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 physiochemical properties have been described.

**MATERIALS AND METHODS**

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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 [³H]-Tdr, no effect on lymphocyte proliferation was observed. This rules out the trivial possibility that the factor just affects uptake of [³H]-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,
containing up to 50% serum-free HT29 supernatant. Proliferation was measured by 3H-Tdr incorporation in microtiter wells for 5 days with PHA (1 pg/ml) in 0.2 ml of medium and inhibition as a percentage of control. The data shown are the mean ± SE of four experiments.

All the cultures were pulsed with 3H-Tdr for 6 hr and then were harvested. The resulting T cell proliferation was measured by 3H-Tdr incorporation, and inhibition was calculated as a percentage of the control. The data shown are the mean ± SE of four 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 < 0.05).

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 (5 × 10⁴/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).

Figure 1. Effect of increasing amounts of HT29 supernatant on the inhibition of T cell proliferation. 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 3H-Tdr for 6 hr and then were harvested. Proliferation was measured by 3H-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).

Figure 2. Kinetics of inhibition by HT29 supernatant. T cells (5 × 10⁴ in 0.1 ml) were cultured with PHA and were supplemented with PHA (1 µg/ml final concentration) and 0, 25, or 50% HT29 supernatant 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 supernatant was added with PHA (1 µg/ml) for 5 days. The resulting T cell proliferation was measured by 3H-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 PHA (1 µg/ml) or without PHA (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 3H-Tdr for an additional 6 hr. Total 3H-Tdr incorporated is expressed as the mean ± SE of four experiments.

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 3H-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 PHA (1 µg/ml) or without PHA (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 3H-Tdr for an additional 6 hr. Total 3H-Tdr incorporated is expressed as the mean ± SE of four experiments.
supernatant than in medium alone (25% ± 6 vs 45% ± 10, respectively, n = 3, p < 0.02). This difference was even more marked after a 3- or 5-day incubation. The effect of the HT29 supernatant on the density of Tac expression on PHA-activated T lymphocytes was then measured by the distribution of the intensity of fluorescence obtained by flow cytometry analysis. When PBL were stimulated for 3 days in the presence of 50% HT29 supernatant, there was both a decrease in the percentage of Tac positive cells (from 79 to 59%), as well as a 10% decline in the mean channel number of fluorescence intensity (Fig. 5). PBL activated by PHA in 25% HT29 supernatant demonstrated no decrease in the percentage of positively stained cells, but there was an 8% decline in mean channel number, indicating a reduction in IL 2 receptor density.

To determine whether the decrease in the percentage of Tac+ T cells represented a specific down-regulation of the IL 2 receptor or a decrease in the percentage of activated cells, the effect of the HT29 factor on the expression of the transferrin receptor (T9), another activation antigen, was quantitated by using a fluorescence microscope. When T cells were cultured with PHA for 5 days, there were fewer T9+ cells in cultures containing 50% HT29 supernatant (10% ± 3) than medium alone (37% ± 3) (n = 3, p < 0.03).

Because PHA-activated T cells are unresponsive to IL 2 after just 24 hr of exposure to the HT29 factor, experiments were conducted to determine whether there was an associated decline in Tac expression. T lymphocytes, stimulated with PHA for 5 days, contained 81% ± 1 Tac+ cells. If the culture medium contained 25% HT29 supernatant during the last 24 hr of incubation, the percentage of Tac+ cells was unchanged (84% ± 1) as determined by using a fluorescence microscope. By flow cytometry analysis, the percentage of Tac+ cells decreased slightly from a control of 94% to 91 and 88% when either 25 or 50% HT29 supernatant was added during the last 24 hr. There was a small but significant mean channel shift of 2% corresponding to a decrease in Tac receptor density with the addition of 50% HT29 supernatant but no decrease with 25% HT29 supernatant.

Although the HT29 factor inhibited T cell proliferation with only minor decreases in Tac antigen expression, it may block the binding of IL 2 to its receptor. To evaluate this possibility, the effect of the HT29 supernatant on IL 2-induced proliferation of activated T cells was determined. PBL were incubated with PHA for 6 days. The resulting “PHA blasts” produced little IL 2 (unmeasurable by CTLL assay), but most expressed IL 2 receptors (75% ± 8) so that they proliferated in response to exogenous IL 2. The proliferation of PHA blasts in response to recombinant IL 2 (0.25 U/ml) was minimally suppressed by 25% HT29 supernatant (Table II), regardless of the amount of IL 2 added to the culture (Fig. 6). In contrast, the same concentration of HT29 supernatant markedly inhibited proliferation of PHA blasts in response to PHA (80% ± 3 inhibition) (p < 0.03) (Table II). Thus the immunosuppressive effect of the HT29 factor is not primarily due to a block in the binding of IL 2 to the IL 2 receptor.

Properties of the HT29 factor. The HT29 factor retained its inhibitory activity after repeated freezing and thawing (Table III). Heating the factor to 56°C for 1 hr destroyed most of the activity. The factor was labile in acidic and alkaline environments. Approximately 80% of the activity was lost after dialysis at 4°C for 18 hr at pH 3.5 or 9.5. After similar treatment at pH 4.5 and 5.5, approximately 80% of the activity was recovered. The
The other protease treatments listed were accomplished with enzymes linked to Affi-Gel 10 beads (100 µg of enzyme/ml). Including two experiments with trypsin. The number of experiments shown in each individual experiment.

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

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Proliferation*</th>
<th>Percent Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>6.504 ± 1.358</td>
<td>1.234 ± 84</td>
</tr>
<tr>
<td>IL 2</td>
<td>4.013 ± 263</td>
<td>3.320 ± 518</td>
</tr>
</tbody>
</table>

* PHA-activated T cells (PHA blasts) were cultured with PHA (1 µg/ml) or IL 2 (2.5 µl/ml) for 2 days with or without 25% HT29 supernatant.

The protein markers used to calibrate the column were duck ovalbumin (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.
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 primarily 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 lysis 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...
suppressor substances derived from solid tumor extracts or cell lines (Table IV). The human colon adenocarcinoma cell line, SKCO-1, produces an immunosuppressive substance that is larger than the one described here (M, > 100,000) and is heat stable (7). However, these investigators also noted a minor peak of inhibitory activity (M, of 45,000) that was not additionally characterized. Soluble extracts from human colonic tumors contain an inhibitor with an M, of 70,000, but unlike the HT29 factor, its effects are irreversible (4). Only the immunosuppressive substance from esophageal tumor extracts resembles the HT29 factor in several ways, although its actions have not been characterized extensively (5). The HT29 factor also differs from immunosuppressive factors produced by leukemia cells. A suppressor substance from adult T cell leukemia cells, with the same apparent M, (50,000 to 70,000) as the HT29 factor, also inhibits T cell proliferation and IL 2 production (25). However, unlike the HT29 factor, its activity is unaffected by heating to 60°C or exposure to pH 9.0. A T leukemia-derived suppressor lymphokine has an M, of 86,000 and is sensitive to heat denaturation and extremes of pH, like the HT29 factor (26). However, its isoelectric point is lower (5.2 to 5.3), and its action is irreversible.

The effects of the HT29 factor are unlike those of many known suppressor substances. Inhibitors of IL 1 can suppress IL 2 production (27) but not mitogen-induced proliferation of thymocytes (28). Inhibitors of IL 2 depress mitogen-induced T cell proliferation, but unlike the HT29 factor, they also block IL 2-induced proliferation of CTLL cells (29). Several substances have been found that depress the activation of T cells but not subsequent cell division. For example, prostaglandin E2 inhibits IL 2 production and the expression of the transferrin receptor, but does not exert an inhibitory effect if added 18 hr after culturing T cells with mitogen (30). Other substances such as gangliosides primarily block the binding of IL 2 to its receptor (31). The HT29 factor blocks both early events in T cell activation, as well as later ones responsible for cell division. Studies of the structure and function of the HT29 factor may lead not only to an additional understanding of T cell proliferation, but also to new approaches in the treatment of cancer.

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REFERENCES
2168 IMMUNOSUPPRESSIVE FACTOR FROM COLON CANCER


