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# Immunomodulatory effect of cyclophosphamide on host humoral immunity in Dunning's R-3327 adenocarcinoma of the prostate

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**Summary.** The object of the present investigations was delineation of the exclusive effects of cyclophosphamide (Cytoxan) on host humoral response to tumor, as evaluated by the level of circulating antigen/antibody complexes (AACs), which may reflect the chemo-responsiveness of hosts and provide a rationale for new therapeutic strategies. Our data, recorded in Copenhagen × Fischer rats bearing Dunning's R-3327 Mat Ly-Lu adenocarcinoma of the prostate, show no modulatory effect of cyclophosphamide at 10 mg/kg, a nonspecific immunosuppressive effect at 30 mg/kg, and a definite immunostimulatory effect on host humoral immunity at 100 mg/kg. Sequential determination of AAC levels at different stages of tumor growth, i.e. from the primary to the metastatic stage, performed with the original purpose of demonstrating that any disturbance in the immunoregulatory mechanism of the host was due to cyclophosphamide rather than to changes in tumor load, revealed that levels of AACs parallel disease progression in the initial stages of primary tumor growth but rapidly decrease to near-normal levels in the presence of heavy tumor burden.

**Key words:** Cyclophosphamide – Humoral immunity – Prostate cancer

Among the many cytotoxic drugs whose immunopotentiating effects have been studied extensively, cyclophosphamide (Cytoxan) is perhaps one of the most frequently used antitumor drugs. Cyclophosphamide may have attained this popularity because of its demonstrated tumoricidal activity against a variety of human and experimental tumors [4, 12, 16, 21, 23, 27]. Studies have demonstrated that for cyclophosphamide to be effective therapeutically, an active response by the host is needed [12, 14, 21]. Earlier investigations suggested that the immunopotentiation by cyclophosphamide should be evaluated by measuring its effect on actual immune responses (cellular or humoral immunity to specific antigen) rather than nonspecific parameters of lymphocyte functions.

Cyclophosphamide's mechanism of immunopotentiation through a selective effect on suppressive T-cells [13, 24] or circulating lymphocytes, affecting their proliferative responses [2], and through its ability to suppress [30] or enhance [3] antibody synthesis, has been demonstrated in many systems. However, its exclusive effect on the humoral response of tumor-bearing hosts, specifically directed towards tumor antigen, have not been delineated. This could be partly because cyclophosphamide is usually given in combination with other drugs and seldom used alone. Cyclophosphamide-mediated changes in humoral responses may be indicative of host chemo-responsiveness and may provide a rationale for the design of new therapeutic strategies.

The present investigations were aimed at evaluating the effect of cyclophosphamide on host humoral attempts at rejecting tumor by producing antibodies directed towards tumor antigen, as judged by the level of circulating antigen/antibody complexes (AACs). The elevated levels of AACs have been identified in a wide spectrum of neoplastic diseases, including prostate cancer [8], and it has been suggested that they may have some value as prognostic indicators of tumor recurrence and development of metastatic disease [28]. We know that adenocarcinoma of the prostate varies widely in its clinical aggressiveness, metastasizing and killing some patients in only a few months while in others it can remain localized for many years [11]. There is no established technique at present by which the metastatic potential of an individual primary tumor can be accurately predicted. We measured AAC levels in Copenhagen × Fischer rats bearing transplantable Dunning's R-3327 addenocarcinoma of the prostate. Because of its similarities to human prostatic cancer and its known growth rate and metastatic ability, Dunning's R-3327 prostatic tumor is a very suitable system [18] for evaluating new solutions to this major clinical problem, which takes a toll of over 26,000 American men annually.

**Table 1.** Antigen-antibody complex (AAC) levels at different stages of tumor growth in Copenhagen × Fischer rats bearing R-3327 MLL adenocarcinoma of the prostate

| Groups[a] | Tumor growth time (days) | AAC levels at O.D. $450 \times 10^3$ (mean ± SE) | $P <$ |
|---|---|---|---|
| Controls (14)[b] | - | 12.1 ± 2.2 | - |
| 1 | 7 | 86.8 ± 1.6 | 0.001 |
| 2 | 14 | 16.4 ± 1.6 | 0.01 |
| 3 | 21 | 52.6 ± 8.0 | 0.05 |
| 4 | 28 | 25.0 ± 4.2 | |

[a] Five rats in each experimental group
[b] Number of rats

## Materials and methods

### *Tumor model*

Seventy-four Copenhagen × Fischer rats, each about 18 weeks of age and weighing 225–260 g, were used. Forty of these rats received implants of Dunning's R-3327 Mat Ly Lu (MLL), a highly metastatic and hormonally insensitive prostatic tumor that metastasizes to lymph nodes and lungs of animals. Each rat received $1 \times 10^7$ cells, prepared from a single cell suspension of collagenase-digested tumor, inoculated s.c. in its left flank. Tumor cells were harvested from growing non-necrotic tumor, and their viability was determined by trypan blue dye exclusion.

### *Experimental design*

In part I of the experiment, 20 rats with inoculated R-3327 MLL tumor were used for sequential determination of AAC levels. This was primarily to demonstrate that the changes in AAC levels in part II of the experiment were the result of cyclophosphamide's immunomodulatory effect on host humoral response rather than of changes in tumor load. These rats were divided into four groups of five rats each, with AAC levels determined during primary tumor growth and at the time of metastasis, i.e. 7, 14, 21, and 28 days after tumor inoculation.

In part II of the experiment, 20 rats with R-3327 MLL tumor and 20 rats without tumor were divided into three groups. In each arm, the rats in group I ($n = 5$) each received cyclophosphamide 10 mg/kg; those in group II ($n = 10$) two doses of 30 mg/kg 1 week apart; and those in group III ($n = 5$), cyclophosphamide 100 mg/kg. ACC levels were also measured in 14 control rats that did not receive cyclophosphamide and were not inoculated with tumor.

### *Chemotherapy*

Cyclophosphamide (Adria Labs, Columbus, Oh., USA) was freshly prepared prior to use by dissolving it in sterile distilled water and was injected i.p. in doses of 10, 30, and 100 mg/kg body weight. The drug was administered when the tumors were palpable (1 week after inoculation) and ACC levels were measured 2 weeks after administration.

### *Antigen/antibody complexes*

Circulating AACs were measured by the polyethylene glycol precipitation technique, as detailed previously [5]. Briefly, serum samples were diluted with 0.1 M borate buffer (pH 8.4) and mixed with 3.75% solution of polyethylene glycol (PEG) (mol. wt 6000) at a ratio of 1:9. Each serum sample had a control mixed with 0.1 M borate buffer (B.B.) at the same ratio. All samples were run in duplicate and kept at room temperature for 120 min. The optical density (O.D.) of each sample was measured at 450 nm with a Beckman Model 25 spectrophotometer.

The results were calculated using the following formula:

$$\text{ACC} = (\text{O.D. } 450 \times 10^3 \text{ PEG}) - (\text{O.D. } 450 \times 10^3 \text{ B.B.})$$

The statistically significant differences between various subgroups were calculated using Student's *t*-test.

## Results

### *Sequential determination of AACs*

In order to demonstrate clearly that the host response to tumor was due to the immunomodulatory effect of cyclophosphamide than to changes in tumor load, and also to reveal any correlation between host response and tumor burden or development of metastasis, AAC levels were measured at different stages of tumor growth; the highest levels (86.8 ± 5.4) were measured during the initial phase of tumor growth, i.e., 1/week after implantation of the tumor cells (Table 1). At the end of day 14, although the tumor volume had increased to more than twice its original size, AAC levels had dropped drastically (16.4 ± 1.6), to near or below the background concentration seen in non-tumor-bearing control animals (12.1 ± 2.2). In the next 2 weeks the primary tumor did not only undergo a further increase in volume; in addition it metastasized to the lungs and lymph nodes. AAC concentrations, although higher than recorded after week 2, were significantly lower than at 1/week post inoculum.

### *Immunomodulatory effects of cyclophosphamide*

The second part of the experiment was conducted primarily to evaluate the effect of cyclophosphamide on the production of antibodies directed specifically towards tumor antigen and resulting in the formation of AAC. Our data, as seen in Table 2, show that among the rats receiving 10 mg/kg cyclophosphamide, AAC levels were higher in those without than in those with tumor.

However, the difference between the two groups was not statistically significant. Comparison with rats inoculated with tumor at the same time (21 days post inoculum) but not receiving cyclophosphamide (Table 1) revealed that the AAC level was almost the same in the two tumor groups, (63.3 ± 6.9 vs 52.6 ± 8.0).

In animals that received 30 mg/kg cyclophosphamide, AAC levels were almost the same in rats with tumor as in those without tumor. Rats that did not receive cyclophosphamide (Table 1) had significantly higher AAC levels (52.6 ± 8.0) after the same inoculum period (21 days) than

**Table 2.** Effect of cyclophosphamide on AAC levels in rats with R-3327 MLL tumor and rats without tumor

| Cyclophosphamide dosage (mg/kg) | AAC level at O.D. $450 \times 10^3$ (mean ± SE) | | $P <$ |
|---|---|---|---|
| | With tumor | Without tumor | |
| 10 | 63.3 ± 6.9 (4) | 72.6 ± 10.2 (5) | N.S. |
| 30 × 2 | 34.3 ± 4.7 (9) | 34.9 ± 5.8 (10) | N.S. |
| 100 | 130.3 ± 36.4 (5) | 53.4 ± 6.8 (5) | 0.05 |

Number of rats is given in parentheses in each case
N.S. = not significant

those that did receive cyclophosphamide either with or without tumor (Table 2).

Among the rats that received cyclophosphamide 100 mg/kg AAC levels were significantly higher in those with tumor than in those without tumor. The difference between the two groups was statistically significant ($P < 0.05$).

Comparison of rats with tumor and no cyclophosphamide (Table 1) and those without tumor with cyclophosphamide (100 mg/kg) revealed no substantial difference in AAC levels (52.6 ± 8.0 vs 53.4 ± 6.8).

## Discussion

Studies have shown that host antitumor immune responses can influence the effectiveness of therapy [12, 20, 21]. Therefore, it is essential that a tumor-bearing host's antitumor immunity and any chemotherapy work together if maximum benefit is to be obtained from the drug. The existence of such antitumor immune responses, whether cellular [7] or humoral [8], have already been demonstrated in patients with prostatic cancer and also in tumor model carrying Dunning's R-3327 prostatic tumor [5]. Patients with hormonally resistant advanced prostatic cancer have a poor prognosis, with 50% dying within 1 year [11]. Studies have shown that cyclophosphamide can result in a 46% response rate if stabilization of disease is counted as a response [25]. Although cyclophosphamide is usually used in combination with other drugs, we evaluated the effects of cyclophosphamide alone on host humoral attempts at rejecting tumor in an experimental tumor system that resembles and behaves very much like human prostatic cancer. In these experiments we have shown that cyclophosphamide at 10 mg/kg administered at early stages of tumor growth proved ineffective in altering host resistance to tumor growth, as seen in host humoral responses towards tumor.

It is possible that lower doses of cyclophosphamide would be more effective in humans, especially in patients with advanced cancer, in whom the immune system is often severely damaged by the combined effects of the disease and prior therapies. We did not measure the effect of cyclophosphamide before the tumor implantation, as the findings could not be correlated with those in prostatic cancer patients, even though we realize that many studies have suggested that immunopotentiation by cyclophosphamide is usually produced by administering the drug 1–4 days before tumor inoculum while administration of cytoxan after the implantation results in no effect or immunosuppression.

At 30 mg/kg, cyclophosphamide appeared to be immunosuppressive rather than immunopotentiating (Table 2). The observation that the immunusuppressive effect of cyclophosphamide, resulting in decreased levels of AAC, was not due to the presence of pre-existing relatively low levels of host antitumor immunity was further borne out when AAC levels in such rats were compared with those that did not receive cyclophosphamide and their post-tumor inoculum period was the same (34.3 ± 4.7 vs 52.6 ± 8). Comparison of AAC levels in rats that had no tumor but had received cyclophosphamide (34.9 ± 5.8) and those that had tumor but did not receive cyclophosphamide (34.3 ± 9.3) demonstrates that the immunosuppressive effect of cyclophosphamide was not tumor-specific. Lower AAC levels in rats with or without tumor that received 30 mg/kg than in those that received 10 mg/kg further reflects the nonspecific nature of the inhibition of antibody synthesis by cyclophosphamide.

The immunopotentiating effect of cyclophosphamide was very obvious in rats that received 100 mg/kg. A comparative look at AAC levels in rats with tumor (130.3 ± 36.4) and those without tumor (53.4 ± 6.8) and those with tumor but no cyclophosphamide (Table 2) clearly shows the enhanced production of antibodies and more aggressive host humoral response to tumor. Our results suggest that high-dose, as opposed to low-dose, cyclophosphamide enhances host humoral immunity. This is in contrast to cyclophosphamide's effect on T-cell-dependent immunity: low-dose cyclophosphamide led to augmented immunity while high doses resulted in suppressed antitumor immunity. We know that in T-cell-dependent immunity, low-dose cyclophosphamide results in toxic effects of the drug and requires cooperation from the host's antitumor immunity, in contrast to a high dose, which does not require cooperation from the host and has a tumoricidal effect. Enhancement of humoral immunity by the increased production of antibodies with high-dose cyclophosphamide is perhaps the host's mechanism of compensation for the suppressed T-cell-dependent immunity. Our data indicate that AACs did not have any tumorcidal effect of their own. It was not possible at this stage to determine what effect high-dose cyclophosphamide and the consequent enhanced humoral immunity have on host cell-mediated immunity; AACs have been implicated in the abrogation of cellular immunity [9, 26] and their presence correlated with poor prognosis of patients [28] and animals [1].

We agree that the PEG technique does not necessarily precipitate AACs related to the tumor. However, the facts that all rats used in the present investigations were bred and raised under controlled conditions, and that these rats did not have any signs or symptoms of infection and that all rats were kept under exact same conditions should minimize, if not totally eliminate, the possibility of the formation of AACs other than those attributable to the

tumor. The AAC levels in control rats with no tumor strongly support the thesis that the AAC levels in tumor-bearing rats were the result of host humoral response to tumor antigen.

The data recorded in part I of the experiment, which was performed mainly to demonstrate the exclusive effect of tumor load on host humoral response, reflect the possibility that host humoral response may decrease with increasing tumor volume. This is not in any way different from what has already been reported in other tumor models [15, 17, 19]. A drop in host humoral response as judged by AAC levels (Table 1) in spite of an increase in tumor load could have been due to: (a) excessive tumor antigen with insufficient production of specific antibodies; (b) an inhibitory effect of circulating AACs [26] or some other nonspecific immunosuppressive factors [1, 6, 29]; or (c) other hematogenous factors altering the pathophysiology of the tumor [10], resulting in the production of fewer antibodies and consequently decreased levels of AAC.

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