Suppressor Lymphokine Produced by Rat T-Cells in Response to Syngeneic Mammary Adenocarcinoma 13762A

Neil D. Christensen, John W. Kreider, and Rick L. Horetsky

Departments of Pathology and Microbiology, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033

ABSTRACT

An antiproliferative suppressor lymphokine was produced from rat T-cells specifically in response to the poorly immunogenic syngeneic mammary adenocarcinoma 13762A. The tumor-induced suppressor lymphokine (TISL) was produced late in culture (peak production on Days 4 and 5) and showed strong but selective inhibitory activity on a variety of immune responses. The immune peritoneal exudate cell response to a highly immunogenic clone from the parental tumor (clone 18A) and the concanavalin A-stimulated response of nonimmune spleen cells were inhibited strongly by TISL. In contrast, the immune spleen cell response to 13762A and the lipopolysaccharide response of nonimmune spleen cells were unaffected. Preliminary molecular weight and physicochemical analysis of TISL indicated that the molecule was large (M, > 350,000); partially sensitive to 75°C treatment for 15 min and to pH 2.0 treatment; only partly degraded by the enzymes trypsin, chymotrypsin, and proteinase K; and completely destroyed by boiling. Although TISL was produced specifically in response to 13762A tumor, prior immunization in vivo was not necessary for the induction of the suppressor lymphokine. These results indicate that populations of rat lymphocytes contain naturally occurring TISL secreting cells, which can be activated specifically by tumor antigens such as those expressed by 13762A.

INTRODUCTION

Several lymphokines which suppress cell proliferation in vitro are products of T-cells (1-26). The best characterized of these, lymphotoxin and immune IFN-γ,3 have been cloned and sequenced (25, 26). Other suppressive lymphokines have not been cloned, but functional assays for their most prominent biological activities have been defined (1-22). The suppressor lymphokines inhibit cell proliferation by either direct cytotoxicity (23-26) or a cytostatic mechanism in which cell death occurs subsequent to proliferative arrest (1-16, 20, 22). Most of these suppressor factors have been produced either constitutively from tumor cell lines (12-14) or from mitogen-stimulated lymphocyte populations (1-11, 15). There are several reports also of antiproliferative suppressor lymphokines that were produced by primed T-cells in response to defined antigens (16, 17) and to tumor antigens (18-22). Two of the factors secreted in response to tumor antigens have been identified as natural killer cell cytotoxic factor (21) and a cytostatic factor similar to leukoregulin (20). Other tumor antigen-induced T-cell suppressor lymphokines are tumor specific, but have not been tested for nonspecific antiproliferative activity (18, 19).

We have been studying the immune response to a weakly immunogenic metastatic rat mammary adenocarcinoma 13762A (27-29), and some preliminary in vitro data have indicated that T cells are activated by 13762A tumor antigens (29). In the studies on immunity to 13762A, a highly immunogenic regressor clone 18A, which was isolated in vitro, provided immunity to parental 13762A following regression of 18A (27). Transfer of immune PEC from 18A regressors provided protection to naive recipients and together with low doses of irradiation eliminated large established 13762A tumors (28). The in vitro responses of immune spleen and PEC may be summarized as follows. Immune PEC proliferated strongly to clone 18A, but poorly to 13762A tumor. High levels of IL-2 were secreted by immune PEC in response to both these tumors. Immune spleen cells proliferated strongly to parental 13762A but did not secrete detectable levels of IL-2. Weaker and variable proliferation of immune spleen cells occurred in response to clone 18A. Cytotoxicity against both clone 18A and 13762A tumor cells was generated from cultures of immune spleen cells stimulated by 13762A tumor.

We have examined the poor responses of immune PEC to 13762A in greater detail and describe here the analysis of a TISL which was produced from rat T-cells specifically in response to 13762A. TISL had strong but selective inhibitory effects on some antigen- and mitogen-driven proliferative responses. Preliminary physicochemical and molecular weight characteristics of TISL are presented.

MATERIALS AND METHODS

Animals. Female F344 rats were purchased from the Frederick Cancer Research Center, Frederick, MD. Female BN rats were purchased from Microbiological Associates, Walkersville, MD.

Cell Lines. The rat mammary adenocarcinoma 13762A was obtained from Dr. A. Bogden, Mason Research Institute (Worcester, MA) and maintained in ascites form and in cell culture as described (27, 29). Clone 18A was isolated from cultures of 13762A by limiting dilution as described (27). The R3230AC rat mammary tumor was obtained from Dr. P. Fritz, Department of Pharmacology, at this institution.

Tumor-immune Rats. Rats immune to a lethal inoculum of 13762A were generated as previously described (27). Briefly, 1 x 10⁶ clone 18A tumor cells were injected i.d. on the right dorsolateral thorax. Tumors regressed completely after 40 days, and rats were challenged with a lethal dose of 1 x 10⁶ 13762A ascites cells. Immune rats were reimmunized 2 to 6 mo after challenge by injecting i.p. 2 x 10⁶ fresh 13762A ascites cells together with 3 ml of sterile mineral oil. The spleen and immune PEC were harvested 5 to 6 days later.

MTLC. MTLC were established as previously described (29). Briefly, 2 x 10⁶ tumor-immune (27) lymphocytes from the spleen, lymph nodes, or peritoneum (glass-adherence depleted) were cultured in 96-well round-bottomed microtiter plates together with 10⁶ mitomycin C-treated tumor cells and 2 x 10⁶ mitomycin C-treated normal spleen cells as fillers. Cultures were assayed for proliferation 5 or 6 days later, by measuring uptake of [³H]thymidine after a 6-h incubation period.

Mitogen Responses. Nonimmune F344 spleen cells were plated into flat-bottomed 96-well microtiter plates at concentrations between 2 and 5 x 10⁵ per well. The mitogens con A at 5 μg/ml or LPS at 10 μg/ml or 50 μg/ml were added to the wells, and proliferation was assayed on Day 2 or 3.

Primary Allogeneic MLR. Nonimmune F344 spleen cells (4 x 10⁵) were plated into 96-well round-bottomed microtiter plates together with
with $2 \times 10^5$ mitomycin C-treated BN spleen cells as stimulators. Proliferation was determined after 6 days of culture.

Detection and Assay of TISL. TISL was assayed by titrating supernatants collected from 5-day MTLC into fresh cultures of immune PEC and 18A. One unit of suppressor factor activity was determined as the volume of supernatant required to give 50% suppression of the 5-day proliferative response of control cultures (immune PEC anti-18A responses). TISL titer was expressed as units of suppressor activity per ml of supernatant containing TISL.

Supernatants containing suppressor material as determined in the above MTLC assay were tested for antiproliferative activity in two other culture systems: (a) in mitogen-stimulated nonimmune spleen cell responses as described above; and (b) in microcultures of tumor cells. Dilutions of supernatants were added to microwells of a 96-well flat-bottomed plate containing $2 \times 10^3$ 18A, 13762A, or R3230AC tumor cells. Duplicate plates were assayed for proliferation on Days 3 and 5. The titer of TISL in these assays was calculated as for the MTLC assay.

Treatment of Cells with Antibody and Complement. Normal guinea pig serum was preabsorbed on F344 red blood cells and thymocytes (30 min at 4°C) and used as a source of complement. The serum was pretested on rat thymocytes to determine effective titer and lack of toxicity. Immune PEC or normal spleen cells were incubated for 30 min with a 1:20 dilution of rabbit anti-rat thymocyte serum (Microbiological Associates) at 4°C, washed once, and then incubated for 30 min at 37°C with a 1:10 dilution of guinea pig serum. (Under these conditions, up to 98% of F344 thymocytes were lysed as determined by trypan blue dye exclusion.) The cells were washed 3 times and plated into MTLC. Supernatants to be tested for the presence of TISL were collected on Day 5, and duplicate cultures were also assayed for proliferation.

AcA34 Size Column Separation of TISL. Supernatants containing TISL obtained from cultures of immune PEC and 13762A were placed on a 30- x 450-mm Ultrogel AcA34 column (LKB Instruments, Inc., Gaithersburg, MD; flow rate, 70 ml/h at 22°C), and 5-ml fractions in PBS were collected. Fractions to be assayed for TISL were filter sterilized through 0.2-µm acrodisc disposable filters (Gelman Sciences, Inc., Ann Arbor, MI) and titrated into fresh MTLC containing immune PEC and 18A. The absorbance at 280 nm for each fraction was measured on an LKB Ultraspec spectrophotometer.

Selected Pretreatment of TISL. Various pretreatments of supernatants containing TISL were carried out to determine some physicochemical properties of the suppressor lymphokine. Aliquots of supernatants were pretreated with RNase (100 µg/ml, 3 h, 37°C), trypsin (500 µg/ml, 3 h, 37°C), chymotrypsin (500 µg/ml, 3 h, 37°C), proteinase K (250 µg/ml, 3 h, 37°C), heat (100°C, 10 min; 75°C, 15 min; 56°C, 30 min; and 37°C, 3 h), and acidic conditions (pH 2.0, 2 h, 4°C, followed by neutralization), and then titrated into fresh MTLC containing immune PEC and 18A. Proliferation was assessed on Day 5 of culture, and the titer of TISL was calculated as described above. The enzymes trypsin, chymotrypsin, and proteinase K were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Positive controls for activity of these enzymes were determined separately by incubating aliquots of enzymes with bovine serum albumin in PBS for various time intervals. Polycrylamide gel electrophoresis of samples showed a decreasing band strength with increasing time, thus demonstrating enzymatic activity. Control cultures containing dilutions of enzymes alone demonstrated that RNase, trypsin, and chymotrypsin did not affect the 18A-induced proliferative response of immune PEC. Proteinase K, however, was found to be toxic at a final concentration of 5 µg/ml. High concentrations of TISL, therefore, were pretreated to enable the toxic effects of proteinase K to be diluted out before dilution of the TISL activity.

RESULTS

Tumor-specific Proliferation of Immune Lymphocytes. Rats immune to a lethal dose of 13762A tumor were used as a source of immune responder cells to 13762A and 18A tumor cells as previously described (29). Tumor-specific proliferation was observed in MTLC containing immune PEC, spleen, and mesenteric lymph node cells, and a representative result is shown in Fig. 1. Immune PEC responded strongly to 18A, but poorly to 13762A. Mitomycin C-treated tumor cells 18A (□), 13762A (○), and R3230AC (△) were titrated into MTLC containing 1 x 10^3 mitomycin C-treated tumor cells and 2 x 10^5 mitomycin C-treated normal spleen filler cells, and proliferation was assessed on Day 6 of culture. Points, mean cpm of triplicate cultures; bars, SEM. Two lines of cultured 13762A established from ascites cells were tested. TdR, thymidine.

Fig. 1. Tumor-specific proliferation of immune PEC, spleen, and mesenteric lymph node cells in response to various stimulators. Immune lymphocytes (2 x 10^5 spleen and PEC, 4 x 10^5 lymph node cells) were cultured with 1 x 10^5 mitomycin C-treated tumor cells and 2 x 10^5 mitomycin C-treated normal spleen filler cells, and proliferation was assessed on Day 6 of culture. Columns, mean cpm of triplicate cultures; bars, SEM. Tumor-induced suppression of an immune PEC response to clone 18A tumor. Mitomycin C-treated tumor cells 18A (□), 13762A (○), and R3230AC (△) were titrated into MTLC containing 2 x 10^5 immune PEC and 1 x 10^6 mitomycin C-treated 18A tumor cells plus fillers on Day 0. Proliferation was determined after 6 days of culture. Points, mean cpm of triplicate cultures; bars, SEM. Immune PEC stimulated by R3230AC (△) and 13762A (●) alone were included as controls. TdR, thymidine.

Fig. 2. 13762A tumor cell-induced suppression of the immune PEC proliferative response to clone 18A tumor. Mitomycin C-treated tumor cells 18A (□), 13762A (○), and R3230AC (△) were titrated into MTLC containing 2 x 10^5 immune PEC and 1 x 10^6 mitomycin C-treated 18A tumor cells plus fillers on Day 0. Proliferation was determined after 6 days of culture. Points, mean cpm of triplicate cultures; bars, SEM. Immune PEC stimulated by R3230AC (△) and 13762A (●) alone were included as controls. TdR, thymidine.
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Fig. 3. Effects of 13762A tumor cells on three different immune responses. A, immune PEC anti-18A response (positive control); B, F344 anti-BN primary allogeneic MLR; C, F344 normal spleen cell con A response. Mitomycin C-treated tumor cells 18A (C), 13762A (O), and R3230AC (△) were titrated into the three responses, and proliferation assessed after 6 days for the MTLC and MLR, and after 2 days for the con A response. Points, mean percentage of positive control cultures; bars, SEM of triplicate cultures. Positive control cpm for the MTLC = 30,860 ± 838, MLR = 24,665 ± 1,926, and con A response = 218,497 ± 2,532.

Fig. 4. Antiproliferative suppressor activity in supernatants collected from 5-day MTLC. Five day cell-free supernatants from various MTLC were titrated into fresh MTLC containing immune PEC and 18A (A, C, and D), or immune spleen cells and 13762A (B) on Day 0. In A and B, tested supernatants were collected from MTLC containing immune PEC and 18A (C), immune PEC and 13762A (O), or immune PEC and R3230AC (△). In C, tested supernatants were collected from cultures containing filler cells and 18A (C), filler cells and 13762A (O), and filler cells and R3230AC (△), and immune glass nonadherent PEC and 13762A (○). Supernatants tested in A, B, and C were collected from the same experiment (one of five similar experiments); those tested in D were from a separate experiment (one of two similar experiments). TdR, thymidine. Bars, SEM.

 peculation in cultures of immune PEC and 13762A despite strong IL-2 production (29) was that 13762A antigens activated tumor-specific T cells. To test this hypothesis, mitomycin C-treated tumor cells were titrated into MTLC containing PEC and 18A (Fig. 2). 13762A tumor cells had a powerful suppressive effect upon 18A-induced proliferation, and as few as 500 cells suppressed the response by more than 50%. Neither added 18A nor R3230AC tumor cells had any suppressive effect upon proliferation at doses of cells up to 10⁴ per culture.

Specificity of 13762A Tumor-induced Suppression. We next examined other in vitro lymphocyte responses to determine whether the observed suppressive effects of 13762A tumor were due to activated tumor-specific T cells, or to nonspecific suppression by other subgroups of lymphocytes, or to 13762A itself. Tumor cells were titrated into cultures of nonimmune F344 spleen cells stimulated by BN spleen cells (primary allogeneic MLR) and a con A-stimulated normal F344 spleen cell response. The results of these experiments are shown in Fig. 3. 13762A tumor cells suppressed both the immune PEC response to 18A tumor (as above) and the primary allogeneic MLR, but not the 2-day con A-stimulated response. These results demonstrated that the observed suppression was selective and was not due to nonspecific suppressor products secreted by 13762A.

Soluble Tumor-inducedSuppressor Lymphokine in MTLC Containing Immune PEC and 13762A. Supernatants from various MTLC were examined next for the presence of soluble factors mediating suppression of tumor-specific proliferation. Five-day supernatants were collected from MTLC containing either immune PEC or spleen cells that were stimulated by 18A, 13762A, and R3230AC, as well as control supernatants from cultures of filler cells and tumor. The cell-free supernatants were titrated into fresh MTLC on Day 0, and these cultures were assayed for proliferation 5 days later (Fig. 4). Powerful suppression of the PEC anti-18A proliferative response was induced by supernatants from MTLC containing PEC and 13762A, but not from the supernatants of MTLC containing 18A and R3230AC (Fig. 4A) or from immune spleen cells and 13762A (Fig. 4D). Suppressive material was not detected also in supernatants obtained from cultures of filler cells and 13762A (Fig. 4C), or immune glass-adherent PEC and 13762A (Fig. 4D). TISL-containing supernatants from cultures of PEC and 13762A did not suppress the immune spleen cell response to 13762A. These results indicated that glass nonadherent PEC were the producers of TISL and that the factor showed selective suppression of tumor-specific proliferation. The results also demonstrated that immune spleens contained few TISL-secreting lymphocytes.

Activity of TISL on Mitogen-stimulated Responses and on in Vitro Mammary Tumor Cell Growth. One batch of TISL was tested simultaneously upon mitogen-stimulated lymphocyte responses and on the growth of rat mammary tumor cells to determine whether the antiproliferative activity was directed...
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Fig. 5. Effect of supernatant containing TISL on mitogenic responses and on the growth of mammary tumor cells. In A, TISL was titrated into cultures containing 2 × 10⁶ normal spleen cells stimulated by 5 μg/ml con A (C), and 5 × 10⁶ normal spleen cells stimulated by 10 μg/ml LPS (Δ) or 50 μg/ml LPS (○). Proliferation was assessed on Day 3 and plotted as the percentage of positive control cultures. Mean positive control cpm was 351,138 ± 21,505 (SEM), 31,765 ± 1,435, and 32,690 ± 1,509, respectively.Suppressive activity of the supernatant on a Day 5 immune PEC anti-18A response was included as a control for TISL activity (●). In B and C, TISL was titrated into cultures containing 2 × 10⁶ 18A (○), 13762A (C), and R3230AC (Δ) tumor cells, and proliferation assessed on Day 3 (B) and Day 5 (C). Results were plotted as the percentage cpm of control cultures.

against antigen-specific proliferation only. Supernatant containing TISL was titrated into con A- and LPS-stimulated normal spleen cell responses and into microcultures of 18A, 13762A, and R3230AC tumor as described in “Materials and Methods.” Proliferation was assessed on Day 3 for mitogen responses and on Days 3 and 5 for tumor cell growth (Fig. 5). TISL significantly suppressed proliferation of T-cells in response to con A, but not B-cell responses to LPS (Fig. 5A). There was also a weak transitory slowing of 18A growth by TISL on Day 3 (Fig. 5B) which was no longer apparent by Day 5 of culture (Fig. 5C). These results indicated that TISL contained predominantly T-cell antiproliferative reactivity that was effective also on mitogen-stimulated T-cell responses.

Time-Course Analysis of TISL Production. Kinetic values of the production of TISL were determined by assaying supernatants collected daily from replicate MTLC containing immune PEC and 13762A. The results shown in Fig. 6 indicated that TISL was not detectable until Day 3 of culture and reached maximum levels between Days 4 and 5.

Cell Type Involved in the Production of TISL. Experiments were set up to determine which population of lymphocytes produced TISL in response to 13762A. Immune PEC and nonimmune spleen cells were pretreated with either guinea pig serum as a source of complement or with rabbit anti-rat thymocyte serum plus complement (as described in “Materials and Methods”) and then plated into MTLC. Proliferation was assessed on Day 5 of culture, and supernatants were collected to be assayed for TISL. 18A-induced proliferation was abolished completely by anti-rat thymocyte serum plus complement, but was unaffected by complement alone (data not shown). The production of TISL from both immune PEC and normal spleen cells was eliminated also by pretreatment of lymphocytes with anti-rat thymocyte serum and complement (Fig. 7). Complement only pretreatment had no effect upon the production of TISL. These results indicated that T-cells were required for the production of TISL.

Preliminary Molecular Size Analysis of TISL. Supernatants containing TISL were placed on an AcA34 column, and 5-ml fractions in PBS were collected as described in “Materials and Methods.” Selected fractions were filter sterilized and assayed for antiproliferative activity in the immune PEC anti-18A response. The results shown in Fig. 8 (one of two similar column separations tested) indicated that TISL in physiological buffers was of apparent high molecular weight, since activity appeared predominantly in the void volume (Mₐ > 350,000).

Physicochemical Properties of TISL. Crude supernatants as well as AcA34 column fractions containing TISL were given various pretreatments as described in “Materials and Methods” to determine some preliminary physicochemical properties of the lymphokine. Crude supernatant containing approximately 600,000 units/ml of TISL was diluted 100-fold in PBS; pretreated with trypsin, chymotrypsin, and proteinase K for 3 h at 37°C; and then titrated into fresh MTLC. The results of these treatments and others are summarized in Table 1. TISL was shown to be (a) resistant to RNase and 56°C treatment for 30 min, (b) partly sensitive to acidic conditions (5-fold reduction of titer) and to 75°C treatment for 15 min (75-fold reduction...
of titer), and (c) completely destroyed by boiling for 10 min. Pretreatment of TISL with the enzymes trypsin and chymotrypsin decreased the activity by a factor of 2–3 (trypsin had no apparent effect in one experiment), and proteinase K decreased the activity by a factor of 3–4. TISL in both crude supernatants and in PBS was stable at 4°C for greater than 6 mo and for 24 h at 37°C (data not shown).

DISCUSSION

In this study we have shown that rat mammary adenocarcinoma 13762A stimulated the release of a soluble T-cell suppressor lymphokine which had antiproliferative activity in vitro. TISL was produced from tumor-immune glass-nonadherent PEC and from normal nonimmune spleen cells specifically in response to 13762A tumor. Neither a highly immunogenic clone of the parental 13762A tumor (clone 18A) nor an antigenically unrelated mammary tumor R3230AC induced the secretion of the suppressor lymphokine from the same population of lymphocytes. The lymphokine was not produced by cultures containing mitomycin C-treated normal spleen filler cells and 13762A tumor cells, thereby demonstrating that the suppressive activity was not due to shed 13762A tumor antigens.

During the development of assays for the detection of TISL, initial experiments were designed to determine why the proliferative response of immune PEC to 13762A tumor was so poor despite strong IL-2 production (29). 13762A tumor cells strongly inhibited both the immune PEC response to 18A tumor and the primary allogeneic MLR, but not a con A-induced normal spleen cell response. The resulting inhibition of proliferation was caused by a soluble suppressor lymphokine retrievable from 5-day MTLC contained 13762A tumor cells. TISL, however, had selective inhibitory effects on a variety of immune responses. Both the con A-stimulated and the PEC anti-18A responses were inhibited strongly by TISL. In contrast, the 13762A-induced immune spleen cell response which is predominantly a T-cell response (29), the LPS response of normal spleen B-cells, and the in vitro proliferation of the tumor cell lines 13762A, 18A, and R3230AC were unaffected by greater than 10,000 units/ml of TISL. (Immune spleen cell proliferation induced by con A, however, was suppressible by TISL; data not shown.) Higher concentrations of TISL may suppress these latter responses, or alternatively, the effects of TISL may be enhanced by other lymphokines such as IL-2, as reported for the antigen-induced T-cell response of rat lymph node cells to soluble antigens (16). This hypothesis is supported by the finding that the immune spleen cell response to 13762A does not produce detectable amounts of IL-2, whereas the immune PEC response to both 18A and 13762A tumor, as well as the con A-stimulated responses produce high amounts of IL-2 (29).

TISL, but not 13762A tumor, significantly suppressed proliferation when added to con A-stimulated normal spleen cell responses at Day 0. Time course analysis indicated that TISL production was undetectable during the first 2 days of culture, which would prevent 13762A-induced TISL from suppressing a 2-day con A response containing 13762A tumor cells. Significantly, not all cultures of 13762A-stimulated lymphocytes produced TISL. Both immune spleen cells and glass-adherent immune PEC did not produce detectable amounts of TISL when cultured with 13762A tumor. Weak production of TISL was observed occasionally from 13762A-stimulated immune spleen cells but only when concomitant high background proliferation of unstimulated immune spleen cells also occurred. Background or spontaneous proliferation of rat T-cells in culture has been reported often (30, 31) and was observed also for the nonimmune spleen cell responses described in Fig. 7. IL-2 has been detected in cultures where spontaneous proliferation occurred (data not shown) and may have contributed to the TISL activity that was found in the 13762A-stimulated non-immune spleen cell response (Fig. 7) as hypothesized above.

Analysis of the cell types involved in TISL production, as determined by antibody plus complement-mediated cytolysis, indicated that T-cells were the producers of TISL. Pretreatment of immune PEC and normal spleen cells with a rabbit anti-rat thymocyte serum plus complement or complement alone as described in "Materials and Methods" and then plated into MTLC. Supernatants from the MTLC were collected after 5 days and assayed for TISL activity by titration into fresh immune PEC anti-18A responses as described in Fig. 4. TISL activity in supernatants taken from complement pretreated only MTLC is shown by open symbols and from rabbit anti-rat thymocyte serum plus complement or complement alone as described in "Materials and Methods" and then plated into MTLC. Supernatants from the MTLC containing ISA (•, D) or 13762A (•, O). Points, mean cpm of triplicate cultures; bars, SEM. S/N, supernatant. TdR, thymidine.

Fig. 7. Effect of rabbit anti-rat thymocyte serum plus complement on TISL production. Immune PEC (A) and nonimmune spleen cells (B) were pretreated with rabbit anti-rat thymocyte serum plus complement or complement alone as described in "Materials and Methods" and then plated into MTLC. Supernatants from the MTLC were collected after 5 days and assayed for TISL activity by titration into fresh immune PEC anti-18A responses as described in Fig. 4. TISL activity in supernatants taken from complement pretreated only MTLC is shown by open symbols and from rabbit anti-rat thymocyte serum plus complement or complement alone as described in "Materials and Methods" and then plated into MTLC. Supernatants from the MTLC containing ISA (•, D) or 13762A (•, O). Points, mean cpm of triplicate cultures; bars, SEM. S/N, supernatant. TdR, thymidine.

Further analysis of the subsets of cells secreting TISL has been determined by indirectpanning experiments using the monoclonal antibodies W3/25 and OX8 (Accurate Chemicals & Scientific Corporation, Hicksville, NY) which identify T-helper and T-suppressor/cytotoxic cells, respectively. The results of these experiments indicate that cells with W3/25

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![Graph showing TISL activity across different conditions](image)

**Fig. 8. AcA34 size column separation of TISL.** Crude supernatant containing 11,000 units/ml TISL (4-ml total) was loaded on an AcA34 column as described in “Materials and Methods,” and 5-ml fractions in PBS (pH 7.0) were collected. Twenty μl of selected fractions were added to MTLC containing immune PEC, and tumor cells. Proliferation was assessed on Day 5. The peak of suppressor activity (Fraction 9) contained 2100 units/ml TISL activity. O, absorbance at 280 nm. BD, bovine serum albumin; PR, phenol red; TdR, thymidine.

**Table 1 Effect of various pretreatments on the activity of TISL**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TISL batch</th>
<th>TISL activity (control)*</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Proteinase K</th>
<th>56°C</th>
<th>75°C</th>
<th>100°C</th>
<th>RNase</th>
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<td>208 (30)</td>
<td>294 (43)</td>
<td>238 (35)</td>
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<td>&lt;0.5</td>
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<tr>
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<td>71 (37)</td>
<td>16.1 (8.4)</td>
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<td></td>
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</tr>
<tr>
<td>4</td>
<td>7</td>
<td>1493</td>
<td>500</td>
<td>300 (34)</td>
<td>0.53 (25)</td>
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<td>5</td>
<td>AcA34 peak</td>
<td>2.1</td>
<td>0.77 (37)</td>
<td>0.50 (24)</td>
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* Supernatants containing TISL were control treated (37°C, 3 h) and tested for TISL activity.

**Surface phenotype are essential for the production of TISL, but the cell producing TISL bore the OX8 differentiation antigen.**

Based on physicochemical properties including relative resistance to acidic conditions, TISL does not appear to be IFN-γ, which is an acid-sensitive cytotoxic molecule. TISL appears to be a cytostatic lymphokine, since no decrease in either the proliferation or viable cell number over controls during the first 24 h of a con A response was observed (data not shown), and TISL had selective antiproliferative activity against a variety of lymphocyte responses.

When compared with other less characterized antiproliferative suppressor lymphokines (1–22), TISL has some properties in common with TLSL (13), SAF (12), an immunosuppressive factor produced from adult T-cell leukemia cells (14), carcinoembryonic antigen-induced suppressor lymphokine (22), and leukoregulin (20, 32). TLSL, SAF, and carcinoembryonic antigen-induced suppressor lymphokine were high-molecular-weight cytostatic lymphokines, but the high molecular weight was due to an aggregation phenomenon in the presence of serum (13). The high molecular weight of TISL is due most likely to this same aggregation behavior. However, TISL in contrast to TLSL was totally destroyed by 56°C treatment for 30 min, and by 75°C treatment for 15 min. TISL activity was degraded only partly by the enzymes trypsin, chymotrypsin, and proteinase K. The cytostatic suppressor lymphokine leukoregulin (32) and carcinoembryonic antigen-induced suppressor factor (22) were shown also to be degraded partially by trypsin, chymotrypsin, and Pronase (the activity was decreased by only 50% of the activity of the controls), whereas lymphotoxin was completely destroyed by these enzymes (32). The immunosuppressive factor produced from adult T-cell leukemia cells was shown also to be resistant to trypsin treatment (14). Proteinase K was used to treat TISL at a concentration which was 50 times more than the amount needed to completely destroy all cells in the microcultures over a 5-day period. It is possible that the biological activity of TISL resides in small peptide fragments and/or in a nonprotein component of the molecule. Carbohydrate was shown to be important for the biological activity of carcinoembryonic antigen-induced suppressor lymphokine, and it was hypothesized that extensive protein glycosylation of the lymphokine prevented degradation by proteolytic enzymes (22). Extensive protein glycosylation may be a property that is common to this family of cytostatic antiproliferative lymphokines. The relative resistance of TISL to trypsin treatment also distinguishes this suppressor lymphokine from stimulated rat T-cell-derived inhibitory factor for cellular DNA synthesis, which was trypsin sensitive (9, 10).

In conclusion, tumor antigen(s) of the poorly immunogenic rat mammary adenocarcinoma 13762A stimulated the release of a soluble antiproliferative suppressor lymphokine (TISL) from rat T-cells. TISL had selective antiproliferative activity against a variety of immune responses and showed predominantly anti-T-cell reactivity. The highly immunogenic regressor clone 18A (27) did not express the suppressor-stimulating
antigen(s) of the parental tumor. Of significance was the finding that 13762A could induce the production of TISL from non-immune spleen T-cells. These data indicate that naturally occurring T- cells exist in rats which can be triggered without prior immunization. Such lymphocytes could provide a potential mechanism for the protection of any tumor cell expressing the appropriate T,-activating surface antigens if the suppressor lymphocytes are activated in vivo.

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Neil D. Christensen, John W. Kreider and Rick L. Horetsky


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