CHARACTERIZATION OF AN IMMUNOSUPPRESSIVE FACTOR DERIVED FROM COLON CANCER CELLS[1]

ELLEN C. EBERT,[2]* ARTHUR I. ROBERTS,* SEAN M. O'CONNELL,[+] FREDIKA M. ROBERTSON,[+] AND HIDEAKI NAGASE*

From the *Department of Medicine, the +Center for Advanced Biotechnology and Medicine, and the +Department of Surgery, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ

The colon cancer cell line, HT29, produces a soluble substance (HT29 factor) that blocks mitogen-induced T cell proliferation and the production of interleukin 2 (IL 2). Inhibition of T cell proliferation by the HT29 factor is reversible and is not due to a decline in cell viability or an alteration in the kinetics of T cell proliferation. It occurs even when the HT29 factor is added only 24 hr before terminating the T cell cultures, indicating that the factor affects cell division after activation of T cells has already occurred. No inhibitory activity was found in medium conditioned by human colonic epithelial cells or fibroblasts. The factor has an apparent m.w. of 56,000 and an isoelectric point of 7.9. It is sensitive to endopeptidases, heating to 56°C, and extremes of pH. The HT29 factor also suppresses IL 2 production by T cells. However, low IL 2 availability alone cannot account for the suppressive effect of the factor on T cell proliferation, because the addition of exogenous IL 2 does not reverse the inhibition. This block in IL 2 responsiveness is not primarily due to a decrease in IL 2 receptors because Tac expression on activated T cells is minimally decreased during a 24-hr exposure to the HT29 factor. In addition, IL 2-induced proliferation of mitogen-activated T cells is inhibited only slightly by the HT29 factor, indicating that a block in the interaction of IL 2 with its receptor is not its main mechanism of action. Thus the inhibition of T cell proliferation is likely to be due primarily to a mechanism independent of IL 2.

The mechanisms by which malignant cells avoid destruction by the immune system of the host are unclear. One contributing factor may be the immunosuppression found in cancer hosts. Whether this precedes or follows tumor growth is unknown. Tumors may have a propensity to develop in patients with mildly abnormal immune systems. Certainly, patients with severe immunodeficiencies, such as the acquired immunodeficiency syndrome or ataxia-telangiectasia, have an increased incidence of malignancies. Alternatively, established tumors may suppress the immune system in the host. For example, soluble suppressor factors have been found in the host's serum (1, 2), malignant ascites (3), or tumor tissue (4-6). In the serum, such factors include circulating tumor antigens, immune complexes, lipoproteins, and acute-phase reactants (1, 2). However, there are disadvantages to the study of factors recovered from the host's tissues. For example, the source of such factors is often uncertain, whether they are from the tumor cells or the host's lymphocytes. Even the tumor itself contains tumor-infiltrating lymphocytes that could contribute suppressor activity. Second, starting material obtained from patients may be heterogeneous due to differences among individuals or tumors. Third, purification of a factor from such sources may be difficult due to the large number of extraneous proteins.

Several investigators have studied suppressor substances in medium conditioned by cancer cell lines in vitro. Such soluble factors from a variety of tumor types had m.w. ranging from 7,000 to 200,000 and suppressed a variety of T cell functions (7-10). This study examines a factor derived from a human colon cancer cell line, HT29, which markedly suppressed mitogen-induced peripheral blood T cell proliferation and interleukin 2 (IL 2) production. Its possible mechanism of action has been proposed, and some of its physicochemical properties have been described.

MATERIALS AND METHODS

Materials. Sources of reagents were as follows: RPMI 1640 medium, L-glutamine, antibiotic-antimycotic, Hanks' buffered salt solution (GIBCO Laboratories, Grand Island, NY); fetal calf serum (FCS) (Microbiological Associates Bioproducts, Walkersville, MD); azocasein, hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), trypsin, α-chymotrypsin, pancreatic elastase (bovine), soybean trypsin inhibitor (STI), thermolysin (Sigma Chemical Co., St. Louis, MO); proteinase K (Boehringer Mannheim, West Germany); Ficoll (Bionetics Laboratory Products, Kensington, MD); Percoll (Pharmacia Fine Chemicals, Piscataway, NJ); phytohemagglutinin-P (PHA) (Burroughs Wellcome Co., Greenville, NC); concanavalin A (Con A) (ICN Pharmaceuticals, Inc., Cleveland, OH); [3H]thymidine (3H-Tdr) (2 Ci/mmol; Schwarz/Mann, Spring Valley, NY); collodion bags (Schleicher and Schuell, Inc., Keene, NH); Affi-Gel 10 (BioRad, Rockville Center, NY); carrier ampholytes, Ultrogel AcA 44, 110-ml electrofocusing column (LKB Instruments, Inc., Gaithersburg, MD); anti-Tac antibody (a gift of Dr. Warner Greene, National Institutes of Health); anti-T9 antibody (Ortho Diagnostics, Raritan, NJ); goat anti-(mouse Ig) IgG conjugated to fluorescein isothiocyanate (GAM-FITC) (Coulter Immunology, Hialeah, FL). The units of ultrapure interleukin 1 (IL 1) (Genzyme, Boston, MA) and recombinant IL 2 (Amgen Corp., Thousand Oaks, CA) were those defined by the producers.

Cell cultures. The HT29, A375, or IMR-90 cell line (American Type Culture Collection, Rockville, MD) was cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 10 mM HEPES, 2 mM L-glutamine, and 1% (v/v) antibiotic-antimycotic and was grown to confluency in 25 cm² flasks. To make conditioned

medium, 1.5 ml of RPMI 1640 medium with or without FCS was added to flasks with confluent cultures and was incubated for 3 days and then was pooled, was filtered, and was stored at −70°C until use (HT29, A375, or IMR-90 supernatant). The cell lines were screened for Mycoplasma contamination every 3 mo by using the Hoechst stain (Crescent Chemicals, Hauppauge, NY) (11). One unit of inhibition in the HT29 supernatant was defined as that amount of activity that produced 50% suppression of PHA-induced T cell proliferation.

Epithelial cells were prepared from freshly minced pieces of human colonic mucosa obtained from two surgical specimens containing diverticulitis. The mucosa was incubated with 0.75 mM EDTA in calcium- and magnesium-free Hanks' buffered salt solution in a shaking hot (37°C) water bath for 135 min. Cells in the buffer solution were separated on a Percoll gradient (12). The 30% layer contained over 90% epithelial cells with viability of at least 85% (13). These cells were cultured for 3 days (2×10^6/ml) in RPMI 1640 medium with 10% FCS, and the conditioned medium was collected (epithelial cell supernatant). The amount of carcinoembryonic antigen in each supernatant was determined by an enzyme-linked immunoadsorbent assay (sensitivity, 0.05 ng/ml); α-fetoprotein levels were measured by a radioimmunoassay (sensitivity, 7 ng/ml).

Proliferative assays. Peripheral blood lymphocytes (PBL) were isolated by layering heparinized whole blood over a Ficoll density gradient, and T lymphocytes were separated by nylon wool columns. T cells or PBL (5×10^4) were cultured for 5 days in flat-bottomed microwells at 37°C, 95% air-5% CO_2 in 0.2 ml serum-supplemented RPMI 1640 medium containing 1 μg/ml PHA or 18 μg/ml Con A. Twelve hours before terminating the cultures, 1 μCi of ^{3}H-Tdr was added to each well, and ^{3}H-Tdr incorporation was measured (12).

IL 2 production. T cells or PBL (1×10^6/ml) were cultured in 1 μg/ml PHA for 24 hr, and the medium was tested for IL 2 activity by using a CTLL II line (12). The units of IL 2 were determined by probit analysis at 50% of the IL 2 standard (14). (The standard was 1 U/ml of recombinant IL 2.)

Immunofluorescence. Indirect immunofluorescence was performed on PBL (1×10^6) by using antibodies to Tac or T9 followed by goat anti-mouse Ig-fluorescein isothiocyanate (GAM-FITC) (12). At least 200 cells were counted by using a fluorescence microscope (Zeiss) to determine the percentage of positive cells. The flow cytometry data was collected by using a Coulter Epics 752 Flow Cytometer/ Cell Sorter equipped with a 5 W argon ion laser run at 488 nm excitation wavelength by using 350 mW of power and a 457/502 nm and 525 nm filter set. The coefficient of variation for integrated green fluorescence was 1.16. A minimum of 20,000 cells, gated on forward angle and right angle light scatter, were accumulated for each histogram. The data analysis was performed by using the Coulter Easy 88 system in conjunction with the IMMUNO and QUAD-STAT programs. For analysis of IL 2 receptor (Tac$^+$) fluorescence, the emission was measured in relative units, corresponding to channel number. Log intensity of fluorescence was analyzed by using the mean channel number for each histogram, as well as a determination of the percentage of positive and negative staining cells. The percentage of Tac$^+$ cells was calculated in comparison with a negative control of GAM-FITC-stained cells.

Sensitivity of HT29 factor to endopeptidases. Serum-free HT29 supernatant was treated with 100 μg/ml trypsin or α-chymotrypsin for 30 min at 37°C. The reaction was stopped with 400 μg/ml soybean trypsin inhibitor. Aliquots of the supernatant were also incubated for 30 min at 37°C with 100 μg/ml of elastase, thermolysin, or proteinase-K, each of which was coupled to Affi-Gel 10 beads. The beads were then removed by filtration. The amount of enzyme coupled to Affi-Gel 10 was quantified by its activity against 6% (w/v) azocasein (15). The inhibitory activities (U/ml) of endopeptidase-treated and untreated samples of HT29 supernatant were determined by their abilities to suppress PHA-induced T cell proliferation.

Isoelectric focusing. A sucrose gradient (5 to 55% w/v) was prepared with 3% (v/v) carrier ampholytes with a pH range of 3 to 10 in a 110-ml LKB ampholine column. Serum-free HT29 supernatant in 5 ml (concentrated 20-fold with a collodion bag apparatus) was placed in the center of the sucrose gradient. Isoelectric focusing was performed at 700 V (constant) for 24 hr at 4°C. The column contents were collected in 2.5 ml fractions at a flow rate of 1.5 ml/min, and pH and absorbance at 280 nm of each fraction were measured at 25°C.

Statistical analysis. Control and test values were analyzed by calculating arithmetic means and SEM for each set of data and by comparing paired data by using the Student *t*-test.

RESULTS

Effect of tumor-derived soluble factors on T cell proliferation. T cell proliferation in response to PHA or Con

A was markedly suppressed when 50% of the medium consisted of supernatants from either the HT29 or A375 cancer lines (Table I). In contrast, PHA-induced T cell proliferation was unaffected by the medium conditioned by normal human colonic epithelial cells or a fibroblast line, IMR-90. Similar concentrations of carcinoembryonic antigen were found in the HT29 and normal epithelial cell supernatants (41 ± 1 and 37 ± 1 ng/ml, respectively), but α-fetoprotein was not detected in the HT29 supernatant. To determine whether the inhibitory effect of the HT29 supernatant is due to a shift in kinetics, T cells were cultured in PHA with or without HT29 supernatant, and the resulting proliferation was measured after 1, 3, 5, 7, or 10 days of incubation. T cell proliferation was suppressed by the HT29 supernatant regardless of the duration of incubation (data not shown). To eliminate the possibility that the inhibitory effect is due to a toxic substance, T cells were cultured in serum-supplemented RPMI 1640 with or without 50% HT29 supernatant for 3 days, and lymphocyte viability and cell number were measured. They were found to be unaffected by the HT29 factor. When the HT29 supernatant was added to T lymphocyte cultures, the inhibition of mitogen-stimulated lymphocyte proliferation increased in a dose-dependent manner (Fig. 1). Serum-supplemented HT29 supernatant contained approximately 60 U/ml of activity, whereas serum-free supernatant averaged 42 U/ml.

The HT29 factor was then added on days 0, 1, 2, 3, and 4 to T cells cultured with PHA, and lymphocyte proliferation was measured on day 5. The inhibitory substance could be added as late as day 4 of a 5-day culture and still significantly suppress proliferation (p < 0.03) (Fig. 2). If the factor was added on day 5 immediately before the 6-hr pulse with ^{3}H-Tdr, no effect on lymphocyte proliferation was observed. This rules out the trivial possibility that the factor just affects uptake of ^{3}H-Tdr.

To determine whether the inhibitory effect is reversible, T lymphocytes were cultured in medium alone or were supplemented with 50% HT29 supernatant. After 2 days, the lymphocytes were washed extensively and then were subcultured with PHA. There was no significant difference in the subsequent proliferative response whether or not the cells had been exposed to HT29 supernatant (45,479 ± 2,327 cpm vs 39,707 ± 5,260 cpm; n = 3), indicating that its effect was reversible. To test for the induction of suppressor cells by the HT29 factor,

TABLE I

Effect of HT29 supernatant on mitogen-induced T lymphocyte proliferation

	Proliferation[a] (^{3}H-Tdr incorporation, cpm)
T cells + PHA[b]	60,272 ± 5,581 (8)[c]
T cells + Con A	58,391 ± 4,115 (5)
T cells + PHA + HT29 supernatant	1,863 ± 864[d] (8)
T cells + Con A + HT29 supernatant	1,013 ± 393[d] (5)
T cells + PHA + A375 supernatant	1,141 ± 863[d] (4)
T cells + PHA + epithelial cell supernatant	73,860 ± 8,537 (4)
T cells + PHA + IMR-90 supernatant	46,019 ± 15,958 (4)

[a] Proliferation after a 5-day incubation was expressed as total ^{3}H-Tdr incorporated (mean ± SE) during a 12-hr pulse labeling period.
[b] T cells (5×10^4) were cultured with PHA (1 μg/ml) or Con A (18 μg/ml) in RPMI 1640 medium alone or supplemented with 50% (v/v) of each supernatant.
[c] The number of experiments performed are in parentheses.
[d] These values are significantly less than the control (T + PHA) (p < 0.001).
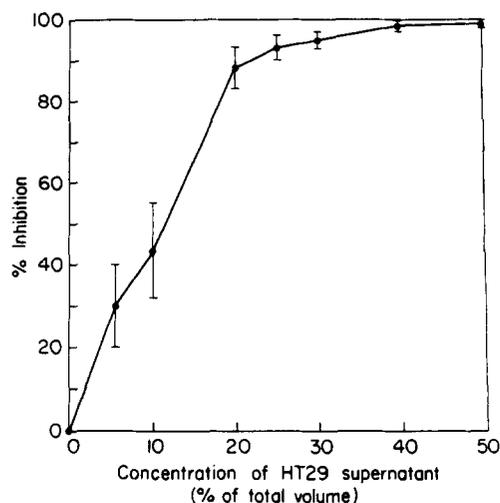
Figure 1. Effect of increasing amounts of HT29 supernatant on the inhibition of T cell proliferation. T cells (5 × 10⁴) were cultured in microtiter wells for 5 days with PHA (1 μg/ml) in 0.2 ml of medium containing up to 50% serum-free HT29 supernatant. Proliferation was measured by ^{3}H-Tdr incorporation, and inhibition as a percentage of control was calculated. The data represent the mean ± SE of four experiments.
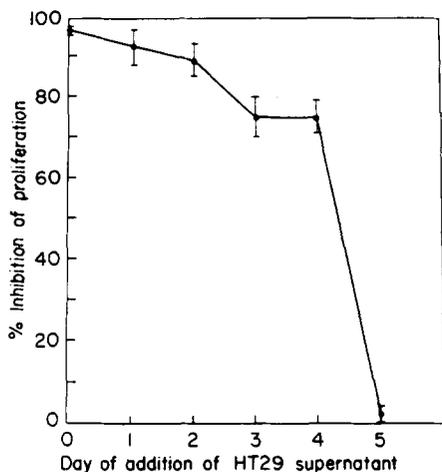

Figure 2. Kinetics of inhibition by HT29 supernatant. T cells (5 × 10⁴ in 0.1 ml) were cultured with PHA and were supplemented daily with either 0.1 ml of serum-supplemented RPMI 1640 medium or HT29 supernatant. The concentration of PHA was maintained at 1 μg/ml. On day 5, all the cultures were pulsed with ^{3}H-Tdr for 6 hr and then were harvested. Proliferation was measured by ^{3}H-Tdr incorporation, and inhibition was calculated as a percentage of the control. The data shown are the mean ± SE of three experiments. When the supernatant was added on days 0, 1, 2, 3, or 4, the percentages of inhibition were all significantly greater than zero (p < 0.03).

T cells were cultured with or without 50% HT29 supernatant for 2 days, were washed, were treated with mitomycin C, and then were co-cultured at a 1:1 ratio with fresh autologous T cells and PHA. There was no significant difference in the subsequent proliferative response whether or not cells exposed to the HT29 factor were present in the culture (49,308 ± 2,909 cpm vs 51,393 ± 2,303 cpm, respectively, n = 3). These results indicate that the effect of the HT29 factor is not due to the induction of T suppressor cells.

Effect of HT29 factor on IL 2 production. Experiments were then conducted to determine whether the HT29 factor affected IL 2 production, IL 2 receptor generation, or the interaction of IL 2 with its receptor. To test the first, T cells (1 × 10⁶/ml) were cultured with PHA (1 μg/ml) in medium alone or were supplemented with 50%

serum-containing HT29 supernatant. After a 24-hr incubation, the medium was tested for IL 2 activity by using the CTLL assay. The HT29 supernatant markedly depressed IL 2 production (0.5 ± 0.3 U/ml, n = 6) compared with a control (5.1 ± 2.0 U/ml, n = 6, p < 0.04). This was not due to a suppressive effect on the CTLL cells themselves, because the proliferation of this IL 2-dependent murine cell line to human or rat IL 2 was unaffected by the presence of HT29 supernatant (data not shown). The addition of IL 1 (5 U/ml) did not reverse the suppressive effect of the HT29 supernatant on IL 2 production. The IL 2 activity produced by T cells cultured in PHA (5.3 U/ml) or PHA and IL 1 (5.9 U/ml) was suppressed by the presence of 50% HT29 supernatant (1.3 U/ml or 1.2 U/ml, respectively).

If the suppression of lymphocyte proliferation by the HT29 factor is due to reduced production of IL 2, then its effects should be reversed by the addition of exogenous IL 2. However, the proliferation of T lymphocytes cultured with PHA and HT29 supernatant (25 or 50%) did not increase significantly with the addition of 25 U/ml of recombinant IL 2 (Fig. 3A). Furthermore, supplementing cultures with IL 1 (5, 10, or 25 U/ml) alone or in combination with IL 2 (5, 25, or 100 U/ml) did not reverse the suppressive effect of 25% HT29 supernatant (data not shown). In addition, IL 2 did not correct the inhibitory action of 50% HT29 supernatant when both were added on day 4 of a 5-day culture of T cells with PHA (Fig. 3B).

Effect of the HT29 factor on IL 2 receptor expression and the interaction of IL 2 with its receptor. The inability of IL 2 to overcome the inhibitory influence of the HT29 factor suggested that the factor may have an additional effect on IL 2 responsiveness. Thus the development of IL 2 receptors, measured by Tac expression, was evaluated. The percentage of Tac$^+$ T lymphocytes enumerated with a fluorescence microscope was no different whether the cells were cultured for 1 day with PHA in the presence or absence of HT29 supernatant (35% ± 9 vs 42% ± 10, respectively, n = 3) (Fig. 4). However, after 2 days of incubation with PHA, fewer T lymphocytes expressed the Tac antigen when cultured in 50% HT29
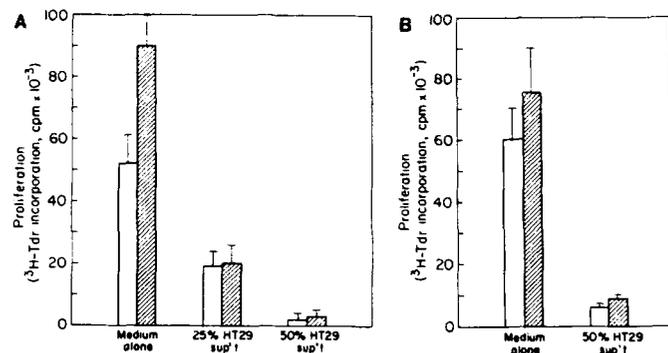

Figure 3. Effect of IL 2 on HT29-induced suppression of T lymphocyte proliferation. In *Panel A*, T cells (5 × 10⁴ in 0.2 ml) were cultured with PHA (1 μg/ml final concentration) and 0, 25, or 50% HT29 supernatant in the presence (▨) or absence (▢) of IL 2 (25 U/ml final concentration) for 5 days. The resulting T cell proliferation was measured by ^{3}H-Tdr incorporation. The results are mean ± SE of total cpm from three experiments. In *Panel B*, T cells (5 × 10⁴ in 0.1 ml) were cultured with 1 μg/ml PHA for 5 days. On day 4, 0.1 ml of serum-supplemented RPMI 1640 medium or HT29 supernatant was added with (▨) or without (▢) IL 2 (25 U/ml) to the cultures with additional PHA to maintain its final concentration at 1 μg/ml. After 18 hr, the cultures were pulsed with ^{3}H-Tdr for an additional 6 hr. Total ^{3}H-Tdr incorporated is expressed as the mean ± SE of four experiments.

TABLE II

Effect of HT29 supernatant on PHA- or IL 2-induced proliferation of PHA blasts

Stimulus[a]	Proliferation[b] (^{3}H-Tdr incorporation, cpm)		Percent Inhibition[c]
	In medium	In HT29 supernatant	
PHA	$6,504 \pm 1,358$	$1,234 \pm 84$	80 ± 3[d]
IL 2	$4,013 \pm 263$	$3,320 \pm 518$	18 ± 9

[a] PHA-activated T cells ("PHA blasts") were cultured with PHA (1 μg/ml) or IL 2 (0.25 U/ml) for 2 days with or without 25% HT29 supernatant.

[b] Values are averages of three experiments, expressed as total cpm, mean ± SE. Background proliferation averaged 138 ± 23 cpm.

[c] Values are averages of the percentage inhibition calculated for each individual experiment.

[d] Inhibition of the PHA-induced response was significantly greater than that of the IL 2-induced response ($p < 0.03$).
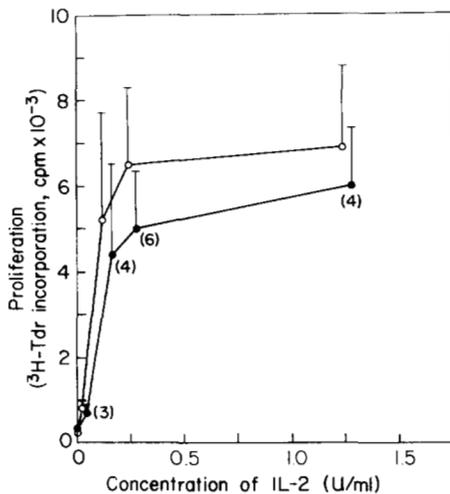

Figure 6. Effect of HT29 supernatant on the IL 2-induced proliferation of PHA blasts. PBL, stimulated with PHA for 6 days, were then washed and were cultured at 2×10^4 cells in 0.2 ml for 2 days with increasing concentrations of IL 2 in the presence (●—●) or absence (○—○) of 25% HT29 supernatant. Proliferation was measured by ^{3}H-Tdr incorporation. The data are the mean ± SE of the number of experiments shown in parentheses. The percentage inhibition was similar regardless of the amount of IL 2 added.

TABLE III

Stability of HT29 factor

Treatment	Percent activity remaining[a]
Heating (56°C for 1 hr)	$8 \pm 8 (3)$[b]
Freeze-thaw (five times)	$100 \pm 0 (3)$
pH 3.5	$21 \pm 1 (2)$[b]
4.5	$79 \pm 2 (2)$
5.5	$82 \pm 2 (2)$
6.5	$100 \pm 0 (2)$
7.5	$105 \pm 3 (3)$
8.5	$113 \pm 5 (2)$
9.5	$23 \pm 1 (2)$[b]
11.5	$17 \pm 3 (2)$[b]
Trypsin	$94 \pm 6 (4)$
Chymotrypsin	$83 \pm 16 (3)$
Proteinase K	$38 \pm 19 (3)$[b]
Thermolysin	$20 \pm 12 (3)$[b]
Elastase	$60 \pm 10 (3)$[b]

[a] Aliquots of serum-free HT29 supernatant were dialyzed overnight at 4°C, first against buffers composed of 0.3 M acetate (pH 3.5, 4.5, and 5.5), 0.3 M phosphate (pH 6.5 and 7.5), 0.3 M Tris (pH 8.5), or 0.3 M glycine (pH 9.5 and 11.5) and then against phosphate-buffered saline (PBS) (pH 7.5). In addition, serum-free HT29 supernatant was exposed to 100 μg/ml trypsin or α-chymotrypsin for 30 min at 37°C, and the reaction stopped with 400 μg/ml STI. The other protease treatments listed were accomplished with enzymes linked to Affi-Gel 10 beads (100 μg of enzyme eq/ml), including two experiments with trypsin. The number of experiments performed are in parentheses.

[b] These values are significantly ($p < 0.05$) less than 100%.

factor was stable from pH 6.5 to 8.5. The factor was resistant to trypsin and α-chymotrypsin treatments, but it was susceptible to thermolysin, proteinase K, and pancreatic elastase, suggesting that the HT29 factor contains a protein. The m.w. of the factor was estimated to be 56,000 by gel permeation chromatography on Ultrogel AcA 44 (Fig. 7). The peak of activity inhibited both PHA-induced T cell proliferation (even when supplemented with 25 U/ml IL 2) and IL 2 production, indicating that a single factor is responsible for both effects. The isoelectric point of the HT29 factor was 7.9 (Fig. 8).

DISCUSSION

This study examines the immunosuppressive effects of a soluble factor produced by the colon cancer cell line HT29. This substance markedly inhibited mitogen-induced T cell proliferation in a dose-dependent manner. The HT29 factor blocked T cell division after activation and required only 24 hr to exert its effect. The inhibition was reversible and was not due to the inducement of suppressor cells. Trivial explanations for this effect were excluded, such as a change in kinetics of T cell proliferation, a decrease in lymphocyte viability, or interference with ^{3}H-Tdr uptake. The HT29 factor was not carcinoembryonic antigen or α-fetoprotein and was not detected in medium conditioned by human colonic epithelial cells or fibroblasts.

When T cells are activated by antigens or mitogens, they produce IL 2 within 24 hr and develop IL 2 receptors on their surface. During the first 24 hr of T cell stimulation by PHA, the HT29 factor blocked IL 2 production but not IL 2 receptor generation. Because T cells require the accessory function of macrophages and/or IL 1 for optimal IL 2 production and receptor generation (16, 17), it is possible that the HT29 factor is primarily directed against macrophages. The effect of the HT29 factor on antigen presentation by macrophages could not be tested
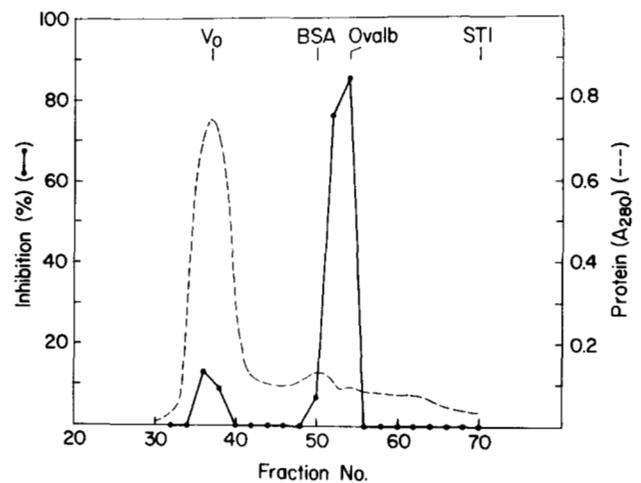

Figure 7. Gel filtration chromatography of HT29 supernatant. Serum-free HT29 supernatant, concentrated 100-fold with a collodion bag apparatus, was passed through a column (1.5 × 110 cm) of Ultrogel AcA 44, equilibrated with PBS. Two milliliter fractions were collected at 0.3 ml/min at 4°C and were tested for absorbance at 280 nm (———). Inhibitory activity against T cell proliferation was measured by culturing T cells (5 × 10^4 in 0.2 ml) for 5 days with PHA (1 μg/ml) in RPMI 1640 medium containing 10 μl of each fraction (●—●). The protein markers used to calibrate the column were duck ovostatin (780,000 daltons), IgG (150,000 daltons), bovine serum albumin (BSA) (68,000 daltons), ovalbumin (ovalb) (43,000 daltons), soybean trypsin inhibitor (STI) (21,000 daltons), and cytochrome c (12,000 daltons). Ninefold purification was achieved with 70% recovery.

Figure 8. Preparative isoelectric focusing of HT29 supernatant. Serum-free HT29 supernatant in 5 ml (concentrated 20 times) was subjected to isoelectric focusing as described in *Materials and Methods*. The pH (O—O) and absorbance at 280 nm (— — —) are shown. Those fractions ranging from a pH of 3 to 10 were dialyzed against PBS overnight at 4°C, were filter sterilized, and were tested for percentage of inhibition of T cell proliferation using 10 μl of each fraction in 0.2 ml of RPMI 1640 medium (●—●). Sixfold purification with 47% recovery was achieved.
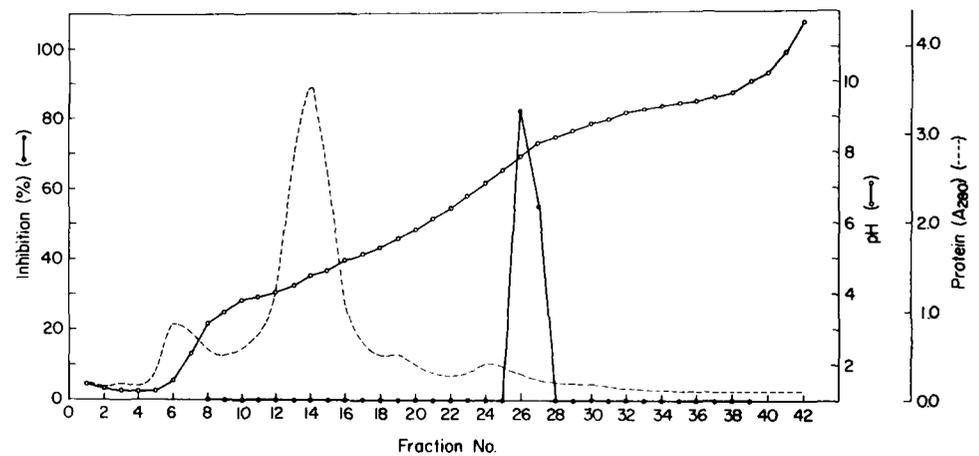
by mixing experiments, because its inhibitory effect on T cell proliferation was reversible by washing lymphocytes. However, the effects of exogenous IL 1 could be assessed. The addition of IL 1 did not reverse the suppressive action of the HT29 factor on mitogen-induced IL 2 production or T cell proliferation, indicating that the immunosuppressive activity of the HT29 factor is not due to low IL 1 production by macrophages.

When T cells are stimulated by PHA, there is a rapid expansion in the percentage of cells expressing Tac, T9, and other activation antigens during the 5-day incubation period. In the presence of the HT29 factor, Tac antigen expression increased during the first 24 hr, but declined thereafter. The percentage of PBL expressing Tac or T9 was markedly depressed if cells were cultured with PHA for 5 days in HT29 supernatant. These changes may be due in part to the low IL 2 availability, because IL 2 upregulates its own receptors (18, 19) and the expression of transferrin receptors may be induced by the IL 2/IL 2 receptor interaction (20). However, the inhibitory effect of the HT29 factor on T cell proliferation was not due solely to the block in IL 2 production. First, the HT29 supernatant could suppress mitogen-induced T cell proliferation even if added during the last 24 hr of a 5-day lymphocyte culture. Such an effect observed late in the proliferative response suggests that the factor involves cell division, as well as the events occurring during activation, such as IL 2 production. In addition, exogenous IL 2 did not reverse the suppressive action of the HT29 factor.

The IL 2 unresponsiveness induced by the HT29 factor was not primarily due to a decrease in IL 2 receptor expression. This was supported by the observation that Tac expression was not affected by a 24-hr exposure to 25% HT29 supernatant, whereas T cell proliferation and IL 2 production were markedly suppressed during this time period. It is possible that T cells that develop Tac on their surface in the presence of the HT29 factor have low affinity or poorly functional IL 2 receptors because anti-Tac antibody can bind to both low and high affinity IL 2 receptors (21). Although our experiments have not evaluated the affinity of IL 2 receptors, this possibility is unlikely because PHA blasts demonstrated only a minimally suppressed proliferative response to IL 2 alone but a markedly depressed response to PHA in the presence of the factor. Similarly, the growth of the CTLL line in response to IL 2 was unaffected by the HT29 factor. These findings suggested that this factor does not pri-

marily block the interaction of IL 2 with its receptor. This factor then affects T cells early during activation (by decreasing IL 2 production) and late during cell division. This second action does not mainly involve IL 2 production, IL 2 receptor generation, or the interaction of IL 2 with its receptor, but may be related to some other structure involved in the proliferative response. The observation that the HT29 factor inhibits PHA- more than IL 2-induced proliferation of PHA blasts suggests that cell cycling is not mainly affected but rather some event required for mitogen- but not IL 2-stimulated proliferation of activated T cells.

The effects of the HT29 factor on cytotoxic and suppressor T cell functions were recently investigated (22). When T cells were cultured with IL 2 in the presence of 50% HT29 supernatant, lymphokine-activated killer (LAK) activity measured 7 days later against the HT29 colon cancer cell line was virtually abolished. Mitogen-induced cellular cytotoxicity and T cell-mediated lympholysis were also abrogated if the HT29 factor was present during the generation of these cytotoxic lymphocytes. However, the HT29 supernatant did not affect the ability of such cytotoxic lymphocytes to destroy their targets (22). Nor did it suppress natural killer activity against the K562 erythroleukemia line (22). To measure its effects on the generation of suppressor cells, the HT29 supernatant was added to T cells cultured with Con A. Although the HT29 factor markedly reduced Con A-induced T cell proliferation, it did not affect the generation of suppressor T cells that inhibit mitogen-induced T cell proliferation. The HT29 supernatant alone did not induce suppressor T cells.

This factor, which inhibited the development of cytotoxic but not suppressor lymphocytes, may favor tumor survival in the host. If the HT29 factor or similar substances circulate in patients with neoplasms, they may suppress the formation of LAK cells capable of lysing a variety of tumor cell types. Such an effect may explain why the in vivo administration of IL 2 alone is not as efficacious as IL 2 with preformed LAK cells in the treatment of cancer (23). Suppressor cells induced by mitogens or alloantigens can block a variety of T cell functions such as T cell proliferation, IL 2 production, or the development of cytotoxic T lymphocytes (24). If such suppressor cells can form even in the presence of the HT29 factor, they would accentuate its immunosuppressive effects.

The HT29 factor differs significantly from most other

4. Remacle-Bonnet, M. M., G. J. Pommier, S. Kaplanski, R. J. Rance, and R. C. Depieds. 1976. Inhibition of normal allogeneic lymphocyte mitogenesis by a soluble inhibitor extracted from human colonic carcinoma. *J. Immunol. 117:1145.*

5. Mohagheghpour, N., B. Parhami, K. Dowlatshahi, D. Kadjehnouri, J. H. Elder, and F. V. Chisari. 1979. Immunoregulatory properties of human esophageal tumor extract. *J. Immunol. 122:1350.*

6. Roth, J. A., B. A. Osborne, and R. S. Ames. 1983. Immunoregulatory factors derived from human tumors. *J. Immunol. 130:303.*

7. Whitehead, J. S., and Y. S. Kim. 1980. An inhibitor of lymphocyte proliferation produced by a human colonic adenocarcinoma cell line in culture. *Cancer Res. 40:29.*

8. Werkmeister, J., J. Zaunders, W. McCarthy, and P. Hersey. 1980. Characterization of an inhibitor of cell division released in tumour cell cultures. *Clin. Exp. Immunol. 41:487.*

9. Hersey, P., C. Bindon, M. Czerniecki, A. Spurling, J. Wass, and W. H. McCarthy. 1983. Inhibition of interleukin 2 production by factors released from tumor cells. *J. Immunol. 131:2837.*

10. Fontana, A., H. Hengartner, N. de Tribolet, and E. Weber. 1984. Glioblastoma cells release interleukin 1 and factors inhibiting interleukin 2-mediated effects. *J. Immunol. 132:1837.*

11. Russell, W. C., C. Newman, and D. H. Williamson. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. *Nature 253:461.*

12. Ebert, E. C., A. I. Roberts, R. E. Brolin, and K. Raska. 1986. Examination of the low proliferative capacity of human jejunal intra-epithelial lymphocytes. *Clin. Exp. Immunol. 65:148.*

13. Ebert, E. C. 1986. Intestinal epithelial cells rosette with sheep red blood cells. *J. Immunol. Methods 91:145.*

14. Gillis, S., M. M. Fern, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol. 120:2027.*

15. Nagase, H., T. E. Cawston, M. DeSilva, and A. J. Barrett. 1982. Identification of plasma kallikrein as an activator of latent collagenase in rheumatoid synovial fluid. *Biochim. Biophys. Acta 702:133.*

16. Gillis, S., and S. B. Mizel. 1981. T-cell lymphoma model for the analysis of interleukin 1-mediated T-cell activation. *Proc. Natl. Acad. Sci. USA 78:1133.*

17. Kaye, J., S. Gillis, S. B. Mizel, E. M. Shevach, T. R. Malek, C. A. Dinarello, L. B. Lachman, and C. A. Janeway, Jr. 1984. Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor: interleukin 1 is required for the expression of receptors for interleukin 2. *J. Immunol. 133:1339.*

18. Welte, K., M. Andreeff, E. Platzer, K. Holloway, B. Y. Rubin, M. A. S. Moore, and R. Mertelsmann. 1984. Interleukin 2 regulates the expression of Tac antigen on peripheral blood T lymphocytes. *J. Exp. Med. 160:1390.*

19. Smith, K. A., and D. A. Cantrell. 1985. Interleukin 2 regulates its own receptors. *Proc. Natl. Acad. Sci. USA 82:864.*

20. Neckers, L., and J. Cossman. 1983. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. *Proc. Natl. Acad. Sci. USA 80:3494.*

21. Robb, R. J., W. C. Greene, and C. M. Rusk. 1984. Low and high affinity receptors for interleukin 2: implications for the level of Tac antigen. *J. Exp. Med. 160:1126.*

22. Ebert, E. C. 1986. A soluble factor produced by colon cancer inhibits the development of cytotoxic T lymphocytes. *Gastroenterol. 90:1403 (Abstract).*

23. Mule, J. J., J. Yang, S. Shu, and S. A. Rosenberg. 1986. The anti-tumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 in vivo: direct correlation between reduction of established metastases and cytolytic activity of lymphokine-activated killer cells. *J. Immunol. 136:3899.*

24. Herscowitz, H. B., T. Sakane, A. D. Steinberg, and I. Green. 1980. Heterogeneity of human suppressor cells induced by concanavalin A as determined in simultaneous assays of immune function. *J. Immunol. 124:1403.*

25. Shirakawa, F., Y. Tanaka, S. Oda, S. Chiba, H. Suzuki, S. Eto, and U. Yamashita. 1986. Immunosuppressive factors from adult T-cell leukemia cells. *Cancer Res. 46:4458.*

26. Santoli, D., D. J. Tweardy, D. Ferrero, B. L. Kreider, and G. Rovera. 1986. A suppressor lymphokine produced by human T leukemia cell lines. *J. Exp. Med. 163:18.*

27. Lotz, M., C. D. Tsoukas, S. Fong, C. A. Dinarello, D. A. Carson, and J. H. Vaughan. 1986. Release of lymphokines after infection with Epstein Barr virus in vitro. *J. Immunol. 136:3643.*

28. Tiku, K., M. L. Tiku, S. Liu, and J. L. Skosey. 1986. Normal human neutrophils are a source of a specific interleukin 1 inhibitor. *J. Immunol. 136:3686.*

29. Maki, T., S. Satomi, M. Gotoh, and A. P. Monaco. 1986. Contra-IL 2: a suppressor lymphokine that inhibits IL 2 activity. *J. Immunol. 136:3298.*

30. Chouaib, S., K. Welte, R. Mertelsmann, and B. Dupont. 1985. Prostaglandin E_2 acts at two distinct pathways of T lymphocyte activation: inhibition of interleukin 2 production and down-regulation of transferrin receptor expression. *J. Immunol. 135:1172.*

31. Robb, R. J. 1986. The suppressive effect of gangliosides upon IL 2-dependent proliferation as a function of inhibition of IL 2-receptor association. *J. Immunol. 136:971.*