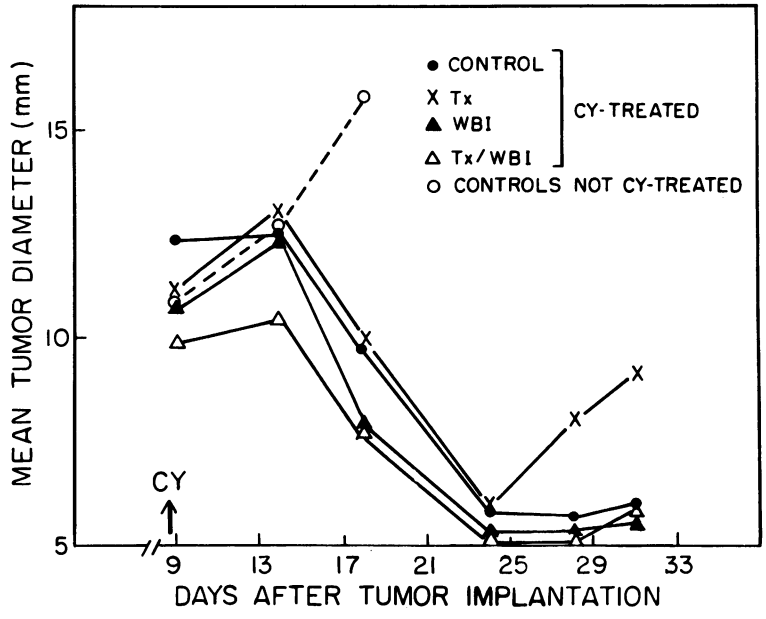
The number of lymphocytes associated with all four tumors, before or after CY treatment, was so low as to be impractical to assess. Consequently, data on lymphocyte changes in the blood or tumor mass are not given.

Influence of Established Immunity on the Antitumor Action of CY

In the preceding experiments, the number of permanent regressions was very low. Although three of the tumors were immunogenic, it was evident that at the time of CY injection of tumor bearers, existing immune mechanisms did not overtly improve the response to CY injection, compared with the response of mice bearing the weakly immunogenic MCA/77-23 sarcoma. To show further that tumor regression occurred as efficiently in immunosuppressed as in normal, immunocompetent mice, the following experiment was carried out. Mice were thymectomized at about 5 weeks of age and irradiated with 450 R WBI 2–3 weeks later. The day following irradiation they were injected with 10^6 MCA/76-9 tumor cells. Experimental groups, 5 mice per group in each experiment, consisted of control untreated animals, animals thymectomized only, animals given WBI only, or thymectomized animals also given WBI. Mice were injected with CY 9 days after tumor implantation. Text-figure 4 summarizes the data. A single control (untreated) growth curve is given. This curve consisted of the pooled data from groups not receiving CY, since no significant differences were seen. After CY injection, regression was seen in 100% of the mice. The reason for the more rapid recurrence of tumors in the groups receiving only thymectomy is not known and was not investigated further. Nevertheless, the overall data indicated that CY-induced regression could occur in immunosuppressed mice.

Discussion

The relevance of tumor-associated, host-derived leukocytes to the biologic behavior of tumors is largely unknown. Whether their presence signifies an attempt to reject the neoplasm, whether the cells contribute to the successful growth of the neoplasm, or whether they accumulate solely in response to an inflammatory stimulus is far from clear.[7] Even though macrophages, lymphocytes, or granulocytes at sites peripheral to the tumor may show cytotoxic activity *in vitro* either directly or indirectly after



TEXT-FIGURE 4.—Cyclophosphamide-induced regression of the MCA/76-9 sarcoma in immunosuppressed mice. O—O, control, untreated; ●—●, control, CY-injected; X—X, thymectomized; △—△, WBI (whole body irradiation, 450 R); △—△, Tx/WBI (thymectomized and irradiated).

reacting with humoral factors,[8] there is no sound evidence that these same tumor-associated cells have comparable activity *in situ*. Indeed, what the precise relationship is between TALs and those in the peripheral circulation is still far from clear. The long-term objective of our present studies is thus to further our understanding of the nature of the changes occurring at the tumor site and to learn whether these relate to or are controlled by leukocyte changes occurring in the blood and bone marrow.

The origin of the macrophages and granulocytes associated with the four sarcomas described in this report is, one assumes, the blood. The parallelism seen in Text-figures 3A and B between the decreased and increased numbers of bone marrow leukocytes and peripheral blood and tumor-associated macrophages and granulocytes after CY injection was highly suggestive of a relationship between TALs and the level of leukocytes seen in the bone marrow and blood. Indeed, it was previously reported that pre-irradiation [2,3] or irradiation of tumor-bearers [2] or treatment with anti-inflammatory drugs, such as azathioprine,[4] reduced the level of tumor-associated macrophages. The mechanism of action was explained, at least in part, on the basis of depressed bone marrow functional activity and reduced levels of circulating monocytes. However, although it seems likely that a continuous replenishment of leukocytes occurs at the

tumor site, the amount of self-multiplication *in situ* is not known. Moreover, on the basis of the histologic evaluation of the regressing tumors in this report, the possibility exists that tumors may be seeded by undifferentiated cells that might be bone-marrow–derived stem cells or precursor cells. The presence of a variety of immature TALs was previously reported when tumor-bearing mice were pretreated with azathioprine.[4] This aspect concerning tumor-associated, bone-marrow–derived stem cells is currently under investigation.

Regarding the mechanism of CY-induced tumor regression, there are several reports suggesting that antitumor immunity plays a dominant role in such drug-mediated regression.[9-14] In a previous report,[6] however, the evidence suggested that established immunity, or the use of immunogenic tumors, was not a prerequisite for obtaining CY-induced tumor regression, and overall the data indicated that the antitumor effects seen were exerted mainly, if not solely, by the action of the metabolites of CY. In the present studies, the drug induced regression of the weakly immunogenic MCA/77-23 sarcoma, as well as of the three immunogenic tumors growing in immunosuppressed mice, would support the contention that established immunity is not a prerequisite for regression per se. This does not imply that immunity may not play an important role under defined conditions: the question is whether the failure of immune or nonspecific effector mechanisms is responsible for obtaining only temporary tumor regression and whether total eradication of tumorigenic cells can occur only in the presence of potent immune effector mechanisms. In preliminary experiments still in progress, it has been possible to eradicate the above immunogenic tumors by the combined action of CY and anti-tumor immunity. We have shown that if pre-immunized mice were given a large enough dose of challenge tumor cells (5×10^6) to overcome antitumor immunity, the subsequent injection of CY induced permanent regression in most cases. When the MCA/77-23 sarcoma was used, no permanent regressions were obtained. These data are similar to those reported by Moore and Williams.[11] Thus, the reason immunity did not play an obvious role during the CY-induced effects obtained in the regular tumor-bearing situation, or why total eradication of the few residual tumorigenic cells did not take place, is not clear, as discussed previously.[6,7] One possibility is that suppressor mechanisms (cell-mediated or humoral), reported to be abrogated by CY[15,16] may have been regenerated fairly rapidly after CY injection of tumor bearers, so that immune effector mechanisms were inoperable. Since the level of suppression might be expected to vary from one tumor system to another, this might account for the variability seen in response to CY injection. Moreover, it has also been reported that CY may

eliminate those cells necessary for the generation of cytotoxic T-lymphocytes.[17,18] Preliminary experiments involving splenectomy of mice before injection of tumor cells indicated that the absence of a spleen did not affect CY-induced tumor regression, suggesting that either splenic generation of suppression in tumor bearers is not important under the conditions of the CY experiments or else suppressor activity is generated at other sites, such as the thymus or lymph nodes. Further studies are under way to investigate this aspect.

The accumulation of macrophages and granulocytes during CY-induced tumor regression raises the question of their relevance to the described events. The entire process from the time CY was injected to that when tumors became very small would appear to involve at least four distinct reactions: *a*) the physical destruction of neoplastic cells; *b*) the formation of a granuloma; *c*) the repair of tissues at the site of tumor regression, and *d*) proliferation of tumorigenic cells. *a*) In view of the formation of giant cells and polykaryons, a reaction similar to that seen when cells are irradiated, it seems unnecessary to evoke immunologically specific or nonspecific mechanisms to account for cell death, on the assumption that such changes were induced directly by the action of CY. Whether such cells were more susceptible to attack by effector mechanisms is, however, a possibility; and their destruction could well have been accelerated by a combined reaction of CY antitumor effects and specific or nonspecific effector mechanisms. *b*) The formation of a granuloma at the site of tumor growth has often been associated with tumor cell destruction, for example, after intralesional injection of bacille Calmette Guérin (BCG).[19] In the present situation, the presence of macrophages and granulocytes was associated with regression, as discussed in *a*. It was clear that whether or not these cells were involved in the destruction of the neoplastic cells, the massive amount of cell death and destruction that occurred was followed by a rapid elimination of dead cells and debris, a function both macrophages and granulocytes are adept at performing. *c*) The final product of this clearance was a fibrous, birefringent tissue containing relatively few cellular elements. Overall, the nature of the changes occurring through *b* and *c* was typical of the wound healing process and the formation of scar tissue. That this situation did not persist for long was because of the final reaction occurring, *d*) the proliferation of tumorigenic cells. As described previously,[5] a biossay indicated that the number of tumorigenic cells remaining after CY injection was reduced to a very low level, but numbers began to increase from about 3 days after treatment; that is, as tumor size was decreasing over a period of 2–3 weeks after drug treatment, the number of tumorigenic cells was actually increasing. Thus, although few neo-

plastic cells were apparent in the final product, these were probably mostly tumorigenic and caused the recurrent tumor.

The overall conclusions from the experiments on these four sarcomas, coupled with previous observations,[5-7] are 1) that CY induces similar changes in all of the tumor models used so far; 2) that since such changes were not confined to a single tumor model system, the findings provide a sound baseline from which to extend these observations, and 3) that peripheral leukocytes have a direct influence on the level of tumor-associated leukocytes. While it is apparent from preliminary data that immunity may play a decisive role during tumor eradication, it is clear that CY can induce potent antitumor effects, as measured by tumor regression, in the absence of overt tumor immunity. The mechanism whereby intratumor leukocytes accumulate at the tumor site has still to be established, but the possibility is that cells accumulated, partly at least, in response to an inflammatory stimulus related to the amount of debris or tissue destruction produced following CY injection. On the basis of these conclusions, we shall continue our investigations into the relationship between peripheral and tumor-associated leukocytes and attempt to provide a rational therapeutic approach to elimination of tumorigenic cells remaining after drug treatment.

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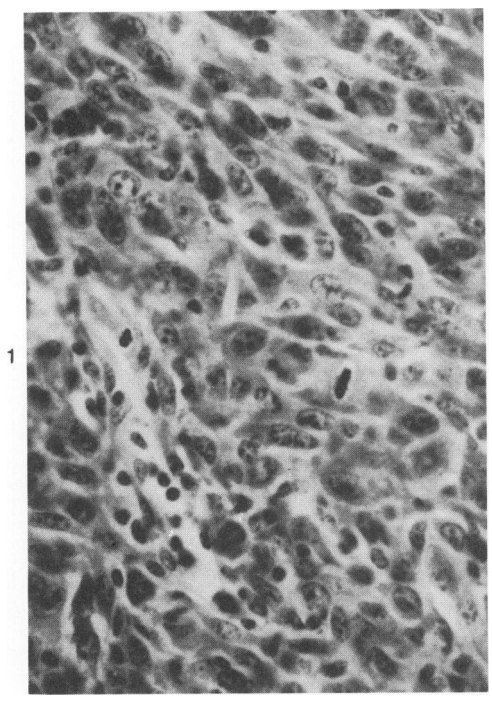

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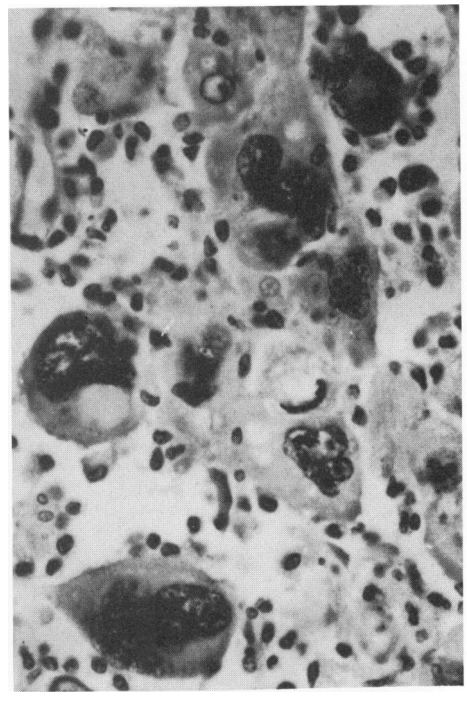

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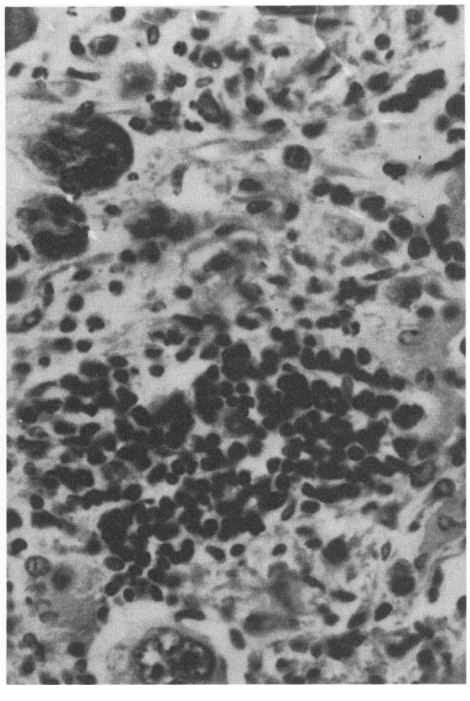

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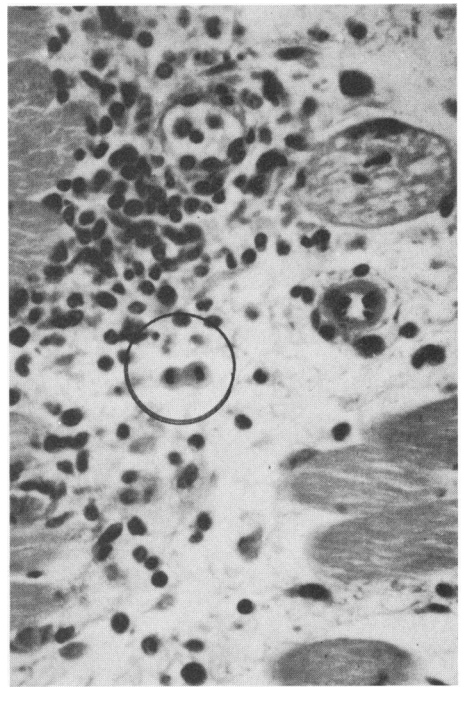
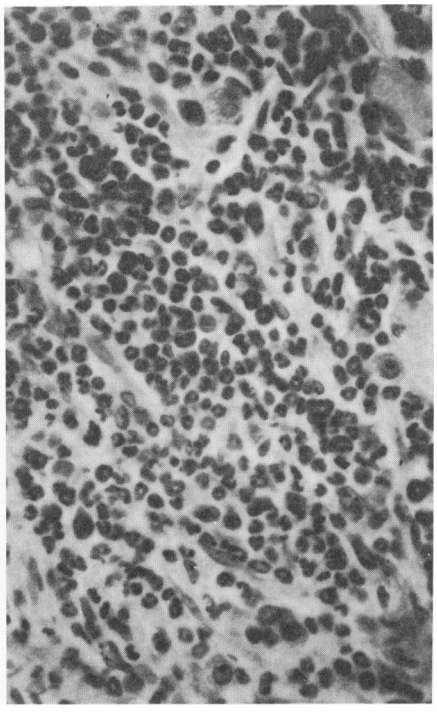


4

Figures 1–6 refer to histologic sections of the MCA/76-64 sarcoma. However, the detailed changes occurring after CY injection apply also to the MCA/76-9, 76-45, and the 77-23 sarcomas. **Figure 1**—Control MCA/76-64 sarcoma. Some mononuclear cells are visible. (×200) **Figure 2**—Five to 7 days after CY injection of tumor-bearing mice. Multinucleate and giant cells with smaller monuclear cells between are prominent. (×200) **Figure 3**—Eight to 10 days after CY injection. An area of hyperchromatic cells showing their relatively undifferentiated state. (×200) **Figure 4**—A similar area of hyperchromatic cells showing a cell in telophase (*circled*). (×200)



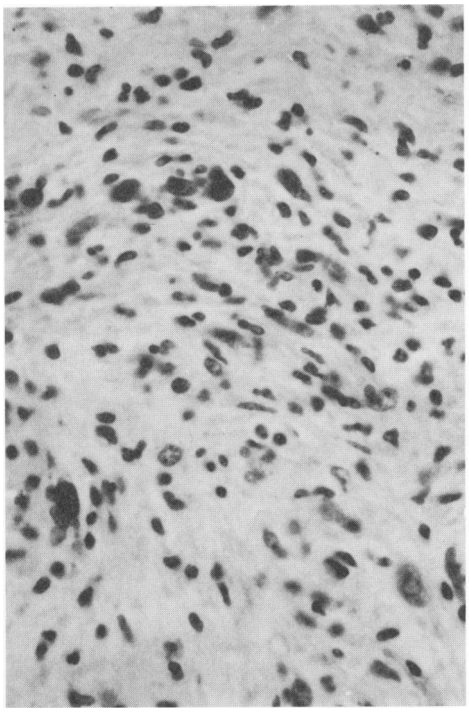


Figure 5—Ten to 14 days after CY injection. Granulocytes are accumulating in the capsule. (×200) **Figure 6**—A section of a regressed tumor showing that most of the tumor mass has been replaced by fibrous material. A few cellular elements, including some neoplastic cells, can be seen. (×200)