

ENHANCEMENT OF CYTOTOXICITY IN MOUSE REGIONAL LYMPH NODES BY LOCAL TISSUE INJECTION OF ACLACINOMYCIN A

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ABSTRACT

The effect of local tissue injection of activated carbon particle adsorbing aclacinomycin A (ACR-CH) on the cytotoxicity in popliteal, inguinal, paraaortic, and axillary lymph nodes was investigated in mice. Aclacinomycin (0.2mg/kg), a potent antineoplastic drug was injected subcutaneously into the footpad of mice in the form of ACR-CH or as an ACR solution. After a single injection of ACR-CH, the regional nodal cytotoxic response against mouse YAC-1 lymphoma cells was markedly increased and sustained for 7-10 days. The immune response was also increased after ACR solution but to a much lesser extent. These effects were found in popliteal, inguinal, and paraaortic lymph nodal effector cells but not in the more remote axillary nodes. Absorption of adherence cells largely abrogated the cytotoxic response.

These results suggest that ACR-CH did not impair but rather stimulated nodal immunoregulatory cells. Potentially ACR-CH may enhance immune responsiveness of regional lymph nodes after subcutaneous administration while concomitantly curtailing neoplastic growth in these same lymph nodes.

The effects of anti-cancer drugs on the immune response are complex. Both lymphocyte proliferation and tumor cell

growth are usually suppressed by administration of these agents, depending on the dose and timing of the drug (1-3). Aclacinomycin A (ACR), an antitumor antibiotic drug, however, seems to increase immunoreactivity (e.g., antibody formation and delayed-type hypersensitivity) in mice over a wide concentration range when injected intraperitoneally (4,5). Because parenteral or oral chemotherapy often depresses the immunoresponsiveness of the host thereby limiting drug therapeutic usefulness, we examined the regional nodal cytotoxic effect of activated carbon particles absorbing Aclacinomycin A (ACR-CH) after local tissue injection.

MATERIALS AND METHODS

Experimental preparation

Six week old male CDF₁ mice (23-25g BW) from Shizuoka Laboratory Animal Agriculture Cooperative Association, Shizuoka, Japan, were housed under standard conditions. Aclacinomycin A (ACR), an anticancer agent isolated from cultures of *Streptomyces galileus* was specially prepared with activated carbon particles (activated carbon Mitsubishi #1500AA, Mitsubishi Chemical Institutes Co., Ltd., Tokyo, Japan) using a specific surface area of 1480m²/g and a diameter of 20nm as an adsorbent. This activated carbon

compound of aclacinomycin A or ACR-CH was composed of 50mg/ml of activated carbon (Mitsubishi Chemical, see above), 20mg/ml of polyvinylpyrrolidone K-30 (PVP, Nakarai Chemicals Co., Ltd. Kyoto, Japan), and 10mg/ml of aclacinomycin A, as described previously (6). Target cells of YAC-1 derived from cultured mouse lymphoma (7) were cultured and cytotoxic experiments were performed in a humidified air atmosphere with 5% CO₂ using RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (complete medium).

Effector cells consisted of whole lymph nodal cells derived from popliteal, inguinal, paraaortic, and axillary lymph nodes, after administration of ACR-CH, ACR solution and saline. Ten mice were used at each data point and a popliteal lymph node, an inguinal lymph node, and a paraaortic lymph node were excised from each mouse. Appropriate regional lymph nodes of ten mice were excised, homogenized, and three kinds of single cell suspensions of each regional lymph nodal subgroup were prepared. In half of the cells suspensions, adherent cells were removed by incubation for 1 hr in medium on plastic culture dishes. The effector cells were counted and prepared in the complete medium to a concentration of 10⁷/ml.

Cytotoxic assay

Cytotoxicity was considered as the reaction in which the effector lymph nodal cells became bonded to and killed the YAC-1 target cells. The extent of killing by the effector cells was determined by the percentage of radioactivity released into the supernatant of damaged or killed target cells (see below).

Aliquots containing 2x10⁶ target cells were labeled with 100μCi of sodium ⁵¹chromate solution (New England Nuclear, Boston, Mass) for 1 hr in 1ml of medium as described by Koren et al (8). The labeled cells were washed three times to remove free ⁵¹Cr from the supernatant. The target cells were then diluted in complete medium to

10⁵/ml. A 100μl aliquot of target cells was added to each well of a MicroTest dish, along with an equal aliquot volume of a target cell suspension. The cell mixtures were incubated for 4 hrs at 37°C. A 100μl aliquot of the supernatant was then removed and counted in a gamma counter (Packard, Auto-Gamma 5000 series). Spontaneous release (background), computed from wells containing target cells and only complete media (no effector cells), was consistently below 5% of the total counts obtained from wells containing target cells lysed with 2% sodium dodecyl sulfate. Three wells were used and the average value was taken for each data point. Percent release was calculated as:

$$\frac{(\text{test cpm} - \text{spontaneous cpm})}{(\text{total cpm} - \text{spontaneous cpm})} \times 100$$

The assay was used to examine three ratios of effector to target cells (100:1; 50:1; and 25:1). Adherent cells were removed by incubation with medium on plastic dishes and non-adherent cells were added to the target cells and cytotoxicity retested.

Subset analysis of lymphocyte populations

Excised mouse popliteal, inguinal and paraaortic lymph nodes were homogenized and nodal lymphocyte subpopulations were examined with T cell differentiating antigens L3T4 and Lyt2. L3T4 is a mouse helper T cell differentiation antigen whereas Lyt2 is a mouse T cell differentiation antigen of suppressor/cytotoxic T cell. Cell suspensions were made in PBS containing 10% fetal calf serum and 0.1% sodium azide and adjusted to a concentration of 2x10⁶ cells/ml. In preliminary experiments, direct fluorescent staining of lymphocytes was observed by fluorescence microscopy. In subsequent experiments, the cell suspensions were analyzed using anti-L3T4 labeled with phycoerythrin (PE), anti-Lyt2 labeled with fluorescein isothiocyanate (FITC) using a fluorescence-activated cell sorter (FACS 400, Becton Dickinson Laboratory Systems, Mechelen, Belgium). The percentage of fluorescent and non-fluorescent cells were

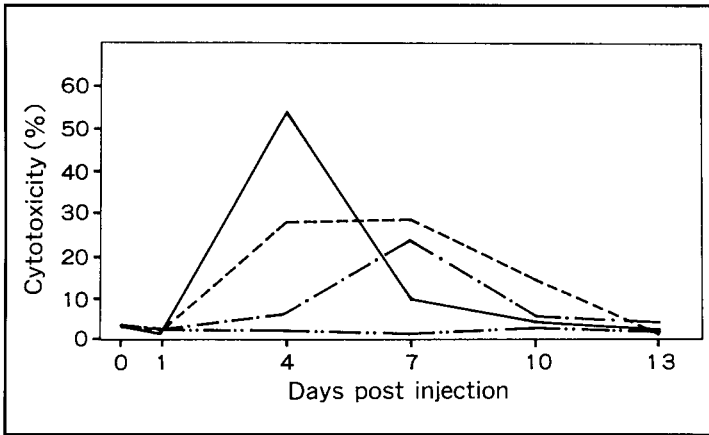


Fig. 1. Chronological changes of cytotoxicity of popliteal lymph node cells, paraaortic lymph node cells, inguinal lymph node cells and axillary lymph node cells after local injection of ACR-CH. Assays were performed at a ratio of whole lymph node cells to target cells of 100:1 on day 1, 4, 7, 10, and 13. —: popliteal lymph nodes; ----: paraaortic lymph nodes; - · - ·: inguinal lymph nodes; · · · ·: axillary lymph nodes.

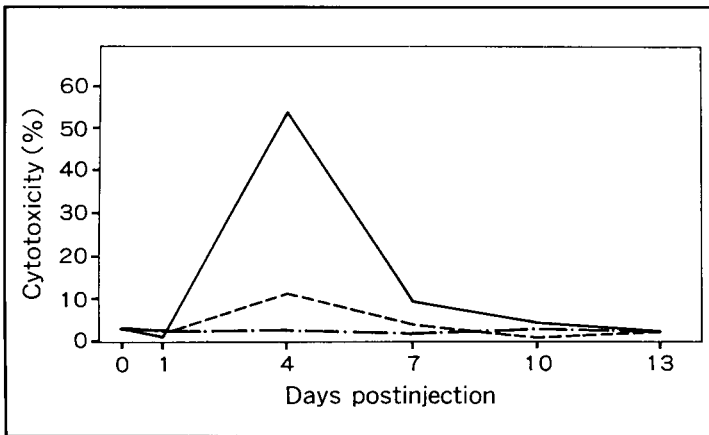


Fig. 2. Chronological changes of cytotoxicity of popliteal lymph node cells in ACR-CH injected, ACR solution injected, only saline injected, and untreated mice on day 1, 4, 7, 10, 13. —: ACR-CH 0.1mg/kg; ----: ACR solution 0.1mg/kg; - · - ·: control (saline).

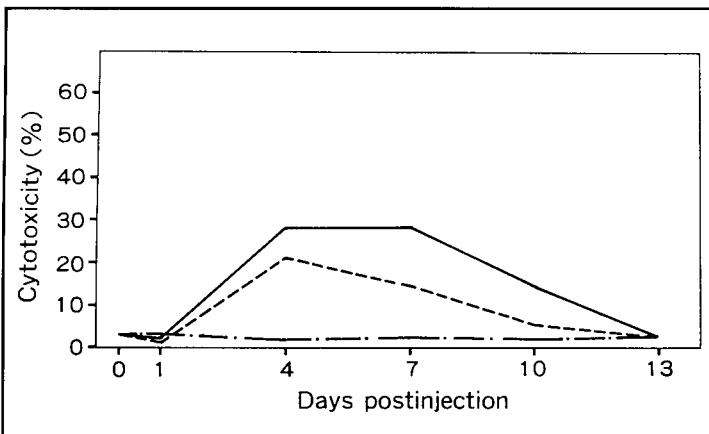


Fig. 3. Chronological changes of cytotoxicity of paraaortic lymph node cells in ACR-CH injected, ACR solution injected, only saline injected, and untreated mice on day 1, 4, 7, 10, and 13. —: ACR-CH 0.1mg/kg; ----: ACR solution 0.1mg/kg; - · - ·: control (saline).

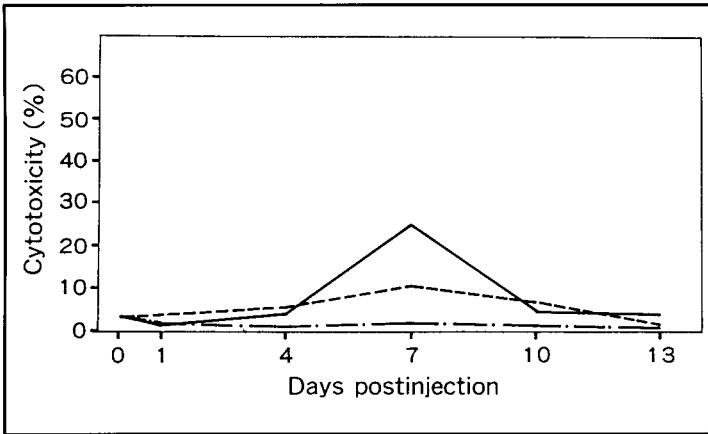


Fig. 4. Chronological changes of cytotoxicity of inguinal lymph node cells in ACR-CH injected, ACR solution injected, only saline injected, and untreated mice on day 1, 4, 7, 10, and 13. —: ACR-CH 0.1mg/kg; - - - - : ACR solution 0.1mg/kg; - · - · : control (saline).

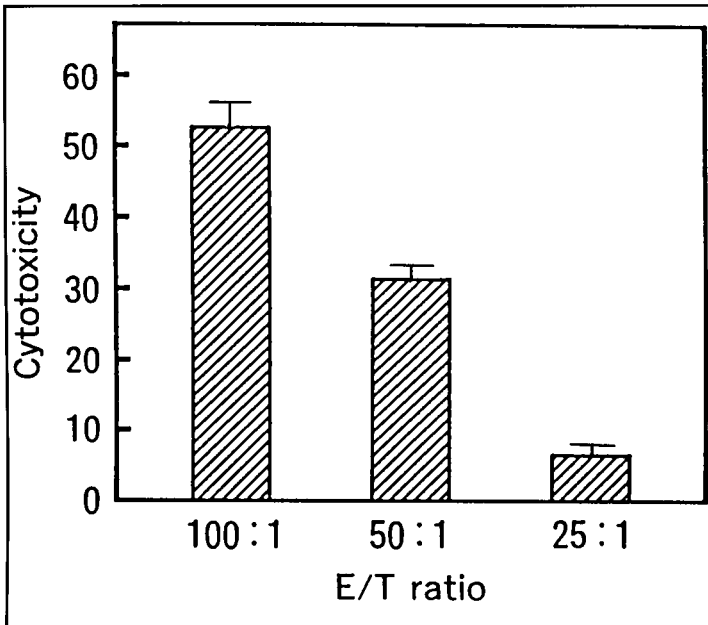


Fig. 5. Effect of varying the ratio of effector to target cells on the level of cytotoxicity. The assay was performed at a ratio of 100:1, 50:1, 25:1 with whole popliteal lymph node cells on day 4 after local injection of ACR-CH. Vertical bars show standard deviation of the mean.

calculated by a computer connected to the fluorescent cell sorter.

RESULTS

Cytotoxic assay

After injection of 0.2mg/kg ACR-CH into the mouse footpad, cytotoxicity of popliteal lymph nodal cells (using a ratio of 100:1 of whole lymph node cells to target

cells) was detectable at day 4 and persisted until day 7 (Figs. 1,2). Cytotoxicity of paraortic lymph nodal cells was also evident at day 4, and persisted at low levels until day 10 (Fig. 3). The cytotoxicity of the inguinal lymph node cells was also detectable at day 7 (Fig. 4). The cytotoxicity of axillary lymph nodal cells remote from the injection site was not detectable for two weeks after ACR-CH administration (Fig. 1). Injection of 0.1ml/kg ACR solution also

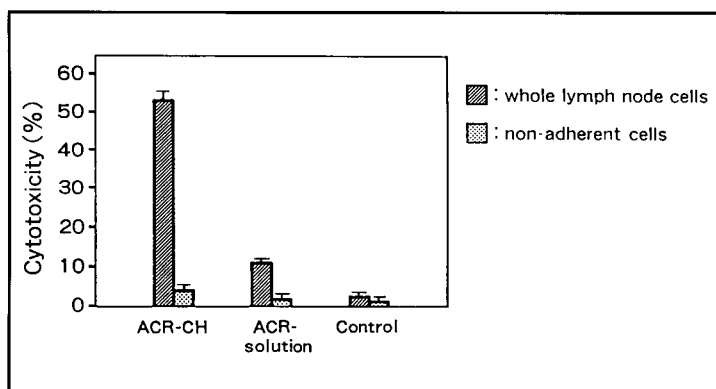


Fig. 6. Change of cytotoxicity of popliteal lymph node cells when adherent cells were excluded after local injection of ACR-CH, ACR solution and only saline on day 4. Vertical bars show standard deviation of the mean.

Table 1 Analysis of the lymphocyte subpopulation of regional lymphnode cells. Percentage of L:T, Lyt cells and ratio of L:T/Lyt; in ACR-CH, ACR-solution, only saline injected and untreated mice

		L:T (%) (helper T cell)	Lyt (%) suppressor/ cytotoxic cell	L:T/Lyt;
ACR-CH (0.2mg/kg)	popliteal LN	61.67	15.61	3.95
	inguinal LN	50.49	17.91	2.82
	paraaortic LN	32.44	10.73	3.02
ACR-sol (0.2mg/kg)	popliteal LN	42.09	14.97	2.81
	inguinal LN	48.14	17.56	2.74
	paraaortic LN	30.47	13.54	2.25
control (saline)	popliteal LN	54.44	25.01	2.18
control (untreated)	popliteal LN	53.14	24.04	2.21

enhanced the cytotoxicity of each lymph node cell, but the cytotoxicity was distinctly less than that of 0.2mg/kg ACR-CH (Fig. 1). Injection of saline was without effect on cytotoxicity of each subset of lymph nodal cells (Figs. 2-4).

Using three ratios of effector to target cells, the magnitude of cytotoxicity increased in proportion to the ratio, with a cell ratio of 100:1 for 4 hr yielding the maximal cytotoxicity. This assay was performed three times and vertical bars show standard deviation (Fig. 5). When adherent cells were removed by incubation in medium on the plastic dishes and only non-adherent cells added to the target cells, the cytotoxicity response was sharply decreased (Fig. 6). This assay was also performed three times, and statistical analysis was performed by student-t test. Statistical differences were found between

cytotoxicity of whole lymph nodal cells and non-adherent cells both in ACR-CH treated group and ACR solution treated group ($p < 0.01$). Vertical bars show standard deviation (Fig. 6).

Lymphocyte subsets

Local injection of ACR-CH and ACR solution produced a decrease in the percentage of Lyt2⁺ cells with the larger decrease in ACR-CH treated mice. In other words, the ratio of L3T4⁺/Lyt2⁺ cells was highest in the ACR-CH treated mouse (Table 1).

DISCUSSION

Increased host immune reactivity after the administration of anticancer agents is unusual. ACR, however, seems to augment antibody formation and delayed-type hypersensitivity over a wide concentration range in mice, possibly by inhibiting suppressor cells selectively or activating macrophages and helper T cell function (4,5,9).

We prepared and tested a new dosage form of this drug consisting of small activated carbon particles, which adsorb Aclacinomycin A (and also Adriamycin, Mitomycin C or Pepleomycin) in order to deliver a greater amount of an anticancer agent to regional lymph nodes directly by lymphatic absorption. Previous animal

experiments and clinical testing showed that the concentration of agents in the lymph nodes after local tissue injection was maintained at a higher level and was more effective in eradicating lymph nodal metastases in the carbonized dosage form than in a simple solution (10,11). In our previous study, ACR-CH appeared in the lymphatics soon after tissue injection with ACR nodal activity maintained much higher than after administration of ACR solution (6). On the other hand, a higher concentration of an anticancer agent may favorably destroy cancer cells but unfavorably impair normal nodal lymphocytes and macrophages thereby depressing local immunity.

In the present experiments in mice, the local tissue administration of ACR-CH enhanced cytotoxicity in the draining regional lymph nodes including popliteal, inguinal, and paraaortic (but not remote axillary) lymph nodes. The ACR solution also enhanced cytotoxicity in these lymph nodes but to a much lesser degree and shorter duration than ACR-CH. These findings suggest that ACR-CH, and to a lesser extent ACR solution, does not impair but rather stimulates immunoregulation.

The cytotoxic effect of ACR-CH was weakened by removal of adherent cells. This finding suggests that adherent cells are important for cytotoxicity in regional lymph nodes and that adherent cells are activated by ACR-CH.

Clinically, anticancer agents such as ACR-CH when administered by local tissue injection, may be expected to enhance cytotoxicity and killing of lymph nodal tumors while concomitantly stimulating nodal immunity.

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