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# Selective Immunosuppressive Action of a Factor Produced by Colon Cancer Cells[1]

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## ABSTRACT

**A soluble substance (HT29 factor) produced by HT29 colon cancer cells markedly suppresses mitogen-induced T-cell proliferation and interleukin-2 production. In this study the range of T-cell functions susceptible to the inhibitory effects of the HT29 factor was evaluated. Serum-free conditioned medium was collected from confluent cultures of HT29 cancer cells, concentrated, and subjected to gel filtration chromatography and anion exchange chromatography, resulting in 24.4-fold purification of the HT29 factor with 31% yield. This factor abolished the development of lymphokine-activated killer cells when present during activation of peripheral blood lymphocytes by interleukin-2 but did not affect the lysis of target cells by normal effectors when added in the lysis stage only. The HT29 factor did not affect the generation of concanavalin A-induced suppressor lymphocytes, even though it markedly inhibited synthesis of DNA, RNA, and protein as well as expression of the CD25 (Tac) antigen during mitogen activation of T-cells. The HT29 factor itself did not induce suppressor cells. These results indicate that the immunosuppressive action of the HT29 factor is selective.**

## INTRODUCTION

The mechanisms by which tumors evade the immune system of the host are unclear. Malignant cells may survive only in those individuals whose lymphocytes are in some way defective. Alternatively, some cancer cells may not be recognized as foreign by the lymphocytes of the host, never activating the immune system. A third possibility is that tumors inhibit lymphocyte function.

Immunosuppressive factors have been described in the serum or malignant effusions of cancer hosts (1–3). Whether these substances originated from the tumor or the lymphocytes of the host is unclear. Suppressor factors also have been isolated from fresh tumors as well as from the medium conditioned by the growth of cancer cell lines. These substances, ranging in $M_r$ from 7000 to >200,000, markedly inhibited mitogen-induced T-cell proliferation (4–8).

We previously described an immunosuppressive substance produced by the HT29 colon cancer line (4). This protease-sensitive compound, with an $M_r$ of 56,000 and an isoelectric point of 7.9, blocks T-cell proliferation by affecting both lymphocyte activation and cell division (4). In the present report the effects of partially purified HT29 factor on the generation of cytotoxic and suppressor lymphocytes are evaluated (9).

## MATERIALS AND METHODS

**Preparation of the HT29-conditioned Medium.** The HT29 cell line (American Type Culture Collection, Rockville, MD) was grown to confluence in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutatmine, and 1% antibiotic-antimycotic solution (Gibco). Cultures were tested every 3 months for *Mycoplasma* contamination using the Hoechst stain (Crescent Chemicals, Hauppauge, NY). To make conditioned medium, 4.5 ml of serum-free RPMI-1640 medium was added to each 75-cm$^2$ tissue culture flask containing confluent monolayers of HT29 cells and incubated for 3 days at 37°C, 95% air-5% $CO_2$. This supernatant contained about 10 units/ml of inhibitory activity. One unit of activity is defined as the amount of activity which, when added to 1 ml of medium, results in 50% inhibition of mitogen-induced T-cell proliferation.

**Partial Purification of the HT29 Factor.** The HT29 supernatant was pooled, filtered, and concentrated about 100-fold using a collodion bag apparatus (Schleicher & Schuell, Keene, NH). The concentrated sample was then applied to a column (1.5 x 100 cm) of Ultrogel AcA 44 (LKB Instruments, Inc., Gaithersburg, MD) and eluted with 0.4 M NaCl phosphate-buffered saline as described previously (4). The fractions with inhibitory activity were pooled, concentrated, dialyzed in 20 mM Tris-HCl (pH 8.6), 20 mM NaCl, and then applied to a DEAE-cellulose column (Whatman, Ltd., Maidstone, Kent, United Kingdom), equilibrated with the same buffer. The inhibitory activity was found in the unbound eluate.

**Immunofluorescence.** Indirect immunofluorescence was performed on PBL[3] ($1 \times 10^6$) using antibodies to CD25 (Tac) (a generous gift of Dr. Warner Greene, NIH, Bethesda, MD), CD4, or CD8 (Coulter Immunology, Hialeah, FL), followed by goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Coulter) (4, 10). At least 200 cells were counted by visual observation using a Zeiss fluorescence microscope to determine the percentage of positive cells (those demonstrating clearly visible epifluorescence when compared to a negative control stained with goat anti-mouse IgG conjugated to fluorescein isothiocyanate alone).

**Measurement of DNA, RNA, and Protein Synthesis.** PBL ($2 \times 10^5$/0.2 ml) were stimulated by 18 μg/ml Con A (ICN Pharmaceuticals Inc., Cleveland, OH) for 2 days at 37°C, 95% air-5% $CO_2$, with or without 4 units/ml of partially purified HT29 factor in microtiter wells. Eighteen h before harvest, the cultures were pulsed with 1 μCi of [$^3$H]Tdr (6.7 Ci/mmol), 0.01 μCi of [$^3$H]Udr (51 Ci/mmol), or 0.1 μCi of [$^3$H]-leucine (42 Ci/mmol) (ICN Radiochemicals, Irvine, CA). Those cultures labeled with [$^3$H]Tdr or [$^3$H]Udr were harvested (PHD cell harvester, Cambridge Technologies, Watertown, MA), while those labeled with [$^3$H]leucine were terminated by TCA (Sigma) precipitation. In brief, cells were resuspended in cold 6% TCA to precipitate protein and then centrifuged. The pellets were washed with cold 6% TCA, and then dissolved in 0.1 N NaOH. Scintillation fluid was added (Scintiverse, Fisher Scientific, Fair Lawn, NJ) and radioactivity counted.

**Con A-induced Suppressor Assay.** PBL ($2 \times 10^6$/ml) were incubated with or without 18 μg/ml Con A for 2 days at 37°C, 95% air-5% $CO_2$, in the presence or absence of 4 units/ml partially purified HT29 factor. In some experiments, T-cells were obtained by nylon wool columns, and CD4$^+$ or CD8$^+$ T-cell subsets were isolated by antibody and complement lysis, as described previously (10). The cells were then treated with 66 μg/ml of mitomycin-C (Sigma) for 30 min at 37°C and washed thoroughly. Each cell type ($1 \times 10^5$/0.1 ml) was then cultured with or without a final concentration of 6 μg/ml Con A for 4 days (indicator culture). The cultures were pulsed with 1 μCi [$^3$H]Tdr for the last 18 h of culture and harvested, and the radioactivity (cpm) was measured. The percentage suppression of proliferation was calculated

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[3] The abbreviations used are: PBL, peripheral blood lymphocytes; Con A, concanavalin A; Tdr, thymidine; Udr, uridine; TCA, trichloroacetic acid; NK, natural killer; LAK, lymphokine-activated killer; IL-2, interleukin-2.

as follows:

$$\% \text{ Suppression} = \left[1 - \left(\frac{\text{cpm } (T_{Sm} + T_F + \text{Con A}) - \text{cpm } (T_{Sm} + T_F)}{\text{cpm } (T_{Cm} + T_F + \text{Con A}) - \text{cpm } (T_{Cm} + T_F)}\right)\right] \times 100$$

where $T_{Sm}$ = cells initially cultured in Con A and then treated with mitomycin-C (suppressor cells), $T_{Cm}$ = cells initially cultured in medium alone and then treated with mitomycin-C (control cells), and $T_F$ = fresh allogeneic PBL cultured with or without Con A (indicator cells).

**Cytotoxicity Assays.** NK activity was measured by a standard chromium release assay. Fresh PBL were mixed with $^{51}$Cr-labeled K562 erythroleukemia cells (American Type Culture Collection) at a 25:1 effector:target cell ratio in the presence or absence of 4 units/ml partially purified HT29 factor. The targets were labeled by incubating K562 cells ($3 \times 10^6$/0.1 ml) with 150 $\mu$Ci $Na_2{}^{51}CrO_4$ (1 mCi/ml) (New England Nuclear, Boston, MA), as described previously (11). To obtain the spontaneous and maximal releases, targets were incubated in medium alone or in 2% cetrimide solution (Fisher).

After mixing effector and target cells in triplicate in round-bottom microwells (0.2 ml total volume), the plate was centrifuged at 400 × *g* for 5 min and then incubated at 37°C for 4 h in 95% air-5% $CO_2$. After incubation, the plate was centrifuged again; 0.1 ml of supernatant was removed from each well and its radioactivity counted. The percentage of cytotoxicity was calculated as:

$$\% \text{ Cytotoxicity} = \left(\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}}\right) \times 100$$

The spontaneous release was always <20% of the maximal release.

LAK cells were generated by culturing PBL ($2 \times 10^6$/ml) at 37°C for 7 days with 25 units/ml of recombinant IL-2 (Amgen Corp., Thousand Oaks, CA; units as defined by producer) with or without 4 units/ml partially purified HT29 factor. The ability of these effectors to lyse $^{51}$Cr-labeled K562 or HT29 cells was determined using an effector:target ratio of 25:1.

**Statistical Analysis.** Control and test values were analyzed by calculating arithmetic means and SEM for each set of data and by comparing data using the paired Student's *t* test.

## RESULTS

**Partial Purification of HT29 Factor.** The results of purification of the HT29 factor are summarized in Table 1. Approximately a 6-fold increase in specific activity was attained by applying the concentrated serum-free conditioned medium on a column of Ultrogel AcA 44. The active material was eluted at the fraction corresponding to $M_r$ 56,000. An additional 4-fold increase in purification was obtained by the DEAE-cellulose step. The overall yield of activity was 31%.

**Effect of the HT29 Factor on DNA, RNA, and Protein Synthesis by PBL.** To determine whether the HT29 factor affects the synthesis of DNA, RNA, or protein, PBL were stimulated with phytohemagglutinin for 5 days and supplemented with either 4 units/ml partially purified HT29 factor or additional medium at various intervals from 0 to 6 h before adding [$^3$H]Tdr, [$^3$H]Udr, or [$^3$H]leucine. All cultures were harvested together on the fifth day, 18 h after the labeled substances were introduced. Inhibition of DNA, RNA, and protein synthesis decreased proportionately as the time of exposure to the HT29 factor was reduced (Fig. 1).

**Effect of the HT29 Factor on Cytotoxic Lymphocyte Functions.** Partially purified HT29 factor was added during either the development of LAK cells or subsequently during the lysis of target cells (Table 2). When the HT29 factor was present during the 7-day culture of PBL with IL-2, there was a marked decrease in the development of cytotoxic lymphocytes directed against HT29 cancer cells (1 ± 1% cytotoxicity *versus* a control of 18 ± 3%, $P < 0.05$). The HT29 factor had no effect on the ability of control LAK cells to lyse the targets. Similarly, NK activity by PBL directed against the K562 target was unaffected by the HT29 factor (24 ± 9% in the presence of 4 units/ml of HT29 factor *versus* a control of 23 ± 6%).

**Effect of the HT29 Factor on Suppressor Lymphocyte Functions.** The generation of Con A-induced suppressor cells was examined with or without the HT29 factor in the culture. When partially purified HT29 factor (4 units/ml) was present during the 2-day culture of PBL with Con A, it markedly reduced the synthesis of DNA, RNA, and protein as well as IL-2 receptor expression on PBL (detected by anti-CD25 antibody) (Table 3). The generation of suppressor cells was unaffected, however. The presence of these cells, after Con A stimulation, was determined by the ability of such cells to inhibit the mitogen-induced proliferation of PBL in the indicator culture. Even when decreasing numbers of suppressor cells (from $1 \times 10^5$ to $2.5 \times 10^4$) were added to the indicator cultures, suppression was reduced to the same extent whether or not the Con A suppressor cells were generated in the presence of the HT29 factor (not shown). The HT29 factor alone, without mitogen, did not trigger the development of suppressor T-cells (4).

To determine whether the HT29 factor favored the development of lymphocytes with the CD8$^+$ cytotoxic/suppressor phenotype after mitogen stimulation, PBL were incubated for 2 days in Con A with or without 4 units/ml of the HT29 factor, and the proportion of CD4$^+$ and CD8$^+$ cells were then quantified by immunofluorescence. The percentage of T-cells in each subset was the same whether T-cell activation was conducted in the presence of the HT29 factor (41 ± 10% CD4$^+$ cells and 28 ± 8% CD8$^+$ cells) or in its absence (45 ± 8% CD4$^+$ cells and 24 ± 11% CD8$^+$ cells, $n = 3$).

The possibility that the HT29 factor may permit preferential activation of the CD8$^+$ subset by Con A was tested by culturing T-cells in Con A for 2 days with or without the HT29 factor. CD4$^+$ and CD8$^+$ subsets were isolated by antibody and complement lysis, and the percentage of CD25$^+$ cells in each subset was then measured by immunofluorescence. Normal Con A stimulation resulted in 59 ± 12% of CD4$^+$ cells and 49± 15% of CD8$^+$ cells expressing CD25. When Con A activation occurred in the presence of the HT29 factor, the percentage of CD25$^+$ cells in each subset was lower by an equal proportion (43 ± 15% of CD4$^+$ cells and 29 ± 13% of CD8$^+$ cells), indicating that the HT29 factor did not selectively affect one

Table 1 *Purification of the HT29 factor*

| Step | Volume (ml) | Concentration of protein ($A_{280}$ units) | Activity | | Purification (fold) | Recovery (%) |
|---|---|---|---|---|---|---|
| | | | Total (units) | Specific activity (units/$A_{280}$ unit) | | |
| Serum-free supernatant | 125.0 | 0.0820 | 1350 | 132 | 0.0 | 100 |
| Ultrogel AcA 44 | 10.0 | 0.1235 | 945 | 765 | 5.8 | 70 |
| DEAE-cellulose | 6.0 | 0.0217 | 419 | 3217 | 24.4 | 31 |

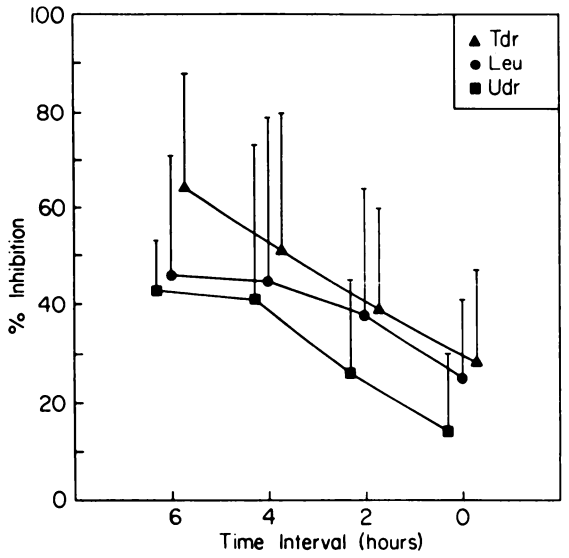


Fig. 1. The inhibition of DNA, RNA, or protein synthesis of proliferating PBL by the HT29 factor added at different time intervals before pulsing with [$^3$H] Tdr, [$^3$H]Udr, or [$^3$H]leucine (Leu). PBL ($5 \times 10^4$/0.1 ml) were cultured with phytohemagglutinin (1 μg/ml) for 5 days. On day 4, 0.1 ml of RPMI-1640 medium with or without 4 units/ml of partially purified HT29 factor was introduced to each well 6, 4, 2, or 0 h before the addition of [$^3$H]thymidine, [$^3$H] uridine, or [$^3$H]leucine. Cultures were terminated 18 h after the addition of the labeled substances using a cell harvester for the cultures containing [$^3$H]Tdr or [$^3$H]Udr or using trichloroacetic acid precipitation for the cultures containing [$^3$H]leucine. Inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{cpm in medium}) - (\text{cpm with HT29 factor})}{(\text{cpm in medium})} \times 100$$

*Points*, means; *bars*, SD.

Table 2 *Effect of the HT29 factor on lymphokine-activated killing*

| Effectors | % Cytotoxicity |
|---|---|
| PBL incubated with IL-2 alone[a] | |
| Assay in medium | 18 ± 3 |
| Assay in 4 units/ml HT29 factor | 15 ± 3 |
| PBL incubated with IL-2 and 4 units/ml HT29 factor | |
| Assay in medium | 1 ± 1[b] |
| Assay in 4 units/ml HT29 factor | 0 ± 0[b] |

[a] PBL ($2 \times 10^6$/ml) were incubated for 7 days with IL-2 (25 units/ml) in presence or absence of 4 units/ml of partially purified HT29 factor and then tested for cytotoxic activity against HT29 colon cancer cells using the chromium release assay.

[b] These values are significantly less than their controls, "PBL incubated with IL-2 alone" ($P < 0.05$).

T-cell subset over the other. Thus, this factor inhibited Con A-induced synthesis of DNA, RNA, and protein as well as IL-2 receptor expression by T-cells, but it still permitted the generation of Con A-stimulated suppressor T-cells.

## DISCUSSION

The HT29 factor, derived from a colonic adenocarcinoma cell line, has a potent antiproliferative effect. It inhibits equally DNA, RNA, and protein synthesis of mitogen-stimulated T-cells, even when added just 18 h before terminating the cultures. The ability of the HT29 factor to block the development, but not the action, of cytotoxic lymphocytes is consistent with this antiproliferative effect. It inhibits the induction of LAK cells by IL-2, an event that is dependent upon lymphocyte proliferation (12), while it does not inhibit the lysis of target cells by LAK or NK cells, an event that is independent of lymphocyte proliferation.

Con A-induced suppressor T-cell activity develops in the presence of the HT29 factor even though the synthesis of DNA, RNA, and protein, as well as the expression of the CD25 antigen on activated T-cells, is inhibited. It is unclear whether cell division is required for the development of suppressor cells. Some investigators, but not others, found that irradiation or mitomycin-C treatment of lymphocytes before Con A activation abolishes suppressor activity, suggesting that proliferation is required during the induction phase (13–15). However, cyclosporin, an inhibitor of the early events of T-cell proliferation, favors the induction of suppressor rather than cytolytic effector cells in a mixed lymphocyte reaction (16, 17). Similarly, heat-treated stimulator cells induce suppressor lymphocytes in a mixed lymphocyte reaction despite minimal [$^3$H]Tdr uptake (18).

Con A-induced suppression may be mediated by a protease-sensitive factor produced after 24 h of lectin stimulation (19, 20). This finding is supported by the inability of lymphocytes to develop into Con A suppressor cells when treated with inhibitors of protein synthesis (15). In contrast, the HT29 factor reduces protein synthesis but permits the normal development of suppressor cells. It is possible that its action may be selective, such that the production of suppressor substances is not blocked. Alternatively, this may just be a consequence of the reversibility of the inhibitory action of the HT29 factor: when PBL are removed from the factor, they can proliferate normally (4).

Some investigators found that suppressor activity is carried out by those activated T-cells that bear the CD25 antigen (21). Suppression may be due to the absorption of IL-2 by available IL-2 receptors (22), although this idea has been refuted (23).

Table 3 *Effect of the HT29 factor on the generation of Con A-induced suppressor cells*

| | DNA synthesis[a] ([$^3$H]Tdr uptake, cpm) | RNA synthesis[a] ([$^3$H]uridine uptake, cpm) | Protein synthesis[a] ([$^3$H]leucine uptake, cpm) | IL-2 receptor expression[b] (% CD25$^+$ cells) | Suppressor activity[c] (% suppression) |
|---|---|---|---|---|---|
| PBL + Con A[d] | 63,054 ± 2,944 | 18,219 ± 2,394 | 31,414 ± 1,303 | 48 ± 18 | 67 ± 14[e] |
| PBL + Con A + HT29 factor | 731 ± 221[f] | 2,604 ± 738[f] | 9,144 ± 1,303[g] | 25 ± 12[g] | 60 ± 13[e] |

[a] DNA, RNA, or protein synthesis was detected by adding [$^3$H]Tdr, [$^3$H]uridine, or [$^3$H]leucine 30 h after initiating the cultures and measuring incorporation of the label 18 h later as described in "Materials and Methods."

[b] IL-2 receptor expression was estimated by the % CD25$^+$ cells measured by immunofluorescence after the 48-h incubation.

[c] Suppressor activity of Con A-stimulated PBL, treated with mitomycin-C, on the proliferation of fresh PBL was determined as described in "Materials and Methods."

[d] PBL were stimulated with Con A (18 μg/ml) for 48 h in medium alone or supplemented with 4 units/ml of partially purified HT29 factor.

[e] NS, not significant.

[f] $P < 0.001$.

[g] $P < 0.05$.

The HT29 factor causes a decline in CD25 antigen expression without a reduction in suppressor cell generation, suggesting that IL-2 receptors are not necessary for suppression.

Whether or not the HT29 factor is secreted by other tumors is unclear. Its physicochemical features distinguish it from most other immunosuppressive factors found in supernatants of tumor lines or in fresh tumor extracts (4). However, the HT29 factor may have homologous regions in common with other tumor-derived factors. This laboratory is in the process of developing antibodies reactive to the HT29 factor which can be used to test other tumor cells or secretions for the presence of this same factor.

The HT29 factor may favor tumor survival in the host. It inhibits the development of cytotoxic lymphocytes but permits the generation of suppressor lymphocytes. A study of the selective immunosuppressive action of this factor may provide information concerning the interaction of tumors with the immune system.

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