Cyclophosphamide-induced Changes in the Cellular Composition of a Methylcholanthrene-induced Tumor and Their Relation to Bone Marrow and Blood Leukocyte Levels

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ABSTRACT

A single i.p. injection of cyclophosphamide (CY) (6 mg) induced regression of a methylcholanthrene-induced fibrosarcoma (MCA/76-9) frequently to a nonpalpable mass. The extent of regression was dependent on dose of drug and time of administration in relation to tumor size, but even under optimal conditions few (<2%) permanent regressions were recorded. Well-marked histological changes were observed after CY injection, involving an initial increase in macrophage numbers followed by an accumulation of mature and immature granulocytes. These cells largely replaced neoplastic cells by 7 to 10 days after CY injection. Changes observed histologically were verified and quantitated after enzymic disaggregation of tumors at various times before and after CY injection. A bioassay was used to assess the number of tumorigenic cells associated with the tumor mass after CY injection by injecting graded doses of cells from enzymically disaggregated tumors into normal recipients. Within 1 hr after CY injection, more than 10^8 tumor cells were required for a take (compared with 10^5 cells from control tumors). However, by 3 days fewer cells (10^4) were required, and by 10 days only 10^3 cells were needed. Bone marrow and peripheral blood leukocyte numbers decreased rapidly after CY injection, but absolute numbers returned to control values by 7 to 10 days. However, differential counts on blood smears indicated that lymphocyte counts required at least 21 days to return to control values, whereas the number of monocytes and granulocytes was elevated above those of control mice by Days 7 to 10. The possibility is discussed that the observed intratumor cellular changes might reflect a variety of different reactions unrelated to tumor regression or recurrence.

INTRODUCTION

During the last few years, there have been numerous reports on the presence of host-derived leukocytes within progressing and regressing tumors. The relevance of these cells to the natural history of the tumor is still obscure, although it has been reported that macrophages and lymphocytes isolated from progressing and regressing tumors are able to destroy target cells or to stimulate proliferation, depending on the conditions (5, 7). Whether such leukocytes perform either activity in situ is uncertain. Thus, one of the major problems during drug-induced antitumor action is not knowing what the precise mechanism of successful antitumor action is or what events are actually occurring at the tumor site. Histological evaluation and electron microscopy (2, 20) have made some inroads by showing that certain events, such as the accumulation of macrophages, may occur during defined forms of treatment, but quantitative information is difficult to obtain under such conditions. Clearly, under conditions where leukocyte numbers may increase or decrease at the tumor site, such as during some forms of drug treatment (4, 22), quantitative and qualitative information regarding their precise functions would be of value.

In the present series of experiments, we used CY as a probe to investigate the possible interrelationship of host cells in the tumor mass and leukocytes in bone marrow and peripheral blood and whether any such relationship may correlate with drug-mediated antitumor effects. CY has well-defined in vivo effects. The drug may immunosuppress recipients; it destroys dividing cells, normal as well as tumor, and has a profound effect on hemopoietic tissue; it destroys suppressor T- and B-lymphocytes (9, 11), and there are reports that CY inhibits the cytotoxicity of T-lymphocytes by destroying the cells responsible for their generation (14, 16). Its overall mechanism of action during therapy is, therefore, complex, on the one hand, facilitating destruction of neoplastic cells by the direct action of its metabolites on DNA synthesis and by removing suppressor cells and, on the other hand, preventing the generation of potent effector cells. Although the duration of CY action is limited to only a few hr, it is evident that a fine balance is created between the reduction of tumor burden, subsequent proliferation of residual neoplastic cells, and the generation of antitumor effector mechanisms.

The present report is a confirmation and extension of previous studies (6, 7). The system to be described involves a murine fibrosarcoma that responded predictably to a single i.p. injection of CY by regressing initially to a small or nonpalpable mass, and then in most cases by regrowing, and finally by overwhelming the host. The purpose of this investigation was to further our understanding of the nature of the events associated with the antitumor effects mediated by CY and with the subsequent recovery and proliferation of cells.

MATERIALS AND METHODS

Mice. Throughout the experiments, female C57BL/6J mice, 6 to 8 weeks old, (18 to 20 g) were used (5 mice/group).

Tumors. The MCA/76-9 fibrosarcoma was induced by Dr. Lisa Prehn using 5% methylcholanthrene. Injection of 10^2 tumor cells consistently produced tumors in at least 60% of control mice. The tumor was immunogenic, immunized mice rejecting an i.m. injection of up to 10^6 cells. Experiments...
described in this paper were spread over about 9 months, utilizing tumors from animal passages 3 through 12. Tumors were maintained by injecting 10⁹ or 10⁸ cells i.m. at regular intervals. “Days of tumor growth” given in the text refers to the time elapsing between injection of tumor cells and measurement of a particular parameter.

Disaggregation Procedure. This has been described fully elsewhere (3). The popliteal lymph node was removed before excision of the tumor mass. The excised tumors were minced and totally disaggregated by using trypsin (250 μg/ml, Sigma type III, twice crystallized; Sigma Chemical Company, St. Louis, Mo.), collagenase (250 μg/ml; Sigma type I), and DNase (1 μg/ml; Sigma). Cells were washed twice by centrifugation at 1000 rpm and resuspended at 10⁶ cells/ml Roswell Park Memorial Institute Tissue Culture Medium 1640. Counts were made in a hemocytometer using phase-contrast optics. Viability (usually >90%) was assessed by combined trypan blue exclusion and phase-contrast microscopy. The number of Fc⁺ cells was assessed by mixing the tumor cell suspension with EA at an EA:tumor cell ratio of 20:1. Rosetting was estimated on the basis of counts from the Coulter Counter. The percentage of Fc⁺ cells (predominantly macrophages) was estimated on the basis of hemocytometer counts. In addition, cytocentrifuge preparations were made using a Perkin-Elmer cytospin (Coleman Instruments Division, Oak Brook, Ill.) before and after EA rosetting. Cytochemical stains were used to identify nonspecific esterase in macrophages or monocytes and specific esterase in granulocytes, using a-naphthylbutyrate and naphthol AS-D chloroacetate (Sigma), respectively, as substrates. Dur-
Analysis of the host-derived leukocytes found associated with regressing and recurring MCA/76-9 tumors

Tumor bearers, 5/group, were given injections of 6 mg CY i.p. on Day 9 of tumor growth. Total cell counts/tumor mess (x 10^6) ± S.D. of Fc* cells, PMN.

<table>
<thead>
<tr>
<th>Days of growth</th>
<th>Total cell yield (x 10^6) ± S.D.</th>
<th>Fc* cells (x 10^6)</th>
<th>PMN (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>31 ± 7.0 (25)</td>
<td>7.8 (25)</td>
<td>3.3 (11)</td>
</tr>
<tr>
<td>9</td>
<td>79 ± 14.4 (18)</td>
<td>14.2 (18)</td>
<td>4.6 (6)</td>
</tr>
<tr>
<td>4</td>
<td>64 ± 6.5 (40)</td>
<td>25.6 (40)</td>
<td>3.2 (5)</td>
</tr>
<tr>
<td>7</td>
<td>77 ± 10.5 (33)</td>
<td>25.4 (33)</td>
<td>7.7 (10)</td>
</tr>
<tr>
<td>10</td>
<td>44 ± 5.5 (30)</td>
<td>13.2 (30)</td>
<td>15.4 (35)</td>
</tr>
<tr>
<td>18</td>
<td>4 ± 2.5 (26)</td>
<td>1.1 (26)</td>
<td>1.6 (40)</td>
</tr>
<tr>
<td>25</td>
<td>234 ± 77 (20)</td>
<td>46.8 (20)</td>
<td>19.2 (8)</td>
</tr>
</tbody>
</table>

- Mean value of 5 tumors.
- Mean ± S.D.
- Numbers in parentheses, percentages of each cell type within the total cell population.

However, when tumors from passages 7 to 12 were used in CY-induced regression experiments, it was not uncommon for the macrophage content to reach levels of 60 to 70% during the 7 days immediately after CY injection. The following histological description is concerned with tumors from passages 7 through 12.

**Histological Evaluation.** The histological appearance of control tumors (Fig. 1) was typically fibrosarcomatous. They were typified by having a relatively high density of mononuclear cells in the subcapsular region (not shown here) with only a modest and scattered infiltration of mononuclear cells elsewhere. PMN were rarely seen outside areas of necrosis and then mainly in low numbers in the muscle. Within 24 hr after CY injection, tumors underwent profound morphological changes, and by 3 days intratumor damage was visible (Fig. 2). Nuclei of neoplastic cells were frequently pyknotic or swollen, and the cytoplasm was often vacuolated. Overall, there was a decrease in staining intensity of sections, presumably due to damage or loss of cells. Mitotic figures were seen, but often these were of a bizarre nature. Mononuclear cells became especially prominent throughout the tumor section. Large cells containing pigment were often seen associated with areas of hemorrhage. These were considered to be macrophages containing hemosiderin. By 7 days (Fig. 3), the outstanding feature of sections was the presence of large numbers of multinucleate and giant neoplastic cells that emphasized the presence of numerous mononuclear cells and to a lesser extent PMN. By 10 days, these giant cells had more or less disappeared leaving few demonstrable residual neoplastic cells. Although mononuclear cells appeared to comprise the bulk of the tumor mass (Fig. 4), distinct, and frequently large, focal points of darkly staining hyperchromatic cells and granulocytes were clearly visible (Figs. 5 and 6). These appeared to be localized in the capsular region and in the muscle. The groups of hyperchromatic cells were sometimes bounded by vascular endothelium. Large numbers of mature and immature granulocytes (ring forms) were associated with these areas (Fig. 7). On Day 14, when regression had evidently ceased, sections showed much fibrous, birefringent material with which fibroblasts were associated. By Day 21, when tumor growth recurred, increase in tumor size was accompanied by an increase in the number of mitotic figures and identifiable neoplastic cells. The pigmented macrophages became localized in the capsular region, which became relatively thick compared with the capsule of tumors seen before CY treatment.

**Bone Marrow and Peripheral Leukocyte Levels.** Charts 2 and 3 summarize the overall data on BMC and WBC when mice were given injections of CY 8 days after tumor implantation. Charts 4 to 6 summarize the blood leukocyte differentials. It is seen that BMC and WBC of control, non-tumor-bearing mice returned to normal values 7 to 10 days after CY injection, and this was essentially similar to the reaction seen in tumor bearers. The differential count showed that monocyte numbers (Chart 4) fell rapidly after CY injection of normal and tumor bearers, but by Day 7 (15 days after tumor implantation) the numbers had risen to above those of the untreated counterparts. Although monocyte levels of treated normal mice returned to untreated control values soon after this, those of tumor bearers returned to normal at a slower rate. The elevated number of monocytes seen in untreated tumor bearers on the 25th day of tumor growth was not seen in the CY-treated tumor-bearing mice. This difference has been seen in 3 separate experiments. Granulocyte numbers (Chart 5) showed a similar decline and rise after CY treatment. However, unlike...
monocyte counts, those of PMN in untreated and treated tumor bearers showed a substantial increase above the control background. Lymphocyte numbers (Chart 6) were largely unaffected by the presence of the growing tumor. After CY injection of control mice or tumor bearers, overall lymphocyte numbers declined and did not return to untreated control values for 21 days.

Tumorigenicity of Neoplastic Cells Recovered after CY Treatment. To assess what proportion of the total cell yield was able to induce tumors in normal recipients, tumors were excised at intervals before and after CY injection and enzymatically disaggregated. Serial dilutions of the total cell yield were prepared, and 0.1 ml was injected into each of 5 mice, which were then observed for tumor growth. Table 2 summarizes the data in terms of the incidence of tumors after injecting graded doses of cells. Within 1 hr of CY injection and up to 24 hr following, there was a significant reduction in the number of

Chart 2. Effect of CY on BMC from tumor-bearing (MCA/76-9) mice. Six mg CY were injected i.p. on Day 8 of tumor growth.

Chart 3. Effect of CY on peripheral blood leukocyte counts of tumor-bearing (MCA/76-9) mice.

Chart 4. The effect of CY on blood monocyte levels of tumor-bearing (MCA/76-9) mice.

Chart 5. The effect of CY on blood granulocyte levels of tumor-bearing (MCA/76-9) mice.

Chart 6. The effect of CY on blood lymphocyte levels of tumor-bearing (MCA/76-9) mice.
tumorigenic cells compared with control tumors which at all stages tested (from 7 to 20 days) showed at least a 60% take rate after injection of $10^7$ cells. By 3 to 7 days after CY injection, however, the number of cells required for a take was clearly decreasing, and by Day 14 there was no difference from that needed for a take in control mice.

**DISCUSSION**

In the above tumor system, we used CY as a probe to investigate the cellular changes induced peripherally and within the tumor mass. The intention was to further our understanding of the nature of the changes occurring during CY-induced tumor regression and tumor recurrence. The approach was designed to cover 2 important aspects of tumor-host relationships, namely, the relationship between peripheral and intra-tumor leukocytes and the effect of the latter on the behavior of the tumor, whether this involved regression or progression.

CY is a well-established antitumor reagent, the mechanism of action of which is mediated basically through the effect of its metabolites on DNA synthesis of the neoplastic cells. That this mechanism alone is insufficient to account for tumor eradication is evident from a number of reports (1, 13, 15, 19, 21), which suggest that immunity directed towards the neoplasm may effectively deal with the tumor burden when this has been reduced. Whether immune reactivity is involved or necessary in order to obtain only temporary tumor regression is still an open question. Radov et al. (19) reported that melphalan or CY-induced tumor regression was dependent on established antitumor immunity. However, a comparison between the antitumor effects of CY in a nonimmunogenic and immunogenic tumor model system (6) indicated that there was no apparent correlation between CY-induced tumor regression, induction of immunity, or the immunogenicity of the tumor. Similarly, in the present system, when mice were thymectomized and irradiated before injection of the MCA/76-9 fibrosarcoma cells, they responded to the antitumor effects of CY as readily as control mice, although none of the groups showed permanent regression. The fact that few permanent regressions of MCA/76-9 tumors were obtained suggests that there was a distinction between the mechanisms involving tumor regression and those required for total and permanent eradication of neoplastic cells.

Injection of CY into MCA/76-9 tumor-bearing mice resulted in the tumors regressing, often to nonpalpable masses. Histologically, well defined and reproducible events were observed beginning with immediate changes in neoplastic cells and ending with the change from a densely packed cellular mass to one consisting of fibrous material and few cells. During these extremes, there was an initial increase in the proportion of Fc+ mononuclear cells, mainly macrophages, as also observed by others. This was followed by an increase in the number of mature and immature granulocytes. At this stage, therefore, the tumor mass was essentially granulation tissue containing relatively low numbers of neoplastic cells. In view of the reports that granuloma formation at the site of tumor growth often leads to tumor regression, e.g., after intrallesional injection of *Bacillus Calmette-Guérin* (20), these, together with the increase in the ratio of macrophages and PMN to residual neoplastic cells, might have led one to expect eradication of residual neoplastic cells. This was rarely seen. Moreover, data from the bioassay indicated that the number of tumorigenic cells was increasing almost in parallel with the increase in macrophages and PMN even though the tumor mass was decreasing in size. These results, therefore, would seem to indicate that the tumor-associated host cells present at this time were not responsible for the observed tumor regression and that regression was probably mediated by the direct effect of CY metabolites. That the antitumor action of CY was of relatively short duration was indicated by the increase in the number of tumorigenic cells within 3 days of CY injection. It would follow that the mechanism required for total tumor eradication was either absent in the present system or inhibited in its action.

The enzymic disaggregation of tumors yielded data that confirmed the histological appearance of tumors in that relatively high proportions of macrophages and PMN were seen at the appropriate times. Cytocentrifuge preparations showed the morphologically different cell types in proportions similar to those expected from the histological appearance. This would indicate that the enzymic disaggregation procedure did not selectively kill cells, although a certain amount of cell death must be expected when this technique is used. Whether the enzymes used affected the physiology, function, or structure of the various cell types was not evaluated. The Fc receptors of the macrophages were clearly intact, although whether the avidity for binding EA had been modified is not known. The failure of most PMN to bind EA, as reported previously (3), might indicate a change induced by enzymic treatment. The neoplastic cells from control-tumor were not dramatically changed in that a high percentage of takes was always obtained from low doses of cells. Although higher doses of tumor cells were required for a take after CY treatment (see Table 2), it seems unlikely on the basis of the histological appearance of tumors that the take rate was unduly influenced by the action of enzymes on the isolated tumor cells. The pattern of events suggested that tumorigenic cell numbers were rapidly reduced to low levels by the antitumor action of CY and that a certain amount of time was required before proliferation occurred and tumors became palpable again. What has yet to be established is whether the macrophages or the PMN contained in the tumor cell inocula were responsible for the poor take rates when tumors were disaggregated soon after CY injection, i.e., within 24 hr. Conversely, the contribution of the high proportion of macrophages and PMN in the inocula of tumors taken 7 to 14 days after CY treatment to the high take rate from small numbers of cells also needs evaluation.

The interrelationship of blood and tumor-associated leukocytes is far from clear. Little is known about the kinetics of the

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**Table 2**

<table>
<thead>
<tr>
<th>Time after CY injection</th>
<th>Lowest dose of cells inducing a tumor</th>
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<tbody>
<tr>
<td>1 hr</td>
<td>$10^6$ (80%)</td>
</tr>
<tr>
<td>4 hr</td>
<td>$2.5 \times 10^6$ (20%)</td>
</tr>
<tr>
<td>12 hr</td>
<td>$2.5 \times 10^7$ (20%)</td>
</tr>
<tr>
<td>1 day</td>
<td>$2.0 \times 10^7$ (20%)</td>
</tr>
<tr>
<td>3 days</td>
<td>$10^6$ (40%)</td>
</tr>
<tr>
<td>7 days</td>
<td>$10^6$ (60%)</td>
</tr>
<tr>
<td>10 days</td>
<td>$10^6$ (60%)</td>
</tr>
<tr>
<td>14 days</td>
<td>$10^6$ (60%)</td>
</tr>
<tr>
<td>Control tumor cells</td>
<td>$10^6$ (60%)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of incidence of tumors.

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4 R. Evans, D. M. Eidlen, and G. J. Baigent, unpublished data.
accumulation of tumor-associated host cells (8), how long they persist within the tumor, whether they proliferate in situ, or whether they move away from the tumor at any given time. It has been suggested that accumulation of monocytes in the blood during tumor growth is the result of a tumor product that inhibits migration to sites of inflammation (17, 18). However, there is no direct evidence that monocytes fail to migrate to the tumor site, even though a monocytes may be demonstrable. Similarly, a granulocytosis is sometimes seen (10, 12), as in the present experiments, but there is no evidence that this is indicative of a loss of migratory potential to the tumor mass. In the present experiments, there was no evidence to support the view that the observed mononcytosis or the granulocytosis resulted in decreased levels of these cells at the tumor site. Unfortunately, until there is more information on the kinetics of infiltration of tumors by host leukocytes, it is not possible to draw conclusions concerning the precise relationship between peripheral and tumor-associated leukocytes. In the above experiments, data were presented for an active accumulation of both macrophages and PMN at the tumor site up to 10 days after CY injection of mice. The accumulation was evident histologically and demonstrable by cell counts on enzyme disaggregated tumors. The distribution of macrophages and PMN was, however, quite different; the former spread throughout the regressing tumor, and the latter more or less localized to the muscle and capsule. The significance of this is unknown. What is also not clear is whether this accumulation was due to proliferation of immature forms in situ or to extravasation from the blood capillaries, or to both. Mitotic figures were seen among the hyperchromatic cell population with which PMN rather than macrophages were usually associated, but as yet there is no evidence that the PMN were derived from these cells. Autoradiography studies should indicate whether DNA synthesis is occurring at these hyperchromatic focal points, and injection of tumor cell suspensions into lethally irradiated mice might indicate whether these regressing tumors contain precursors cells for the macrophage and PMN lineage. Since the results of the above experiments suggest that the tumor-associated host cells were not directly involved in the overall mechanism of CY-induced regression, the question arises as to the reasons for their presence. A speculative discussion on this aspect has been presented elsewhere (7). An aspect that should not be ignored is that, during the process of regression, other basic biological reactions were presumably taking place. For example, the localized degeneration of the majority of neoplastic cells implies that there was a need to eliminate debris or dead cells from the site. Perhaps the observed granuloma formation fulfilled this requirement. In addition, the initial growth of the i.m. tumor and its subsequent regression must have involved severe local tissue damage. Since mice regained the use of the limb in which the tumor had regressed, wound healing and formation of scar tissue must have ensued. Such reactions are characterized initially by the presence of granulation tissue. Moreover, the possibility that the accumulating leukocytes contributed towards the successful proliferation of tumorigenic cells during the period of granuloma formation cannot be ignored.

Protocols involving the use of chemotherapeutic agents, such as CY, are largely empirical and based on observations derived from a combination of in vitro data and in vivo trial experiments that have been designed to obtain maximal effects. Often a high degree of success is obtained, if not in totally eradicating the tumor mass, then in temporarily checking the tumor growth. Little is known about the nature of the events occurring at the level of the tumor mass, which must ultimately be the focal point of investigation. This report demonstrates changes occurring within the tumor and in the blood. It forms the beginning of a series of experiments that will determine the functional capacity of tumor-associated host cells during regression and tumor recurrence and also whether the findings can be utilized and extended to rationalize certain practical aspects of adjuvant therapy.

REFERENCES

Fig. 1. Histological section of a control MCA/76-9 fibrosarcoma (passage 9). × 200.
Fig. 2. Histological section of MCA/76-9 fibrosarcoma (passage 9) 3 days after mice had received 6 mg CY i.p. × 200.
Fig. 3. Histological section of MCA/76-9 fibrosarcoma (passage 9) 7 days after CY injection. Multinucleate, giant cells, and smaller mononuclear cells are visible. × 200.
Fig. 4. Histological section of MCA/76-9 fibrosarcoma (passage 9) 10 days after CY injection. Few neoplastic cells are visible among the prominent mononuclear cells. × 200.
Fig. 5. Same as Fig. 4, showing dense hyperchromatic foci in the region of the capsule and muscle. × 50. The circled area is enlarged in Fig. 6.

Fig. 6. Enlargement of circled area of Fig. 5 showing densely stained cells and granulocytes in capsule. × 200.

Fig. 7. Histological appearance of the MCA/76-9 fibrosarcoma 10 days after CY injection. The capsule and muscle show the intensive accumulation of mature and immature granulocytes. × 200.
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