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Modulation of Immune Response and Tumor Development in Tumor-bearing Mice Treated by the Thymic Factor Thymostimulin

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ABSTRACT

Thymostimulin (TS), a partially purified thymic factor, has a significant impact on tumor development in C57Bl/6 mice inoculated with Lewis lung carcinoma (3LL) cells, as judged by its effect on time of tumor appearance after tumor cell transplantation. In a previous study, we determined the conditions under which survival rate of the tumor-bearing mice can be significantly increased by TS treatment. In the study communicated here we analyzed host defense mechanisms that are modified by TS treatment in the tumor-bearing mice. In general, immune parameters that were increased or stimulated by the presence of the tumor were further increased in the TS-treated animals (number of lymphoid spleen cells, their response in mixed lymphocyte tumor cultures, their natural killer cell activity, and their ability to produce colony-stimulating factor), or reached earlier maximum levels (spontaneous [^{3}H]thymidine incorporation, a reflection of *in vivo* spleen cell activation). Responses which reflect tumor-induced immunosuppression (proliferative response induced by phytohemagglutinin or concanavalin A stimulation) were restored to normal level by TS. Specific tumor-related reactions (specific cell-mediated cytotoxicity) were preserved in the TS-treated animals. The wide spectrum of TS effects had, nevertheless, certain elements of selectivity; *e.g.* colony-stimulating factor, but no interferon production is enhanced by TS in the tumor-bearing mice in diametric contrast to TS effect in Mengo virus-infected mice. The spectrum of TS effects was also dependent on the type of tumor cell used. The results indicate that the significant effect of TS on 3LL tumor development in mice is associated with a strong, multifaceted effect of TS on the immune system.

INTRODUCTION

The potential of thymic hormones for the treatment of cancer has been suggested by several studies, both experimental and clinical (1–12). In a previous study (4) we were able to show that the thymic factor TS[2] (13) has a significant impact on the development of a transplanted tumor, Lewis lung carcinoma (3LL) in its syngeneic host, the C57Bl/6 mouse. TS by itself caused a significant delay in time of tumor appearance, but did not change ultimate survival rate of the tumor-bearing mice. However, when TS treatment was combined with either chemotherapy [lomustine 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] or resection of primary tumor or both, a synergistic, highly significant effect of these combinations on survival and cure rates of the animals was observed (4).

The remarkable antitumoral effect observed in that study was in all probability related to the immunomodulatory properties of TS. Indeed, in previous studies, TS was shown to modulate several immune functions, including proliferative response of human peripheral blood mononuclear cells in MLTC (14, 15) CMC of such cells (16) and their capability to produce immune interferon (17). Moreover, in a randomized clinical study performed by us on TS treatment of chemotherapy-treated patients with advanced gastrointestinal cancer (9), TS was shown to have a significant, strong lymphocytopoietic effect and to convert negative delayed type hypersensitivity skin tests to dinitrochlorobenzene to positive ones. This effect was observed against the immunosuppression caused by both the developing tumor and the cytotoxic drugs used to treat the patients. No clear-cut effect of TS on the survival of these patients could be observed. The lessons learned from all these studies were implemented into another randomized clinical study, which is still ongoing, on TS treatment of stage I and II melanoma patients during their disease-free interval. An interim review of the results (1) indicate that TS has an effect on the duration of disease-free interval and survival.

Returning to the experimental model of 3LL in mice, we felt that it offers us the opportunity to analyze host defense mechanisms involved in changing tumor development after TS treatment, in a more systematic way. We designed a multistage analysis, first, of TS effect on animals treated by a cytotoxic drug like lomustine 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (18), second, on TS effect on animals inoculated with 3LL cells and, finally, the combined effects of TS and chemotherapy or amputation. Having the repertoire of changes caused by TS, we would probably be able to evaluate which one is responsible for the observed control of tumor development.

The study communicated here is, accordingly, the second part of this plan, namely the effect of TS *per se* on tumor development and on some immune functions in mice inoculated with 3LL tumor cells. The transplanted 3LL tumor is known to cause significant immunosuppression of the host (19). This is not an exception, since nonspecific immune suppression, affecting mainly the T-cell compartment of the immune system, was reported in several experimental tumors in animals as well as in clinical cancer (20–23). We report here that TS-induced immunomodulation encompasses both tumor-related and -unrelated immune responses.

MATERIALS AND METHODS

Mice. Six to 10-week-old male C57Bl/6 mice were purchased from Lewonstein Animal Farm, Yokneam, Israel. Mice were kept under standard conditions during experiments and weighed, on the average, 25 g at the start of the experiments.

Thymostimulin. Thymostimulin (TS or TP-1), a partially purified thymic extract of bovine origin, was prepared by Istituto Farmacologico Serono, Rome, Italy, and kindly provided to us by their producer as a lyophilized, sterile, endotoxin-free preparation (4, 9, 13). The material was kept at 4°C and dissolved in PBS before use.

Cells and Cell Cultures. The following cell lines were used in this study: 3LL cells; two clones (U and Z) of different tumorigenicity isolated in our laboratory from the 3LL parental line (24); L_{929} mouse cells (for titration of antiviral activity of mouse interferon); YAC-1 mouse lymphoma cells (for measurement of NK activity). Cells were cultured in plastic tissue culture dishes in Dulbecco's modified Eagle's medium, high glucose (GIBCO, Grand Island, NY) with 10% fetal calf serum (GIBCO). Routine passage of anchorage-dependent cells (3LL and L_{929}) was made once per week with 0.25% trypsin solution (1:300) or by dilution for cells in suspension (YAC-1). There was no detectable

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[2] The abbreviations used are: TS, thymostimulin (TP-1 Serono); PHA, phytohemagglutinin; ConA, concanavalin A; NK, natural killing; CMC, cell-mediated cytotoxicity; MLTC, mixed lymphocyte tumor culture; CSF, colony-stimulating factor; IFN, interferon; PBS, phosphate-buffered saline.

bacterial, fungal, or mycoplasmal contamination of cultures used in this study.

Study Design. Mice were inoculated with 1×10^5 3LL cells (usually parental line; in some experiments U or Z clones) into the hind footpads. TS (4 mg/kg) or PBS control was injected i.p. six times per week starting on day of tumor inoculation and continuing until animals were sacrificed. The final TS injection was always 1 day before animal sacrifice.

At several time intervals after tumor inoculation (usually 3 to 18 days) animals were sacrificed by cervical dislocation, their spleens removed and dissociated into single cell suspensions by teasing. Nucleated cells were counted in a hemocytometer, their viability determined by eosin Y exclusion, and their morphology assessed after Giemsa staining. Usually, more than 90% of the dissociated spleen cells were found to be viable (suspensions with less than 85% viable cells were discarded).

The spleen cell suspensions were used either immediately for the different assays (see below) or, in some experiments after *in vitro* incubation with TS (1 μg/ml serum-free medium for 1 h at 37°C).

Controls included tumor-bearing animals treated with the diluent (PBS) only or tumor-free animals of the same lot treated by a similar schedule of either TS or its diluent.

Proliferative Responses. These responses were determined on either unstimulated spleen cells ("spontaneous incorporation") or on spleen cells stimulated by either PHA, ConA, or 3LL cells (mixed lymphocyte tumor culture, MLTC). All these assays were performed as previously described (14) in microwell plates (flat bottomed, Nunc, Denmark) using RPMI 1640 medium containing 10% fetal calf serum (GIBCO). One μCi of [^{3}H]thymidine was added to each well 18 h before harvesting. Harvesting was performed in a semiautomatic harvester and results read in Packard's liquid scintillation spectrometer. For spontaneous [^{3}H]thymidine incorporation, 2×10^5 freshly dissociated spleen cells were distributed into microwell plates together with [^{3}H]thymidine and harvested 18 h later.

Lectin stimulation was performed with PHA (Burroughs-Wellcome) 2.5 μg/ml or ConA (Bio-Yeda, Rehovot, Israel) 2 μg/ml. The cultures were incubated for the time indicated below in a humidified CO_2 incubator at 37°C. Cultures were kept for 72 h, with the addition of [^{3}H]thymidine for the last 18 h of incubation.

MLTC was performed using mitomycin C (Sigma, St. Louis, MO) treated 3LL cells (4×10^6 cells with 100 μg mitomycin in 1 ml medium for 1 h at 37°C). The 3LL cells were washed after mitomycin treatment and 1×10^4 cells were mixed with 1×10^5 viable nucleated spleen cells in a final volume of 0.2 ml RPMI 1640 containing 5% human AB serum for 3–7 days.

Assays for Lymphocytotoxicity. The assays for natural killer (NK) cell activity and specific cell-mediated cytotoxicity (CMC) were performed using ^{51}Cr-labeled YAC-1 or the 2 3LL clones (U and Z), as target cells, respectively, as previously described (16, 25). Briefly, labeling with chromium-51 was done by incubation of 1×10^6 target cells with 100 μCi chromium-51 for 2 h (YAC-1) or overnight (U or Z clones). The target cells were washed 3 times in PBS and 1×10^4 cells of each line were mixed with 10, 30, or 100 times more nucleated viable spleen cells in 0.2 ml (final volume) of RPMI 1640 with 2% fetal calf serum. The mixed cultures were incubated for 4 or 18 h in a humidified CO_2 incubator at 37°C. The results with 4 and 18 h were essentially similar in our system. The time of incubation for each experiment is specified in the results. After incubation, 0.1 ml of medium were collected and radioactive emission read in Packard's Automatic Gamma Counting System. Cytotoxicity was calculated as percent lysis according to the formula:

$$\% \text{ Specific chromium-51 release} = \text{exp. cpm} - \text{spont. cpm}/\text{max. cpm} - \text{spont. cpm} \times 100 \quad \text{(A)}$$

where spontaneous (spont.) cpm was the reflection of chromium-51 release in cultures containing target cells only and maximal (max.) cpm was based upon chromium-51 release in similar cultures after the addition of 1 N HCl.

Assays for Lymphokine Production. For the assessment of CSF production, spleen cells of sacrificed animals (1×10^7 cells/ml) were cultured for 4 days in RPMI 1640 medium containing 10% pooled human serum and 5×10^{-5} M 2-mercaptoethanol, but without the addition of a mitogen. The medium conditioned in these cultures was tested for its colony stimulating activity on BALB/c bone marrow cells using the bilayer agar method for growing granulocyte macrophage colonies (26). The colonies were counted after 7 culture days. Our previous experience with this method indicates a linear correlation between concentration of CSF and number of colonies.

Serum IFN was measured by a plaque reduction assay, and by an immuno enzymatic assay as previously described (27).

Statistical Analysis. All experimental points were in triplicates and each experiment was repeated at least three times. The results are generally from one representative experiment and reported here as a mean ± one standard deviation. Significance was tested by paired Student's *t* test.

RESULTS

Effect of TS on Tumor Development

Although this part was studied in detail previously (4), we repeated the experiments related to the effect of TS *per se* (not combined with chemotherapy or amputation), as a preparatory step for the present study. Indeed, TS had an effect on tumor development, expressed as a significant delay in time of tumor appearance (Fig. 1). The effect was dose- and schedule-dependent. No effect could be seen on the kinetics of tumor development after its appearance or on ultimate survival. For further experiments, we selected the 4 mg/kg dose, six times per week, as a dose schedule representing a significant biological effect. The results also indicated that the best time to look for TS effect on the immune system in these animals should be the first 20 days after tumor inoculation.

Appearance of Spleen and Spleen Cell Number

The spleen size of tumor-bearing animals gradually increased as a function of time after tumor inoculation and this was reflected in about 20% increase in the number of nucleated cells in the spleen (Fig. 2) which were primarily of lymphoid morphology. At later stages (more than 20 days after tumor inoculation), increasing numbers of tumor cells started to occupy the spleen, as judged from experiments in which 3LL cells were cultivated from the spleen. This was an additional indicator for us to confine our experiments to the first 20 days or less.

Effect of TS on spleen cell number could be clearly demonstrated from day 10 after tumor inoculation (Fig. 2). There was

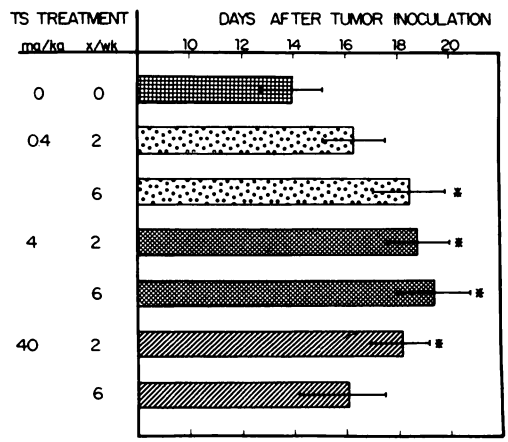

Fig. 1. Determination of the effect of TS on tumor development as measured by time of tumor appearance. C57Bl/6 mice were inoculated with 1×10^5 3LL cells to footpad and TS treatment with the indicated doses was started 1 day after tumor inoculation, six times or twice per week. *Columns*, mean ± SE of results with 12 mice; *, $P < 0.05$.

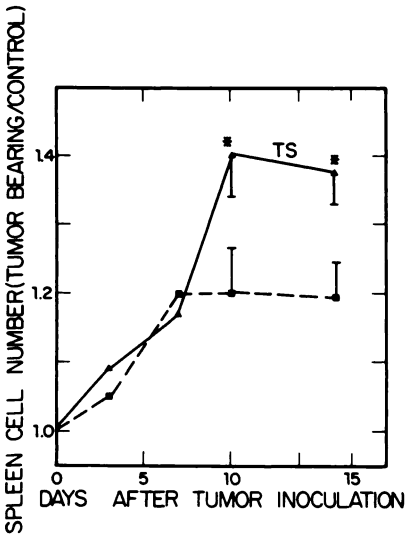

Fig. 2. Effect of TS on spleen cell number of mice inoculated with 3LL cells. ▲——▲, TS treated mice; ■- - -■, untreated controls. Results expressed as ratio of spleen cell number in tumor bearing *versus* control (nontumor bearing) mice. Spleen cell number on day 0 = $1.15 \times 10^6 \pm 0.9 \times 10^7$. *Points*, mean ± SD of the results with four mice; *vertical bars*, ±1 SD; *, $P < 0.05$.

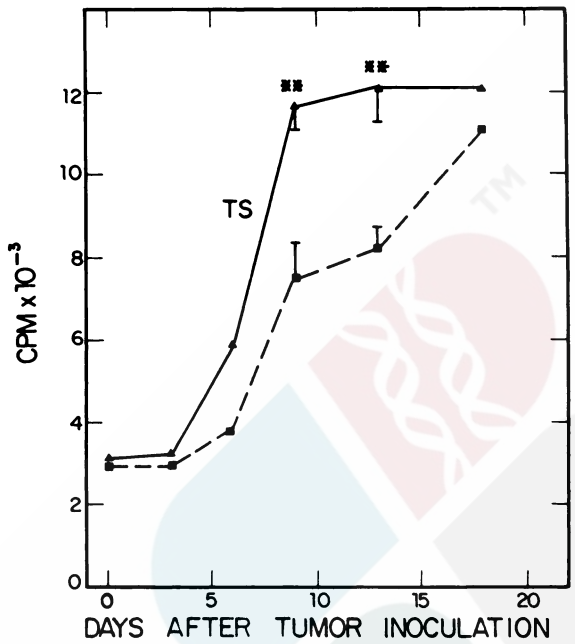

Fig. 3. [^{3}H]Thymidine incorporation in freshly isolated, unstimulated spleen cells ("spontaneous incorporation") taken from mice inoculated with 3LL cells and treated with TS (▲——▲) or diluent (PBS) control (■- - -■). Four mice per point. **, $P < 0.01$.

about a 15–17% increase in spleen cell number after TS as compared to untreated, tumor-bearing mice. No significant effect of TS on spleen cell number could be observed in normal, tumor-free animals.

Proliferative Responses

Several types of proliferative response were tested.

Spontaneous [^{3}H]Thymidine Incorporation (Fig. 3). This simple test, which consists of overnight incubation of freshly isolated unstimulated spleen cells with [^{3}H]thymidine, most probably reflects *in vivo* activation of the lymphoid system. Indeed, a gradual 3-fold increase in [^{3}H]thymidine incorporation could be observed from day 6 to 18 after tumor inoculation. The increase was, however, much steeper with TS and reached maximal level after 9 days. No TS effect on this test could be observed in tumor-free animals.

Response to Lectin Stimulation (Fig. 4). The response to PHA or ConA were profoundly suppressed by the growing tumor, response to PHA being more affected than to ConA. TS treatment caused restoration of the response to ConA to virtually normal levels, and prevented suppression of PHA responses.

Mixed Lymphocyte Tumor Cultures (Figs. 5 and 6). Spleen cells of either tumor-bearing or tumor-free mice respond to the syngeneic 3LL tumor cells in MLTC. The response peaked at 6 days (sometimes 5 days) and was significantly stronger (>2-fold) in the tumor-free animals, as compared to the tumor-bearing ones (Fig. 5). Because of these differences, we tested TS effect both *in vitro* (Fig. 5) and *in vivo* (Fig. 6). Incubation of spleen cells with TS (1 μg/ml) for 1 h before MLTC resulted in opposite effects in spleen cells of tumor-bearing (Fig. 5*A*) or tumor-free mice (Fig. 5*B*) enhancing it in the former and suppressing it in the latter.

The ability of spleen cells of tumor-bearing mice to respond to the tumor cells in MLTC *in vitro* increased slowly with time after tumor inoculation (Fig. 6). It was, however, much augmented by TS treatment *in vivo*. This effect peaked at 13 days. No TS effect could be observed in tumor-free mice.

Lymphocytotoxicity

Two types of cytotoxic activities were tested: natural killing and specific CMC.

Natural Killer Cell Activity (Fig. 7). NK activity was tested using ^{51}Cr-labeled YAC-1 cells as targets. The results indicate that the presence of the tumor by itself enhances NK activity

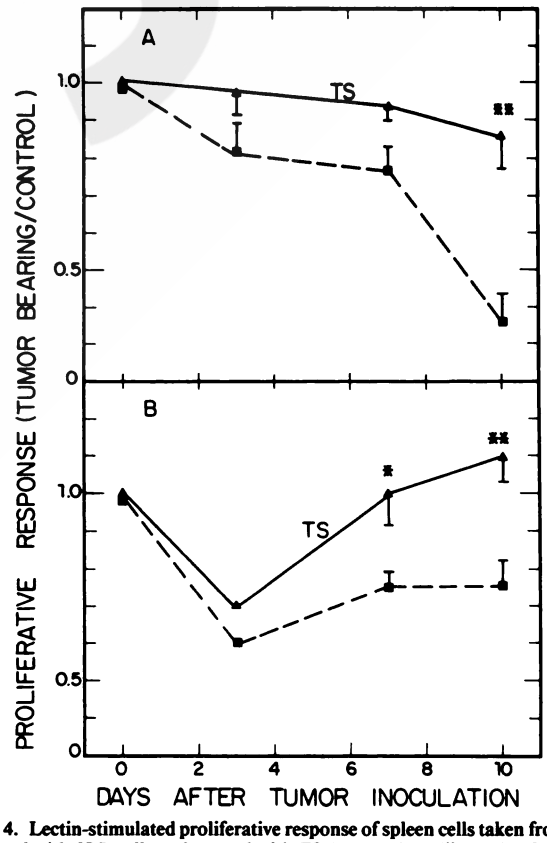

Fig. 4. Lectin-stimulated proliferative response of spleen cells taken from mice inoculated with 3LL cells and treated with TS (▲——▲) or diluent (PBS) control (■- - -■). *A*, cultures stimulated with PHA; *B*, cultures stimulated with Con A. Results expressed as ratio of cpm ([^{3}H]thymidine incorporated) in spleen cells of tumor bearing *versus* control mice. cpm on day 0 = $67 \times 10^3 \pm 7.3 \times 10^3$ (PHA); $92 \times 10^3 \pm 8.5 \times 10^3$ (ConA). Four mice per experimental point. *Vertical bars*, mean ± 1 SD; *, $P < 0.05$; **, $P < 0.01$.

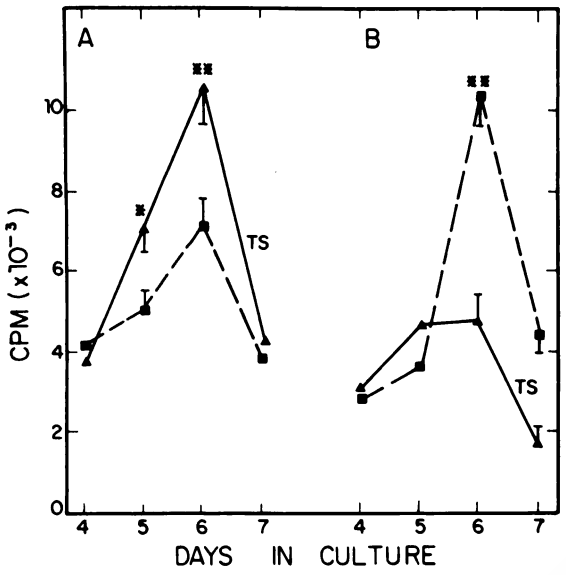

Fig. 5. Mixed lymphocyte tumor culture (MLTC) with spleen cells of tumor-bearing (*A*) or tumor-free (*B*) mice as responders and mitomycin-treated 3LL cells as stimulators. Responders were preincubated with TS [1 μg/ml in serum-free medium for 1 h at 37°C (▲——▲)], or medium control (■- - -■). Tumor-bearing mice were used 9 days after tumor cell inoculation. *Bars* and *P* values as in Fig. 4.

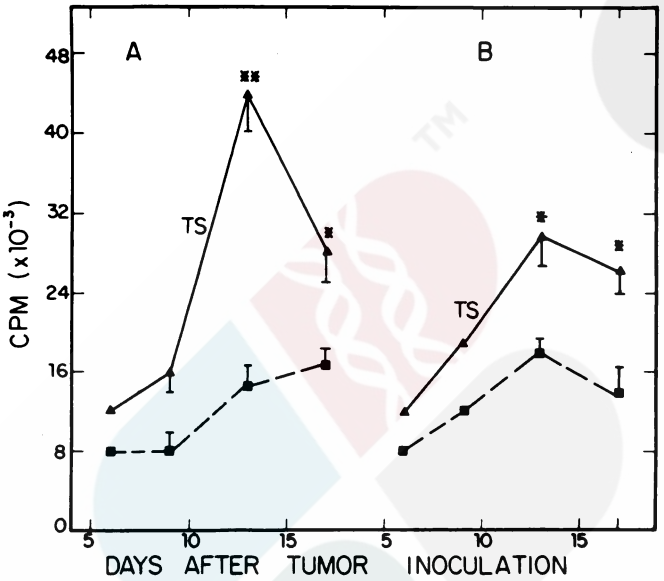

Fig. 6. MLTC with spleen cells taken from tumor-bearing mice as responders and mitomycin C-treated 3LL cells as stimulators. Mice were treated with TS (▲——▲) or diluent (PBS) control (■- - -■). *A*, 5-day cultures; *B*, 7-day cultures. *Bars* and *P* values as in Fig. 4.

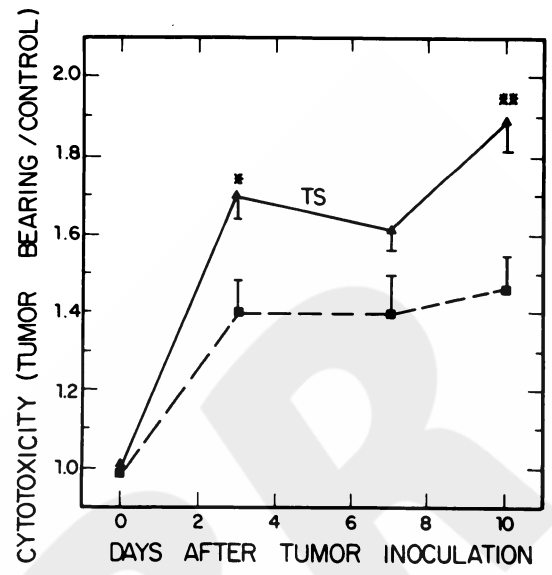

Fig. 7. Natural killer cell activity of spleen cells taken from tumor-bearing mice against ^{51}Cr-labeled YAC-1 cells in an 18-h assay. Mice were treated with TS (▲——▲) or diluent (PBS) control (■- - -■). Results expressed as ratio of % specific lysis of YAC-1 cells by spleen cells taken from tumor-bearing mice *versus* those taken from control mice. Effector:target ratio, 30:1. Percentage of specific lysis on day 0: 22 ± 4%. Four mice per experimental point. *Bars* and *P* values as in Fig. 4.

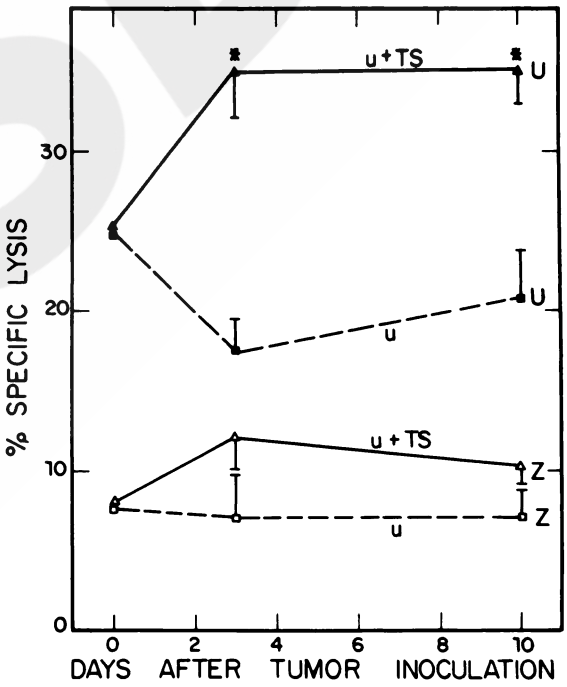

Fig. 8. Cytotoxicity of spleen cells taken from mice inoculated with 3LL-clone U cells and tested against ^{51}Cr-labeled U or Z cells in a 4-h assay and using effector:target ratio of 30:1. Mice were treated with TS (△——△; ▲——▲) or diluent (PBS) control (□- - -□; ■- - -■). *Bars* and *P* values as in Fig. 4. See text for more details.

of spleen cells. However, TS treatment further increased NK activity significantly.

Specific Cell-mediated Cytotoxicity (Fig. 8). In this part of the study we took advantage of the 3LL clones of different tumorigenicity previously developed by us (24). We selected two clones, clone Z with low tumorigenicity (no tumor take 120 days after inoculation of 1×10^4 cells) and clone U (40% take under these conditions). These two clones also manifested other biological differences (*e.g.*, different doubling time and saturation density in culture, different appearance in scanning electron microscopy and different karyotype). Animals were inoculated with U clone cells, treated with TS (or a diluent control), and their spleen cells were used in a cytotoxicity assay against either U or Z cells (Fig. 8). The results of day 0 reflect probably NK cell activity and indicate that U cells are more sensitive to NK lysis than Z cells. Indeed, competition experiments with cold YAC-1 cells added in excess to ^{51}Cr-labeled target tumor cells (30:1) significantly reduced target cell lysis. In contrast, the increase in cytotoxicity observed on day 10 in mice inoculated with clone U and treated with TS resulted in specific cytotoxicity which was not reduced by YAC-1 cell competition. The competition results on day 3 were intermediate between those of day 0 and day 10. The cytotoxicity against Z cells was not significantly changed by TS (Fig. 8). The assays were performed for 4 and 18 h, with similar results.

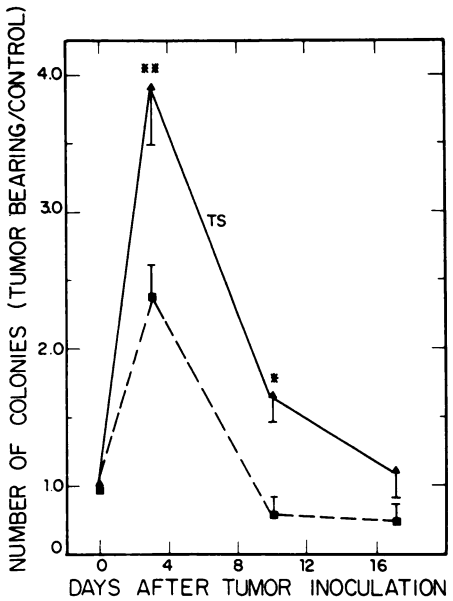

Fig. 9. CSF activity in supernatants of spleen cells taken from mice inoculated with 3LL cells and treated with TS (▲——▲) or diluent (PBS) control (■- - -■). *Bars* and *P* values as in Fig. 4. Results expressed as ratio of number of colonies induced by 10% supernatant of spleen cells taken from tumor-bearing *versus* control mice. Number of colonies on day 0: 72 ± 16.

Lymphokine Production

Two lymphokines were tested in this study:

Colony-stimulating Factor. There is a sharp increase in the ability of spleen cells to produce CSF 4 days after tumor inoculation with return to normal levels subsequently (10 days). Spleen cells of TS-treated tumor-bearing animals had a higher capacity of CSF production (Fig. 9).

Interferon. No effect of TS on the production of IFN could be observed in either the tumor-bearing or tumor-free animals, as measured by serum interferon levels. In fact, there were barely detectable levels of IFN in the serum (<16 IU/ml) under all the experimental conditions tested in the present study (data not shown). In contrast, in another experimental system studied by us, that of Mengo virus-inoculated mice (27), serum interferon levels were significantly increased by TS, whereas no effect on CSF production by spleen cells could be observed.

DISCUSSION

In the study communicated here we demonstrated the effect of TS on some immune functions in mice bearing the transplantable 3LL tumor. Several conclusions may be drawn from these data.

In general, TS effect on spleen cell functions is stronger and earlier than its effect on spleen cell number. The latter effect (Fig. 2) is, nevertheless, of interest. Tumor-induced splenomegaly is a well-documented phenomenon (28, 29). As with splenomegaly induced by microbial infections, it is related to the intensification of the activities of the immune system. TS apparently enhances this trend. We try now to analyze the changes in lymphoid spleen cell population as a kinetic function of tumor development and TS effect and to relate it to the respective changes in function documented here. It will also be of interest to analyze TS effect on the tumor-induced thymic atrophy (30–31) and on intratumoral and regional (lymph nodes) immune responses.

There is a wide spectrum of TS effects on T-cell functions. However, TS activity has certain elements of selectivity. Thus, CSF production, but not IFN serum levels, is increased by TS. This selectivity is further emphasized by the fact that in another experimental system, that of virus-inoculated mice, we found a strong effect of TS on serum IFN levels (27), but not on CSF production by spleen cells.[3] We observed TS effect on production of type II IFN *in vitro* (17) or type I IFN *in vivo* (27). Our assay methods (27) can detect both types of IFN. TS augmented NK activity (Fig. 7) in the absence of a detectable effect on IFN levels [which was also found in another study (32)]. However, there is no direct effect of TS on NK activity *in vitro*, thus reducing the likelihood of a direct effect *in vivo*. Local intrasplenic effects of low levels of IFN (undetectable in the serum) on NK activity can be envisaged, or alternatively, TS may regulate the production of a non-IFN, NK-cell regulatory factor.

The spectrum of TS effects was also dependent on the type of tumor cells. Thus, with two isolated clones of 3LL (M and T), TS caused different effects on the two types of lymphocytotoxicity tested, *i.e.*, augmentation of NK activity in the M and not the T clone-inoculated mice and augmentation of specific CMC in the T and not the M clone-inoculated mice.[4]

Although most of the tests used by us reflect the expected nonspecific effect of TS in this experimental system, it was nevertheless of interest to evaluate TS effect in an immune specific test. The availability to us of different clones of 3LL, which differ considerably in their biological characteristics, was of great advantage in this connection. Indeed, we found that the CMC activity in the tumor-bearing animals was specific to the inoculated clone and that TS-induced enhancement of CMC preserved this specificity (Fig. 8). A similar finding related to lymphocytotoxicity of human peripheral blood mononuclear cells was previously reported by us (16).

In summary, TS effect on tumor development (Fig. 1 and Ref. 4) is associated with strong, widespread effect of TS on the immune system. 3LL is known to combine high tumorigenicity with low immunogenicity and immunsuppressive effects, resulting in weak host response and relentless tumor development. TS can apparently modify this situation, to a more favorable one.

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