Concomitant Inhibition of Tumor-associated Inflammatory Responses and Rapid Enhancement of Cyclophosphamide-induced Tumor Regression by Hydrocortisone

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ABSTRACT

We have demonstrated that hydrocortisone (HC) enhances the antitumor action of cyclophosphamide (CY). When sarcoma-bearing C57BL/6J, BALB/cByJ, and C3H/HeJ mice were given a single injection i.p. of CY (300 mg/kg), followed 1 to 3 days later by a s.c. injection of HC (10 mg in 0.1 ml of incomplete Freund's adjuvant), there was a dramatic acceleration of the reduction in tumor size compared with CY treatment alone. Without prior treatment of mice with CY, HC alone did not influence tumor growth. Histological evaluation and an analysis of the cellular composition of tumors after treatment with CY alone indicated a reduction in the neoplastic content of the tumors, a concomitant increase in the proportion of tumor-associated macrophages, and a marked neutrophil influx 7 to 9 days after CY injection. Similar changes were seen following CY-HC treatment except that a neutrophil infiltrate was not evident. The increase in the proportion of diploid host cells was confirmed by cytofluorimetric analysis of the DNA content of cells obtained by enzymic disaggregation of the tumors. After either CY or CY-HC treatment, the G1 peak of aneuploid cells disappeared with a concomitant increase in the proportion of cells accumulating in G2-M between days 2 and 4. After this, the G2 peak disappeared, at which time diploid cells comprised most of the cell yield (70 to 80%). When tumors regrew, the original DNA profile seen in control tumors was obtained. Although CY-HC treatment of tumor bearers resulted in a dramatic reduction in tumor size and cellularity, there was no significant prolongation of life when compared to CY treatment alone, indicating that HC was probably not acting on those cells surviving the antitumor effects of CY.

The data are discussed in the context of the relative importance of the CY-induced acute inflammatory response occurring at the tumor site, as well as the chronic inflammatory response that is a constitutive part of progressing tumors.

INTRODUCTION

Progressive tumor growth in animal model systems is accompanied by a chronic inflammatory response, during which a variety of host-derived leukocytes may infiltrate the tumor site (9). Of these leukocytes, the monocyte-derived macrophage has received much attention and indeed forms a constitutive part of many different tumors (5). The question has been raised whether infiltrating leukocytes play any role in the natural history of the tumor, whether this involves spontaneous regression or progression. In an attempt to resolve this question, we have developed a tumor model system involving both sarcomas and carcinomas in which tumor regression is induced by a single injection of CY (7, 10, 12). However, permanent regressions are rare (<5%) and tumors ultimately regrow, thus giving a system where the potential involvement of host-derived leukocytes may be investigated during both tumor regression and progression.

Previous observations indicated that during CY-induced tumor regression distinctive morphological intratumor changes occurred irrespective of tumor types (10, 12). These changes included: progressive deterioration in the appearance of neoplastic cells and their almost total disappearance within 7 to 10 days of giving the drug; the rapid emergence of mononuclear cells throughout the tumor within 2 to 3 days resulting in a sharp increase in the percentage (ratio) of macrophages to other cells; the infiltration of granulocytes 7 to 9 days after drug treatment; and the deposition of fibrous material following the clearance of the bulk of the neoplastic cells. However, during this active phase of granuloma formation, it was demonstrated by a bioassay for tumorigenesis that proliferation of the few residual tumorigenic cells began shortly after the dissipation of CY-antitumor activity. In short, recovery of tumor growth potential was occurring during the overt period of tumor regression. These findings led to the tentative conclusion that this intense acute inflammatory response might have been responsible not only for the elimination of drug-damaged tumor cells but also for the stimulation of the proliferation of the residual tumor cells. The latter possibility was based on reports that TAM have the capacity to stimulate tumor growth (4, 6, 8, 11, 14, 18, 22).

In this report, we describe experiments that elucidate the relationships between the acute inflammatory response, granuloma formation, CY-induced tumor regression, and the subsequent proliferation of residual tumorigenic cells. Using the antinflammatory reagent HC to inhibit both the inflammatory response (22) and granuloma formation (3, 16, 19, 20), we show that HC enhanced the antitumor action of CY in a dramatic manner in the absence of the acute inflammatory, granulomatous response. However, intratumor changes still involved the aforementioned increase in the ratio of TAM, and the enhancement of tumor regression had no apparent effect on those tumorigenic cells resistant to the action of CY.

1 Supported by USPHS Grant CA 27523 awarded by the National Cancer Institute, Department of Health and Human Services.

2 To whom requests for reprints should be addressed.

3 The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Received May 17, 1982; accepted August 10, 1982.

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MATERIALS AND METHODS

Mice. Male and female C57BL/6J, BALB/cByJ, and C3H/HeJ mice, 8 to 10 weeks old, 5/group, were used in the various experiments.

Tumors. The C57BL/6J, BALB/cByJ, and C3H/HeJ sarcomas, MCA/76-9, 79-83, and 79-1, respectively, were methylcholanthrene induced in female mice. Each of the tumors was passed at intervals of 2 to 3 weeks by injecting into the gastrocnemius muscle (i.m.) 0.1 ml of cell suspension obtained by enzymic disaggregation of tumor fragments.

Disaggregation of Tumors and Identification of Cell Types. These techniques have been fully described elsewhere (6, 12). Briefly, tumors were sliced and agitated in a mixture of papain, collagenase, and DNase until fragments had been totally dispersed. Tumor cells were identified on the basis of morphology, macrophages by the presence of Fc receptors, phagocytosis of antibody-coated sheep RBC, and staining for nonspecific esterase, and granulocytes by morphology and positive staining for chloroacetate esterase or peroxidase.

Drugs. CY (Cytoxan; Mead Johnson, Evansville, Ind.) was dissolved in distilled water and injected i.p. at a concentration of 300 mg Cytoxan per kg. The optimal conditions for use of this drug in tumor regression experiments were previously reported (10, 12). HC (Sigma Chemical Co.) was suspended in IFA as a vehicle at a concentration of 100 mg/ml and injected in 0.1-ml volumes s.c. Assessment of its antiinflammatory properties was carried out by injecting HC-treated mice with thioglycollate medium into the gastrocnemius muscle or the peritoneal cavity, 0.1 and 1 ml, respectively. Three days later, the total number of cells yielded by each site was estimated. Muscles were excised, minced, and disaggregated with enzymes by the same technique used for tumors.

Histology. Tumors were routinely sectioned at 3 levels to gain an in-depth impression of intratumor changes. Thin sections (5 to 6 μm) were stained with hematoxylin and eosin.

Cytotluorimetry. The DNA content of cells was assessed using the method of Krishan (17) by staining cells with propidium iodide followed by measurement of the fluorescence using the Ortho 50H cytofluorograph.

Statistical Analysis. Data were analyzed using the unpaired Student's t test, by which p < 0.05 was considered significantly different.

RESULTS

HC as an Antiinflammatory Agent. To show the efficacy of HC as an antiinflammatory agent, C57BL/6J mice were injected i.m. or i.p. with thioglycollate medium 1 or 4 days after the s.c. injection of HC. Three days later (on Day 4 or 7) the number of cells in the muscle or in the cavity was counted. Isotonic sodium chloride or IFA was used to control the experiment. Table 1 summarizes the data that demonstrate that HC exerted a potent antiinflammatory effect, the effect appearing to be stronger at the i.m. site. Blood leukocyte counts (not shown) were depressed for at least 14 days after HC injection, monocytes and lymphocytes showing a 90% reduction over this period; granulocyte numbers on Day 14, although still depressed, were showing evidence of recovery.

Enhancement of CY-mediated Tumor Regression by HC. Preliminary experiments using C57BL/6J, BALB/cByJ, and C3H/HeJ sarcomas indicated that when mice that had received an i.p. injection of CY were given injections of HC 1 to 3 days later, there was a dramatic reduction in tumor diameter within 48 hr compared with about a 10-day period to effect regression after CY treatment alone.

These data were confirmed using tumor weights instead of diameters. Several experiments were carried out to investigate whether HC produced similar effects if given 1, 2, or 3 days after CY injection. Chart 1 summarizes data from one such experiment and illustrates the effect of CY and HC treatment on tumor weights. In this experiment, tumor burden was greater than the optimal size necessary to obtain strong CY-mediated antitumor effects and, as seen, the antitumor effect of CY treatment alone was minimal. However, injection of HC 1, 2, or 3 days later produced a significant reduction in tumor weight (p < 0.01) 24 hr later, and this reduction persisted for about 14 days until tumors began to grow rapidly. On Day 24 (14 days after CY injection), tumor weights were either not significantly different (p > 0.05) when comparing the CY alone and the CY-HC (Day 1) groups, or marginally so (p < 0.05) when comparing the CY alone with the CY-HC (Days 2 and 3) groups. The CY-HC (Days 1, 2, 3) groups were not significantly different. The magnitude of the immediate decrease in tumor size after HC injection appeared to be independent of the tumor size at the time of CY injection since small and large tumors were similarly affected. HC injection without prior CY injection or IFA injection with or without prior CY treatment did not influence tumor growth or regression.

Cellular Composition of Tumors. Following CY-HC treatment of MCA/76-9 tumor-bearing mice, tumors were excised at intervals and disaggregated with enzymes into single cell suspensions. Total cell yields per tumor were assessed, and

Table 1

<table>
<thead>
<tr>
<th>Compound injected s.c. into mice</th>
<th>Peritoneal inflammatory response (total cell yield × 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.p. challenge with thioglycollate</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>0.9% NaCl solution/IFA</td>
<td>43.6 ± 2 (4)</td>
</tr>
<tr>
<td>Hydrocortisone (HC)</td>
<td>9.9 ± 2 (8)</td>
</tr>
</tbody>
</table>

a C57BL/6J B6 mice were given s.c. injections of 0.1 ml HC in IFA, IFA alone, or 0.9% NaCl solution. After 1 or 4 days, mice were given injections i.m. of 0.1 ml or i.p. of 1.0 ml of thioglycollate medium. Three days later, total cell yields from the muscle or peritoneal cavity were estimated.

b Mean ± S.E.

c For each point compared with control values, p < 0.001 by the unpaired Student's t test.

d Numbers in parentheses, inflammatory index, calculated as

\[
\text{Inflammatory index} = \frac{\text{No. of exudate cells in stimulated test mice} - 1}{\text{No. of exudate cells in unstimulated test mice}} \times 100
\]

\[
\text{No. of exudate cells in stimulated controls} - 1
\]

\[
\text{No. of exudate cells in unstimulated controls}
\]
the macrophage content of the cell populations was estimated. Chart 2A summarizes the cell yield data, which show that injection of CY alone on Day 10 of tumor growth resulted in a progressive decline in cell numbers until Day 7 after CY, after which time the number increased significantly above that seen on Day 7 (p < 0.01). When mice received both CY and HC, there was a rapid reduction in tumor-associated cells, paralleling the decrease in tumor diameters and weights (see above). By 24 hr after injecting HC, cell yields were less than 50% of the CY alone group and less than 10% of the progressing control groups. Unlike the CY alone group, there was no significant increase in cell yields from 7 days after CY injection (p > 0.05), even though at 14 days post-CY the mean number of cells was higher. Chart 2B shows that the macrophage numbers declined initially in both CY alone and CY-HC groups, but this stopped by Day 4 in the CY alone group. There was a marked increase in numbers from 4 to 10 days after CY injection. In contrast, the CY-HC group showed a continuous decline in macrophage numbers until at least 8 days after CY injection. However, the percentage of macrophages, shown in parentheses, showed a progressive increase from the control value of 30% irrespective of treatment, reaching a peak value of about 70% by 7 days after CY treatment. As tumors began to regrow, the percentage declined and eventually returned to control tumor levels (not shown).

The increase in the percentage of host-derived cells after CY injection was confirmed by cytofluorimetry. Cell suspensions were stained with propidium iodide, and DNA fluorescence was measured on the Ortho 50H cytofluorograph. Chart 3 summarizes some of the changes occurring in the DNA profile after CY injection. No data are included from HC experiments since the information obtained on the DNA content of cells was identical. The most salient features of the overall data were as follows: control tumor cells consisted of about 40% diploid...
cells (Chart 3A), the rest being aneuploid (Chart 3, B and C). Of the aneuploid cells, about 80% were estimated to be in G0-G1 (Chart 3B) and the rest in S and G2-M stages (Chart 3C) of the cell cycle. After injection of CY, the G0-G1 peak (Chart 3B) disappeared within 1 to 2 days with a concomitant increase in the percentage of cells in G2-M (Chart 3C), such that by Day 4 about 48% of the aneuploid cells had accumulated at this stage. The diploid population accounted for 70 to 80% of all cells 4 to 7 days after CY injection. When tumors began to regrow in this experiment (about 14 days after CY injection) the G0-G1 aneuploid peak (Chart 3B) reappeared, the diploid population (Chart 3A) having been reduced to about 40% of the total.

Other cellular changes occurring involved mainly the influx of neutrophils into the tumor 7 to 9 days after the injection of CY, as fully quantified previously (10, 12). While these were readily identifiable in the cell suspensions derived from tumors from the CY-treated mice, in which neutrophils comprised 20 to 25% of the total cell suspensions by Day 7, few (less than 5%) could be discerned on the basis of morphology, and chloroacetate esterase or peroxidase staining after HC injection. When tumors regrew, the neutrophil content represented about 6% of the total cell yields in both groups.

Histological evaluation of sectioned tumors confirmed the analysis of the cellular composition of tumors, as well as the data fully reported previously in this same system (10, 12). That is, CY treatment induced distinctive changes in the morphology of neoplastic cells, which became large and sometimes vacuolated and contained abnormal nuclei. The extent of these changes depended on tumor size, although similar localized morphological changes occurred irrespective of tumor size. Mononuclear cells became prominent throughout the tumor mass, unlike their distribution in control tumors where they were mainly confined to the subcapsular region. Under optimal conditions, the bulk of the tumor mass consisted of mononuclear cells by Day 7 after CY treatment. Granulocytes started to make their appearance in the tumor at about this time. In tumors from mice receiving both CY and HC, there were no major observable histological differences, except that the relative number of cells per unit area of section appeared to be smaller. This was exemplified by disaggregation data which clearly showed differences between CY treatment alone and the combined treatment. After CY treatment alone, the number of cells per g of tumor increased from about 30 x 10^6 on Day 3 to 150 x 10^6 per g by Day 7 and to 200 x 10^6 per g when tumors regrew. After the combined treatment, the number remained low (<30 x 10^6/g) until Day 14 after CY. There were also few neutrophils in HC-treated mice, particularly during the 7- to 9-day period after CY injection. In addition to these intratumor differences, the marked edema seen to be associated with tumors in the CY-treated mice was absent in the HC-treated mice, although observations were qualitative rather than quantitative. These data in toto, together with those in Charts 1 and 2, indicated that HC had effectively inhibited the acute inflammatory response defined by the marked accumulation of macrophages, neutrophils, and fluid, thereby preventing the formation of the granulomatous lesion.

**Influence of the CY-HC Combination Treatment on Other Tumors.** The general applicability of the HC adjuvant effect to other sarcoma systems is shown in Table 2, in which the response of the MCA/76-9, 79-83, and 79-1 sarcomas (C57BL/6J, BALB/cByJ, and C3H/HeJ, respectively) to the combined treatment of CY and HC is compared. It is seen that, although starting tumor weights were substantial in all cases, the injection of HC caused a significant reduction as measured by weights and total cell yields 4 days after CY injection and 2 days after HC injection.

**DISCUSSION**

The above experiments were performed in an attempt to answer the question of whether the CY-induced acute inflammatory response leading to the formation of the granulomatous...
**Enhancement of Tumor Regression**

The natural text reads:

The changes induced by the action of CY, which caused the cell cycle of control tumor cells and no modifications of tumor injection and not the time at which the tumors were well induced a reversible block at the G2-M phase (2). Therefore, this response was a side effect of CY treatment and not directly causally related to tumor regression. Therefore, if tumor regression was dependent in any way on host-derived leukocytes, then only those already resident in the tumor mass at the time of CY injection could have been involved in the subsequent elimination of drug-damaged tumor cells. Preliminary evidence from experiments in which macrophages were isolated from either progressing or regressing tumors indicated that both types of TAM were able to lyse mitomycin C-treated target cells but unable to lyse untreated targets. On this basis, therefore, it seems feasible to suggest that the cells damaged by drug action may be expeditiously eliminated from the tumor site by the ability of macrophages to recognize damaged cells, as they would remove effete or dead cells from normal tissues. Since the ratio of macrophages to tumor cells increased after injection as a result of tumor cell loss, whether or not CY-injected mice received a subsequent injection of HC, this mechanism could be active in both situations. The regrowth of tumors after the CY or CY-HC treatment occurred at almost identical times, even though tumors in the latter group had been significantly more reduced in size. This, together with the findings that there was no significant prolongation of life after CY-HC treatment, would seem to indicate that HC did not directly affect the surviving tumorogenic cells. Moreover, the same reasoning as above may be applied concerning the CY-induced acute inflammatory response since its absence after HC treatment indicated that the proliferation of the tumorogenic cells was not dependent on it. Whether the resident TAM were involved in their proliferation directly or indirectly is currently under investigation. There are reports suggesting this possibility (4, 6, 11, 14, 18, 22). The actual cause of the dramatic decrease in tumor size following HC injection of CY-treated mice is unclear at this time. As a general category of agents, corticosteroids have been reported to exert antitumor effects in vivo and in vitro and indeed are among the most widely used chemotherapeutic agents, usually in combination with other drugs (1, 2). It has been reported that methylprednisolone administered after CY or Adriamycin reversibly inhibited the initiation of proliferative recovery of the surviving cell population (1). However, these studies are not directly comparable to those described above since they were more concerned with the application of drugs at the same time as tumor injection and not the time at which the tumors were well established. Moreover, corticosteroids have been reported to induce a reversible block at the G1 phase of the cell cycle (2).

In our cytofluorimetry experiments, HC exerted no effects on the cell cycle of control tumor cells and no modifications of those changes induced by the action of CY, which caused the disappearance of the G1 peak and a temporary block at the G2-M phase. This G2-M block is what would be expected after CY injection (15). It thus seems unlikely that HC was exerting a direct effect on tumor cells in general and almost certainly no effect on the residual tumorogenic cells. As a tentative explanation, we would like to propose that the antiinflammatory properties of HC reduced vascular permeability at the tumor site (and elsewhere) and impaired the extravasation of bloodborne nutrients, either in the soluble form or those derived from infiltrating cellular elements, as well as oxygen, resulting in the rapid death of CY-damaged cells. This, coupled with the possible failure of catabolic wastes to diffuse away from the site because of the changes in vascular permeability, would exacerbate the noxious conditions imposed by dying or dead cells causing an across-the-board death of both neoplastic and normal host cells, with a concomitant shrinkage of the tumor mass. Since drug-damaged tumor cells would probably be more sensitive to these conditions than TAM, relatively fewer TAM would be killed during this process so that the relative proportion would still rise as after treatment with CY alone. This explanation would imply that residual tumor cells as well as perhaps the TAM were more resistant to such conditions and were probably existing under anoxic or hypoxic conditions. These events would not be expected to occur in HC-treated control tumor bearers since such a mechanism of tumor cell destruction would presumably occur only under conditions of stasis (i.e., after CY treatment) or when considerable previous intratumor damage had been inflicted.

Although HC-enhancement of CY-induced tumor regression did not result in prolongation of the life of the mice, the rapid debulking of the tumor mass might be used to facilitate access of immuno- or chemotherapeutic agents to the residual tumorogenic cells. In particular, the high ratio of TAM might be used to the benefit of the host if agents that render macrophages tumoricidal can be localized in or transported to the tumor site. For example, North (21) has recently demonstrated that i.v. infusion of CY-treated tumor bearers with tumor-sensitive T-cells resulted in permanent tumor regression. While the mechanism of action was clearly T-cell dependent, the mechanism of tumor rejection could depend on the availability of macrophages at the site. In addition, i.v. injection of liposomes carrying macrophage-activating factor or muramyl dipeptide (13) effectively destroyed metastatic lung and lymph node lesions, the liposomes localizing in the TAM. Approaches such as these, in combination with cytoreductive chemotherapy, would seem to offer encouragement for future therapy of cancer.

**ACKNOWLEDGMENTS**

The authors express their appreciation to Greg Baigent, Manager of the Cell Sorter Service, for his cooperation in the use of the cytofluorograph and interpretation of data.

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