Preliminary *in vivo* evaluation of the protein transduction domain-modified ATTEMPTS approach in enhancing asparaginase therapy

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Abstract: Asparaginase (ASNase) is an enzyme drug presently approved for the induction of remission in the treatment of patients with acute lymphoblastic leukemia (ALL). The cytotoxic effect of ASNase is derived from its ability to deplete asparagine, an essential amino acid required by certain types of leukemia cells for protein synthesis and survival. Despite its efficacy in enhancing disease remission rate and prolonging complete remission duration in ALL patients, ASNase therapy is nevertheless confounded by a number of serious toxic effects, particularly to organs associated with high protein production (e.g., liver, pancreas), due to the systemic depletion of asparagine. Presented herein is a modified version of our previously established ATTEMPTS protein delivery system that carries the potential to permit a tumor specific, intracellular delivery of ASNase, thereby allowing for a significant reduction of ASNase-induced systemic toxicity. In a previous paper, we already demonstrated the *in vitro* feasibility of this heparin/protamine-regulated, TAT-mediated system in delivering ASNase directly into ASNase-sensitive murine lymphoma cells. In this article, we further validated the *in vivo* applicability of this system in animals harboring ASNase-encapsulated L5178Y lymphoma cells. Preliminary results showed that animals inoculated with L5178Y cells containing TAT-ASNase exhibited an extended survival rate of ~13% over those harboring L5178Y cells without the encapsulation of ASNase. Furthermore, the TAT-ASNase-treated mice also displayed a significantly improved hematological and liver histological status than the control groups. These findings bring promise to the use of the modified ATTEMPTS delivery system in achieving enhanced ASNase therapy. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 91A: 209–220, 2009

Key words: ATTEMPTS approach; asparaginase therapy; prodrug; targeting; protein transduction domain (PTD); peptide

INTRODUCTION

Asparaginase (ASNase) is an enzyme drug presently approved for the induction of remission in the treatment of patients with acute lymphoblastic leukemia (ALL).[1,2] Its mechanism of action is mainly attributed to the hydrolysis of nonessential amino acid asparagine (ASN) into aspartic acid and ammo-

nia, resulting in depletion of serum ASN. Leukemic cells respond to ASNase treatment because they lack the ability to synthesize ASN themselves, therefore, an extracellular supply of ASN is crucial for protein synthesis.[3] Shortage in ASN supply would lead to delayed inhibition of DNA and RNA syntheses and hence an impairment of cellular functions and cell death in leukemic cells.[1–3] Presently, two major bacterial sources of ASNase are used: *Escherichia coli* (*E. coli*) and *Erwinia chrysanthemi* (*Erwinia*). In addition, PEG-ASNase has also been approved for use in patients who have had clinical hypersensitivity reactions to both *E. coli*- and *Erwinia*-derived ASNase.[4] ASNase is usually used in combination with other

chemotherapeutic drugs such as prednisone and vincristine.[5] The inclusion of ASNase in combined drug treatment has significantly enhanced disease remission rate; ASNase-included combination chemotherapy was associated with an initial remission rate of 95%,[6] and has also led to a significantly longer complete remission duration compared with groups of patients without ASNase treatment.[7] However, ASNase therapy is confounded with systemic toxic effects, because it is administered either intravenously or intramuscularly, allowing it to gain access to the systemic circulation. Because ASNase depletes systemic ASN, the organs with high protein or peptide production such as liver and pancreas are also highly affected. Side effects associated with ASNase therapy include thrombotic and hemorrhage complications,[8] immune hypersensitivity,[9] hemorrhagic pancreatitis, hyperglycemia, and neurological disorders.[10] Although PEG-ASNase offers a safer alternative to conventional ASNase therapy in patients who have had clinical hypersensitivity reactions to native ASNase preparations, the systemic toxicity associated with ASNase therapy has not yet been remedied.

In an earlier paper,[11] we described a modified version of our previously established ATTEMPTS approach[12] that potentially could lead to a tumor-specific, intracellular delivery of protein drugs. In brief, this system was comprised of a protein complex made of a targeting component containing an antibody coupled with a heparin (Hep) molecule, as well as a drug component consisting of the protein drug covalently linked with a cell-penetrating TAT peptide via a disulfide bond (see Fig. 1 in Ref. 11). These two components were associated together automatically by a charge–charge interaction between the anionic heparin (on the targeting component) and the cationic TAT (on the drug component). Following intravenous administration, the prodrug-behaved (meaning that the cell-penetrating activity of TAT was inhibited by heparin binding) complex would spare interaction with normal tissues during tumor targeting, thereby aborting the drug-induced toxic effects towards nontargeted tissues. After reaching the target, protamine sulfate, a clinical heparin antidote that binds to heparin more strongly than TAT, would be administered to dissociate the TAT-drug part from its Hep-Ab counterpart. Once relieved from heparin inhibition, TAT would resume its potent cell-penetrating activity, translocating the TAT-drug conjugate into targeted tumor cells. Inside the cells, the drug would be self-detached from TAT because of degradation of S-S linkage by elevated level of glutathione in the cytosol, inducing apoptosis to only tumor tissues. *In vitro* results presented in the previous paper already successfully demonstrated the feasibility of this modified ATTEMPTS system in delivering ASNase into L5178Y leukemic cells. Based on the promise of these find-
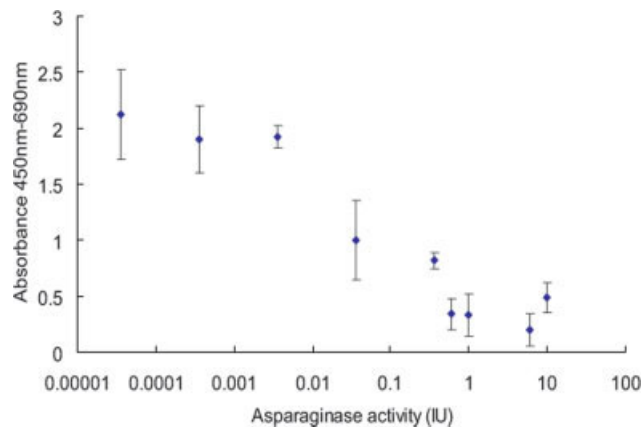

Figure 1. *In vitro* dose dependence curve of asparaginase (ASNase) against L5178Y cells. Cell viability was measured using the XTT assay. Lower absorbance indicates less cell viability. A plateau for maximum cytotoxicity for 106 L5178Y cells was achieved at a dose of 0.5 IU ASNase activity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ings, it was hypothesized that targeted and intracellular delivery of ASNase into tumor cells would render these cells to carry their self-killing agent, thereby inhibiting or, at least, retarding tumor progression. Furthermore, as ASNase would be entrapped inside the tumor cells, it presumably should not elicit a systemic depletion of ASN, therefore aborting organs and tissues from ASNase-associated toxic effects.

In this article, we carried out preliminary animal studies to assess the *in vivo* utility of this delivery system to ASNase therapy. To evaluate the effect of local depletion of ASN on tumor growth by this modified ATTEMPTS approach without a specific tumor targeting component (e.g., an antibody) in hand during the course of experiments, we purposely adopted an alternative strategy by implanting ASNase-encapsulated tumor cells into the animal to mimic the events of tumor targeting and intratumoral uptake of ASNase. Following intraperitoneal injection of ASNase-encapsulated tumor cells, the survival rate and hematological and histological statuses were examined to determine the effects of this new ASNase therapy.

MATERIALS AND METHODS

Conjugation of ASNase to TAT peptide

TAT cell transduction peptide *CGGGYGRKKRRQRRR* was synthesized at the University of Michigan Protein Structure Core Facility. This peptide contained 11 amino acid transduction peptide sequence (italicized sequence) known to be effective in inducing cell transduction.[11]

ASNase was conjugated to TAT using the heterobifunctional linker 3-(2-pyridyldithio)-propionic acid N-hydroxy-succinimide ester (SPDP; Sigma, USA) according to a previously established method.[13]

Purification and characterization of the TAT-ASNase conjugates

The TAT-ASNase conjugates were purified from unreacted ASNase and TAT using a HiTrap Heparin column (Supelco, Bellefonte, PA) connected to a HPLC system (Alltech 526 HPLC pump, Deerfield, IL) through a gradient elution (0.15–2.0M NaCl; flow rate: 1 mL/min). Successful conjugation and the purity of the TAT-ASNase conjugates were confirmed by SDS-PAGE on ready-made 12% Tris-HCl mini gels (Bio-Rad Laboratories, Hercules, CA).

Tumor cell line

L5178Y, a murine T-lymphoma cell line derived from methylcholanthrene-induced lymphoma in DBA/2 mice, was purchased from ATCC (Manassas, VA). The cells were maintained and propagated in vitro using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Determination of optimal ASNase dose for in vivo studies

To determine the optimal dose of ASNase employed in subsequent in vivo studies, varying ASNase doses ranging from 10^{-5} to 10 IU were incubated with L5178Y cells for 48 h, followed by assessing the cell viability using the standard XTT assay.[14]

Encapsulation of ASNase into L5178Y cells

L5178Y cells were suspended to a concentration of 10^6 cells/mL. The cells were then incubated for 2 h with the TAT-ASNase conjugates in a 37°C, 5% CO_2 atmosphere incubator at various doses ranging from 0.025 to 10 IU of equivalent ASNase activity per 10^6 cells. Following three washings with RPMI-1640 containing 50% FBS, the cells were resuspended to a concentration of 10^6 cells/200 μL. As a control and also to examine if ASNase by itself could be taken up by the tumor cells, similar experiments were conducted by incubating free ASNase with the L5178 cells under exactly the same doses and experimental conditions described earlier.

Animal preparations

Five-week-old female DBA/2 mice were purchased from Charles River Laboratories (Raleigh, NC). The mice were housed in animal facilities and were fed standard mouse chow and provided water ad libitum. They were 6–7 weeks old at the beginning of each experiment and the average mouse weight ranged from 14 to 19 g.

All animal experiments were conducted according to the protocol approved by the University of Michigan Committee on Use and Care of Animals that conforms to the standards in the "Guide for the Care and Use of Laboratory Animals," DHEW Pub. No. (NIH) 80-23 (revised 1985).

In vivo experiments

Six-week-old DBA/2 mice were injected intraperitoneally with L5178Y cells (10^6 cells/mouse) pretreated with either PBS solution (i.e., positive control), free ASNase (another control, see reasons given later), or TAT-ASNase (i.e., the treatment group). Six mice were used in each testing group and three additional mice were used as the negative control (where the mice were injected with the RPMI-1640 solution only, without tumor cells). Animals were weighed to monitor weight change and the survival times recorded.

Eight days after tumor implantation, blood was removed by retro-orbital puncture. One hundred fifty microliters of blood was collected from each mouse, and blood from the same group was pooled together. Complete blood count was performed by the hematology laboratory at the University of Michigan Hospital.

At the end of experiments, mice were sacrificed by cervical dislocation and peritoneal lavage was performed according to the procedures of Liu et al.[15] Briefly, the peritoneal cells were removed in two successive 5-mL peritoneal washouts with PBS. These cells were then pelleted, resuspended in 4 mL MEM, and counted using a hemocytometer.

To detect metastases, the axillary, cervical and inguinal lymph nodes (LNs) were removed and placed in 2 mL of Hanks-FBS-azide solution. The LNs were then teased open to release the cells and were passed through a filter to remove fibrous connective tissues. LN cells were washed three times with the same solution.

To monitor tumor cell growth, both the peritoneal and LN cells were stained with antibodies. Antibodies were the following cell surface markers were used: CD4, CD8, CD3, Thy-1, GR-1, TCR-a/b, either in the form of fluorescein-labeled (FITC) conjugate or phycoerythrin (PE) conjugate. In addition, the mouse liver was removed, fixed in 10% formalin, and then stained with hematoxylin–eosin. Histology analysis was performed at the Animal Medicine Facility at the University of Michigan.

RESULTS AND DISCUSSION

The underlying principle of utilizing the modified ATTEMPTS approach in intracellular ASNase delivery was that these tumor cells would carry their own killing agent during migration, eventually leading to their self-destruction. Via this mechanism, the elimination of nutrient ASN would presumably occur only within the tumor cells, thereby aborting

the toxic side effects on the high protein production organs (e.g., liver, pancreas) resulting from systemic depletion of ASN. To provide a quick proof-of-concept demonstration of the feasibility of this drug delivery system, while no suitable murine antibody was available at the time of experiments, we chose to take an alternative yet genuine approach in mimicking the tumor uptake of ASNase, by directly injecting the ASNase-encapsulated tumors cells into the testing animals. The L5178Y tumor cell line was selected for ASNase encapsulation, because it had been well documented in the literature to be highly sensitive to ASNase therapy[3] and also it is commercially available. The DBA/2 mouse was specifically chosen to be the host for the implanted tumor cells because L5178Y cells had been demonstrated to be tumorigenic in this mouse strain.[16] In addition, previous investigations had used this cell line and mouse combination to examine the efficacy of various ASNase therapy, and, therefore, a history of success using this combination has already been demonstrated and documented.[4,17] Moreover, another advantage of using DBA/2 mouse strain was that it did not require any special food or housing conditions associated with immune-deficient mice such as NOD/SCID.

To determine the optimal dose of ASNase to be used in subsequent *in vivo* studies, varying dose of ASNase ranging from 10^{-5} to 10 IU was incubated with L5178Y cells (10^6 cells per dose) for 48 h, followed by cell viability measurement using the standard XTT assay. As shown in Figure 1, increasing ASNase dose resulted in enhanced cytotoxicity towards the L5178Y tumor cells, until a plateau of cytotoxicity was reached at a dose of ~0.5 IU/10^6 cells. This ASNase dose was therefore selected during initial experiments to prepare the ASNase-encapsulated L5178Y tumor cells for subsequent animal studies.

To examine if ASNase by itself could be taken up by the tumor cells, and also to ensure that the encapsulated TAT-ASNase conjugates (i.e., mediated by the cell transduction function of TAT) would still retain their cytotoxic effects towards the host tumor cells, both free ASNase and the TAT-ASNase conjugates (1–5 TAT per ASNase as confirmed by SDS-PAGE and MALDI) were incubated separately with 10^6 L5178Y cells at various doses ranging from 0.025 to 10 IU (in terms of equivalent ASNase activity) for 2 h; a time period that was established previously to allow for a complete cell uptake of the TAT-ASNase conjugates (data not shown). The free ASNase- or TAT-ASNase-treated cells were then thoroughly washed and further incubated in the culture medium for another 2 h, followed by cell viability measurement using the XTT assay. As shown in Figure 2(A), without the additional washing step, free
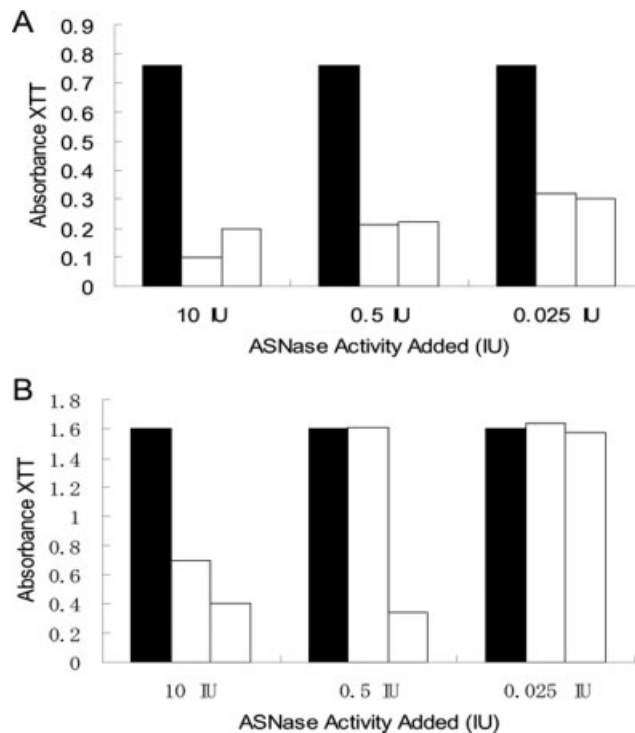

Figure 2. Effect of washing on ASNase (shaded bar) and TAT-ASNase (white bar) cytotoxicity on L5178Y cells. ASNase and TAT-ASNase at equal doses were added to a pool containing 106 L5178Y cells and allowed to incubate for 2 h (black bars: control medium). Cells were (A) not washed, and (B) washed with 50% FBS in RPMI-1640, and then allowed further incubation for another 2 h prior to conducting the XTT assay.

ASNase (shaded bar) and TAT-ASNase (white bar) exhibited an almost identical viability at all the dose range studied. However, when the cells were incubated with ASNase or TAT-ASNase for 2 h followed by washing with 50% FBS in RPMI-1640 to remove extracellular ASNase, significantly higher cytotoxicity was observed in the TAT-ASNase-treated group after an additional 2-h incubation [see Fig. 2(B)]. Obviously, this phenomenon was due to the fact that both the extracellularly (i.e., free) or intracellularly (i.e., internalized) delivered ASNase could elicit the same kind of cell-killing effect, by depleting the ASN substrate in the media that was essential for tumor cell growth. The greatest difference was observed at the ASNase dose of 0.5 IU, whereas the free ASNase did not yield any detectable effect on cell variability before being washed away, and yet the internalized TAT-ASNase that dodged the removal by the washing step exhibited a completely retained cytotoxic activity. It should be pointed out that at an ASNase dose of 0.025 IU, the cytotoxicity was too weak to be observed regardless of whether an additional washing was adopted. Lysis of the ASNase-encapsulated tumor cells followed by quantitation of NH_3 produced by direct nesslerization

showed that ~10% of the initial dose of TAT-ASNase was successfully encapsulated into the L5178 cells.

Based upon the above *in vitro* dose response curve, an ASNase dose of 0.5 IU or equivalent (i.e., in the TAT-ASNase samples) was employed. During the very initial animal survival study, three cell suspensions, each with a total of 10^6 L5178Y cells, were incubated with PBS solution (positive control), free ASNase (another control, see reasons given later), or TAT-ASNase for 2 h, followed by thorough washing with 50% FBS in RPMI-1640. Immediately after washing, each of the three pools of cell suspensions was injected into a mouse intraperitoneally. Six mice were included in each of the tumor-injected testing groups, and an additional three mice were injected with the RPMI solution without tumor cells to function as a negative control. Five days after tumor cell implantation, the abdominal area of the three tumor-injected mice showed visible distention whereas the negative control mice injected with only the RPMI-1640 solution exhibited normal abdominal size and shape. Furthermore, the maximum weight gain for the tumor-injected mice was ~16% while that of the negative control was only 4.4% of the original body weight; a clear sign of rapid tumor progression. As tumor grew, the L5178Y-implanted mice showed a visible decrease in mobility and activity levels. The survival rate of the mice was illustrated in Figure 3. As seen, the mean survival time of the positive control mice (tumor implantation, no drug treatment) and TAT-ASNase-injected mice were found to be 9.83 and 11.17 days, respectively. To ensure that the injected TAT-ASNase-encapsulated L5178Y cells consisted of "live" tumor cells only and thus the animal's survival results were not overestimated, a group of mice were injected with tumor cells pretreated with 0.5 IU of free ASNase for 2 h (see description of the experimental groups given earlier), despite that previous *in vitro* results already demonstrated the lack of cytotoxic effect toward L5178Y cells by free ASNase at this dose after 2 h of incubation [see Fig. 2(B)]. In agreement with these *in vitro* findings, a survival time of 10.0 days, which was almost identical to that of the positive control (i.e., 9.83 days), was observed for this group of animals (see Fig. 3), implicating that the majority of tumor cells pretreated with TAT-ASNase at the same ASNase dose and incubation period should remain alive prior to the administration of the tumor cells into the test animals. An analysis of variance was performed using SPSS to determine if the mean survival rates between all the treatment groups were significantly different. ANOVA analysis suggested that there were no statistically significant differences in the mean survival rates between the positive control, free ASNase, and TAT-ASNase groups at the
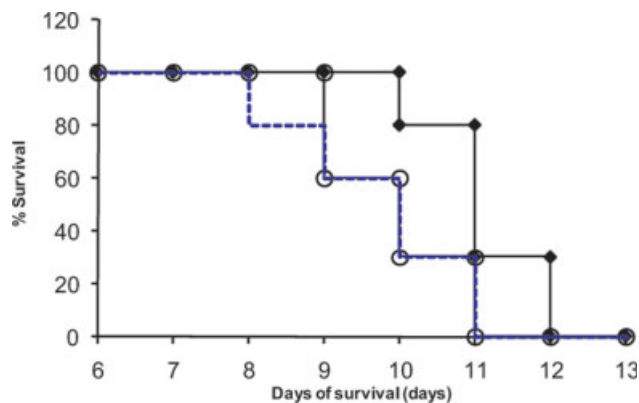

Figure 3. Survival curve of DBA/2 mouse. Each pool of 106 L5178Y cells was incubated with 0.5 IU of either ASNase (open circles), TAT-ASNase (filled diamond), or RPMI-1640 solution (positive control; dotted line only) for 2 h, followed by washing three times with 50% FBS in RPMI. The DBA/2 mice of 6 weeks old were then injected intraperitoneally with one of these three treated tumor cell samples (106 cells/sample). Mouse survival times were recorded. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

significance level of $p = 0.05$ ($F_{2,5} = 3.476$, $p = 0.058$). By performing multiple pairwise comparisons using the Tukey's and Dunnett's test, however, it was discovered that while there were no statistically significant differences between any of these three groups at $p = 0.05$, the difference between the positive control and TAT-ASNase group was approaching a statistically significant level, as evidenced by the Tukey's and Dunnett's statistics of $p = 0.07$ and $p = 0.052$, respectively. These results suggested that if more appropriately designed experimental conditions were selected, statistically significant difference between the control and TAT-ASNase groups could have been detected.

It should be pointed out that a total ASNase dose of ~0.05 IU was estimated to be administrated to each of the TAT-ASNase-treated mice, based on an encapsulation rate of ~10% of the initial ASNase dose (0.5 IU) employed during incubation with the tumor cells, as discussed previously. Although this was exactly the optimal ASNase dose determined from the *in vitro* experiments on cultured cells (see Fig. 1), it was nevertheless 16-fold lower than the reported ASNase dose of approximately 8 IU used by other investigators in their animal experiments.[4] Apparently, a significant difference between *in vitro* and *in vivo* dose assessment did exist. Hence, a second trial of animal studies were carried out using an increased TAT-ASNase dose of 6 IU during incubation with 7×10^5 L5178 cells, in an attempt to magnify the overall survival time of the tumor-injected mouse thereby enabling a clearer detection of the difference in survival rate among all test animals. All other experimental conditions were kept same as
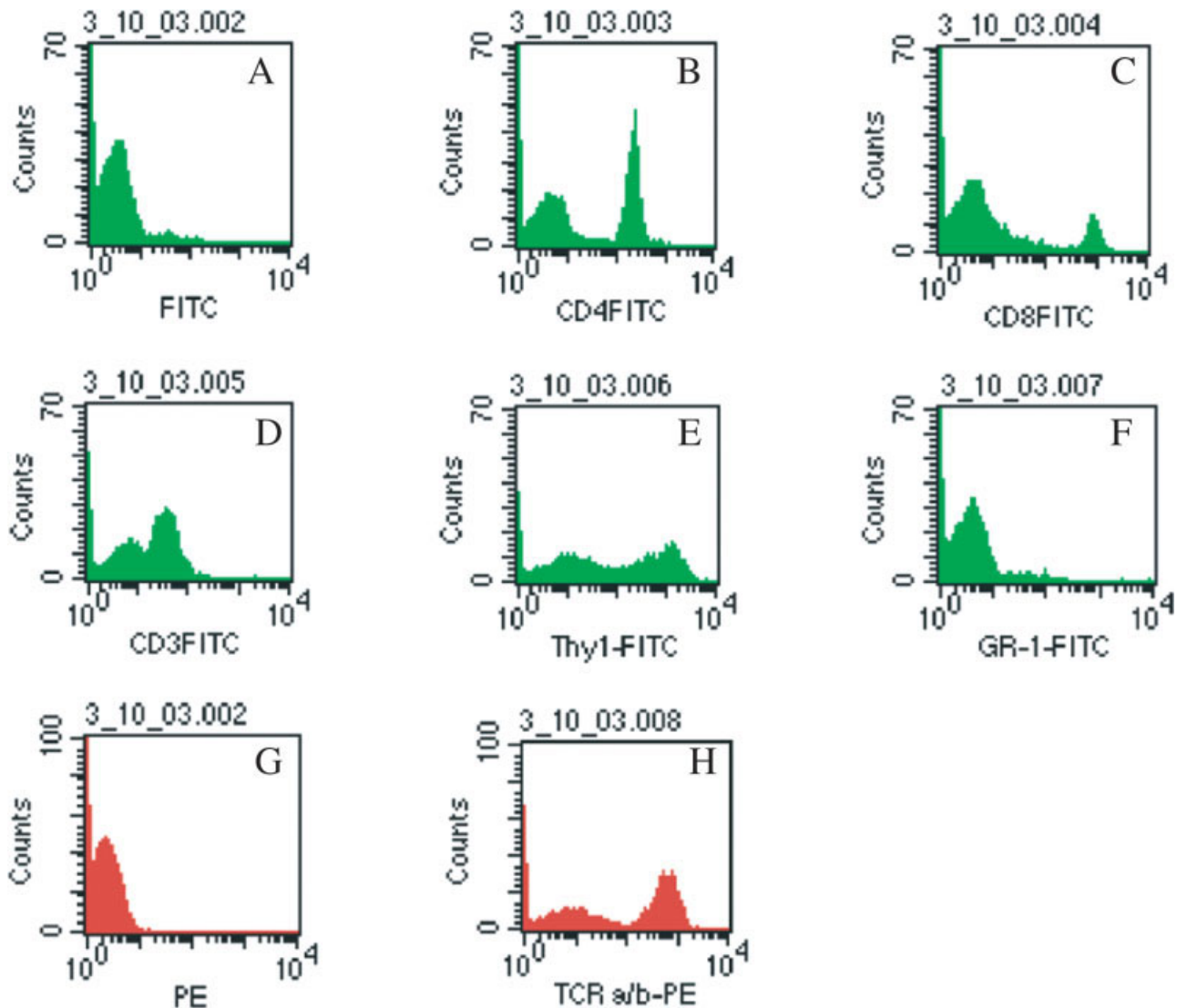
Figure 4. Flow cytometry analysis of surface markers of lymph node cells. Lymph nodes were removed from DBA/2 mice and lymph cells were extracted. Cells were stained with different anti-mouse cell surface monoclonal antibodies. Panels A and G represent unstained cells acquired under FITC and PE filter. Antibodies used to stain panels B–F and H are labeled on the X-axis of each histogram. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

those used in the previous trial, including the positive control group in which animals were injected with 7×10^5 L5178 cells without ASNase treatment as well as the experimental group in which animals were injected with 7×10^5 L5178 cells preincubated for 2 h with the TAT-ASNase conjugates containing 6 IU ASNase equivalent dose. Also similar to the first trial, six mice were used in each of the tumor-injected test groups, and three mice were used as the negative control where RPMI-1640 solution without any tumor cells was injected. Results (to avoid repetition, data were not plotted) showed that the mean survival time for the positive control and TAT-ASNase-treated group were 13.9 and 15.6 days, respectively. ANOVA analysis displayed a statistically significant difference in the mean survival rate between the treatment groups at $p = 0.05$ ($F_{2,12} = 6.478$, $p = 0.012$). In addition, the Tukey and Dunnett test also yielded a statistically significant difference in the mean survival rate between the positive control and the TAT-ASNase-treated group, with Tukey's and Dunnett's statistics of $p = 0.009$ and $p = 0.007$, respectively. Albeit preliminary, these findings clearly demonstrated the plausibility of utilizing the modified ATTEMPTS approach in enhancing the existing ASNase therapy.

To further validate the *in vivo* results, detection of tumor cells in the intraperitoneal cavity using antibody staining and determination of the number of cells in the peritoneum were carried out to better monitor tumor progression. It should be noted that L5178Y tumor cells were of T cell lineage and
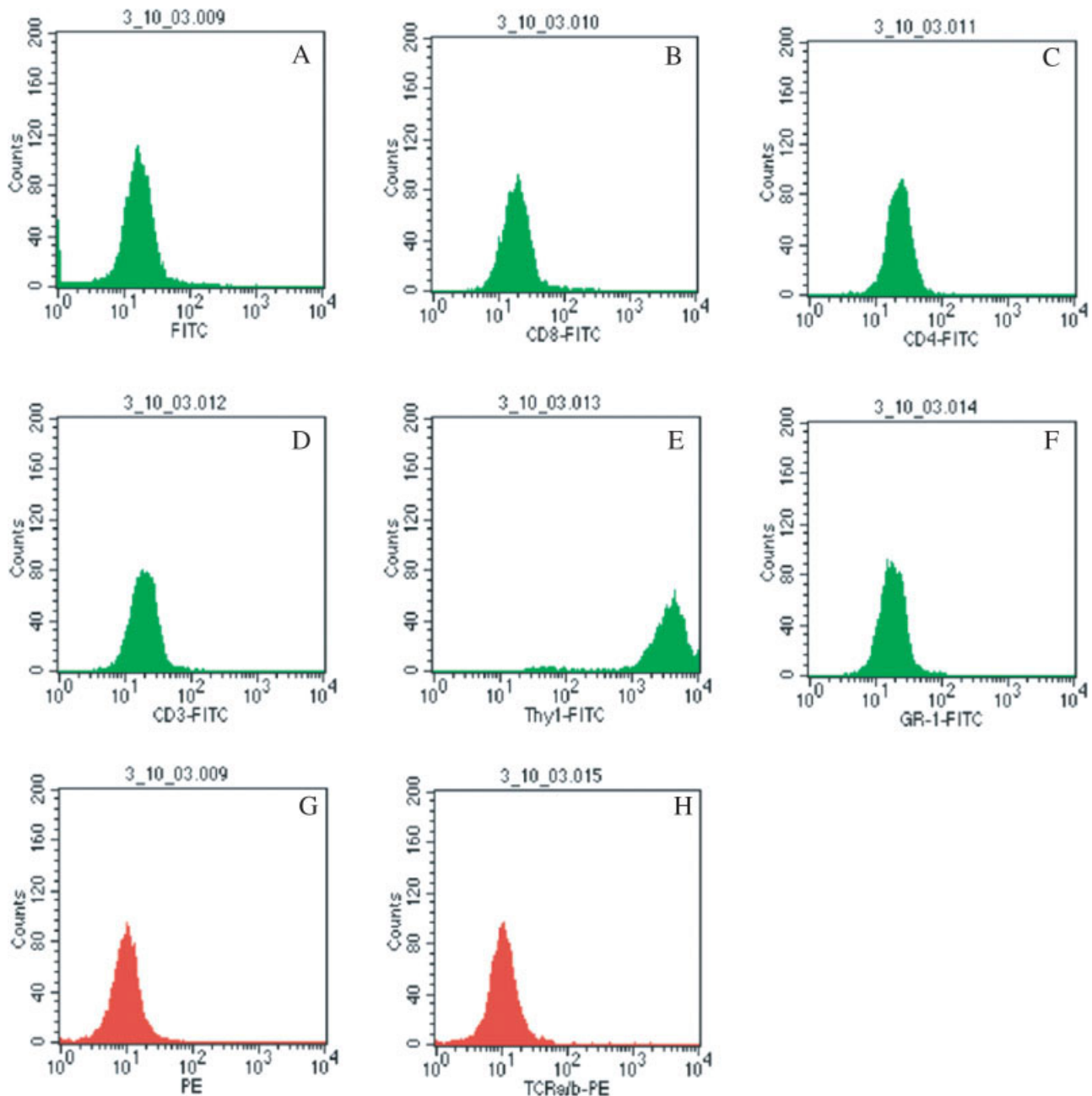
Figure 5. Flow cytometry analysis of surface markers of L5178Y tumor cells. L5178Y cells were stained with different anti-mouse cell surface monoclonal antibodies and analyzed by flow cytometry. Panels A and G represent unstained cells acquired under FITC and PE filter. Antibodies used to stain panels B–F and H are labeled on the X-axis of each histogram. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

derived from DBA/2 mice. As a result, normal T cells of the DBA/2 mouse and the tumor cells could share similar antigen expression, thus yielding some obstacle in tracking down the L5178Y cells *in vivo*. Various antibodies were first screened to determine which antibody or antibody combination could be utilized to differentiate tumor versus normal T lymphocytes. In these experiments, the LNs were removed from a DBA/2 mouse to provide a representative population of normal DBA/2 T lymphocytes. Both LN cells and L5178Y cells were then

stained with various FITC- or PE-labeled monoclonal antibodies, followed by detection of the expression of a certain antigen by using the flow cytometry technique. Figure 4 depicts the staining pattern of normal LN cells with the various antibodies. From flow cytometry analysis, LN cells stained positive for all tested T-cell–associated antibodies, as indicated by an increase in fluorescence intensity relative to the control. As expected, these LN cells did not stain for Gran-1 antibody (Panel F) which would be normally expressed on neutrophils, eosinophils, baso-
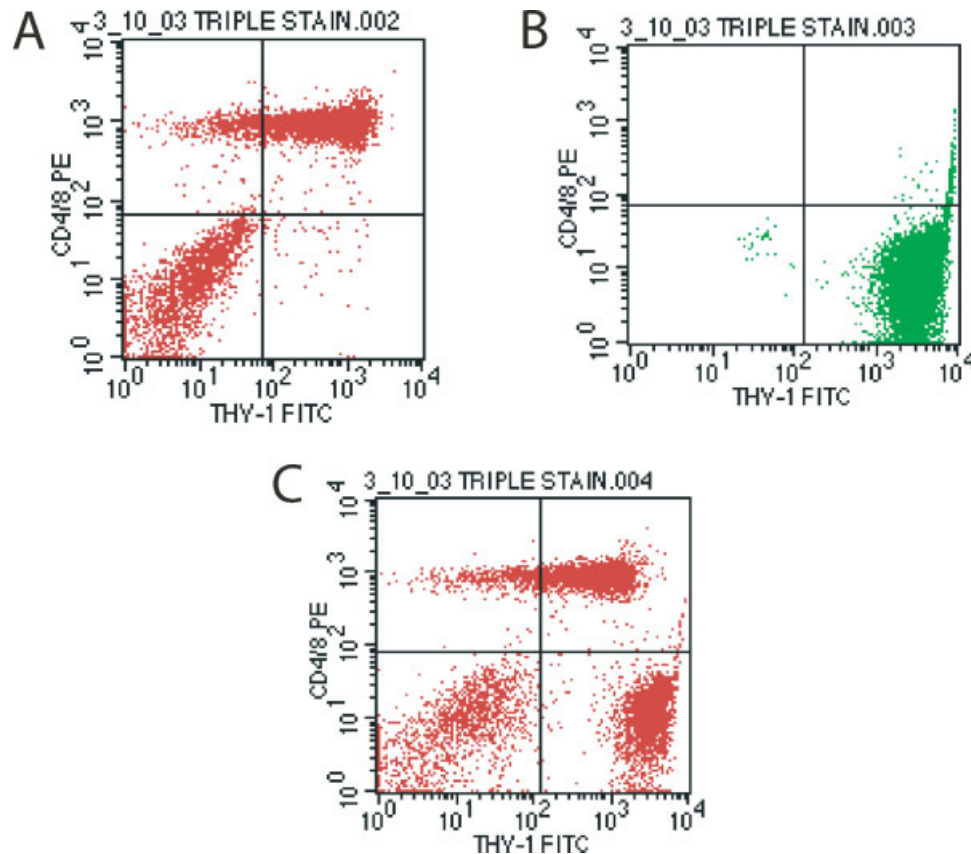
Figure 6. Flow cytometry analysis using triple antibody staining. (A) Lymph node (LN) cells, (B) L5178Y cells, and (C) LN + L5178Y mixture were stained with FITC-Thy 1, PE CD4/CD8 and analyzed for antigen expression using flow cytometry.

phils, and macrophages. On the other hand, Figure 5 shows that L5178Y cells stained positive only with the Thy-1 antibody, as increase in fluorescence intensity was only detected in Panel E. Based on these findings, it was determined that using a combination of CD4 and CD8, along with Thy-1 antibodies would enable the differentiation of normal DBA/2 T cells from L5178Y tumor cells. By applying this strategy with the use of PE-labeled CD4 and CD8 as well as FITC-labeled Thy-1, it was seen in Figure 7 that normal T cells, which would stain for both CD4/CD8 and Thy-1, resided in the upper right quadrant of the flow cytometry scatter graph with PE-CD4/CD8 being located on the *Y*-axis and FITC-Thy-1 on the *X*-axis. Because L5178Y would only stain for the Thy-1 antibody, it resided in the lower right quadrant of the same flow cytometry scatter graph. To mimic physiological conditions where tumor and normal T cells would coexist, normal LN cells and L5178Y cells were mixed first and then stained with PE-CD4/CD8 and FITC-Thy-1. As shown by the flow cytometry results in Figure 6, these two different cell populations were clearly identified in the scatter graph.

To monitor tumor progression among the treated animal groups, tumor cells in the intraperitoneal cavity were examined. A mouse from each treated animal group was sacrificed, and peritoneal lavage was performed to collect peritoneal cells. Peritoneal cells were then stained with FITC-Thy-1 and PE-CD4/CD8 according to the procedures established above, and the percentage of peritoneal cells that expressed tumor antigen was determined. As shown in Figure 7, staining of peritoneal cells revealed a significant tumor cell occupation in the peritoneum. For the positive control mice [Fig. 7(B)], 50% of the cells in the peritoneum exhibited the tumor phenotype, whereas for the TAT-ASNase group [Fig. 7(C)], only 15% of the cells displayed tumor phenotype. In contrast, no tumor phenotype cells were detected in the peritoneum [Fig. 7(A)] for the negative control animals. These results clearly suggested a significantly slower tumor progression in mice injected with L5178Y cells that were preincubated with the TAT-ASNase conjugates.

Aside from peritoneal cavity, LNs were also examined as an indicator of tumor metastasis; because lymphocytes would pass through the LNs for activation and antigen presentation to mediate an immune response. By staining the LN cells with FITC-Thy-1 and PE-CD4/CD8, the percentage of LN cells exhibiting the tumor phenotype among the different treatment groups was determined (Fig. 8).
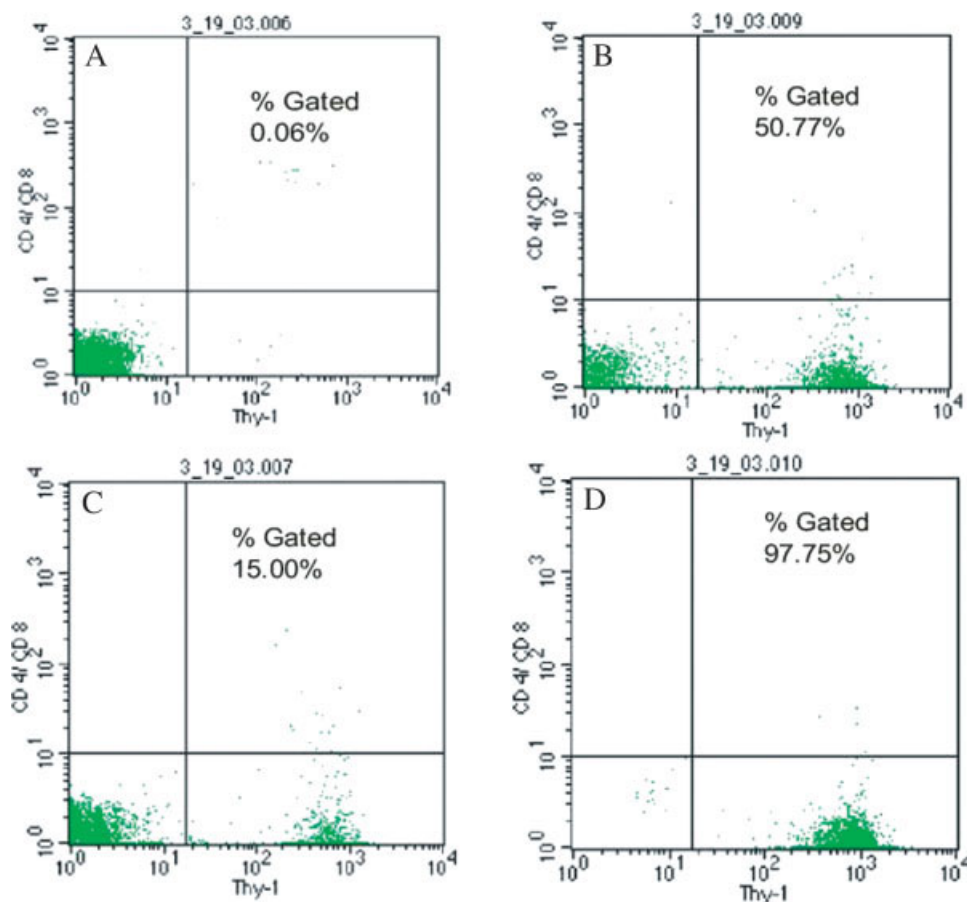
Figure 7. Antibody staining of tumor cells in the peritoneum. A: Negative control (i.e., mice were injected with RPMI-1640 solution only). B: Positive control (i.e., mice were injected with L5178Y cells pre-incubated with PBS buffer). C: TAT-ASNase-treated group. D: Standard cultured L5178Y cells. The "% Gate" represents the percentage of peritoneal cells in the lower-right quadrant of the scatter graph which are the cells expressing the tumor phenotype. Please refer to the text for detailed information. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

As demonstrated in all scattered graphs, there was a population of cells stained for both CD4/CD8 and Thy-1 representing that of normal T lymphocytes, as well as a population of cells which did not stain for either antibody representing that of B cells. No cell population was detected in the lower right quadrant of any of the treatment groups, indicating no tumor cells were detected in the LNs. These results suggested that by day 8, the L5178Y had not yet metastasized and proliferated in the LNs.

Furthermore, the number of tumor cells in the peritoneum was also determined by using a hemocytometer. In agreement with findings reported by Wheelock and Trainer in their studies on tumor emergent mice involving the L5178Y murine tumor dormant state, which displayed that greater than 10^7 peritoneal cells were in the peritoneal cavity,[18] our results in Table I showed that more than 10^8 cells were discovered in the peritoneum of tumor-injected mice, compared with 10^6 cells in the peritoneum observed in the negative control mice (i.e., no injection of tumor cells).

Moreover, blood samples were drawn from the mice 8 days after the initial tumor implantation, and were measured for white blood cell (WBC) count, red blood cell count, and hematocrit. As shown in Table II, the positive control animals exhibited a much greater increase in WBC count and decrease in hematocrit, when comparing with the results of the TAT-ASNase-treated mice. As known, an increase in WBC count would reflect the elevation of the animal's immune system in combating tumor progression. In addition, tumor growth was also reported to cause significant decrease in red blood cell count and hematocrit.[19] Based on these hematological results, it was concluded that the TAT-ASNase tumor-injected animals encountered the slowest tumor progression, as reflected by the much smaller decrease in hematocrit and increase in WBC count when compared with results of the positive control animal group. It should be noted that although, on the day 8 in the survival curve, there are no differences between groups (i.e., tumor bearing animals were still alive), differences between groups were
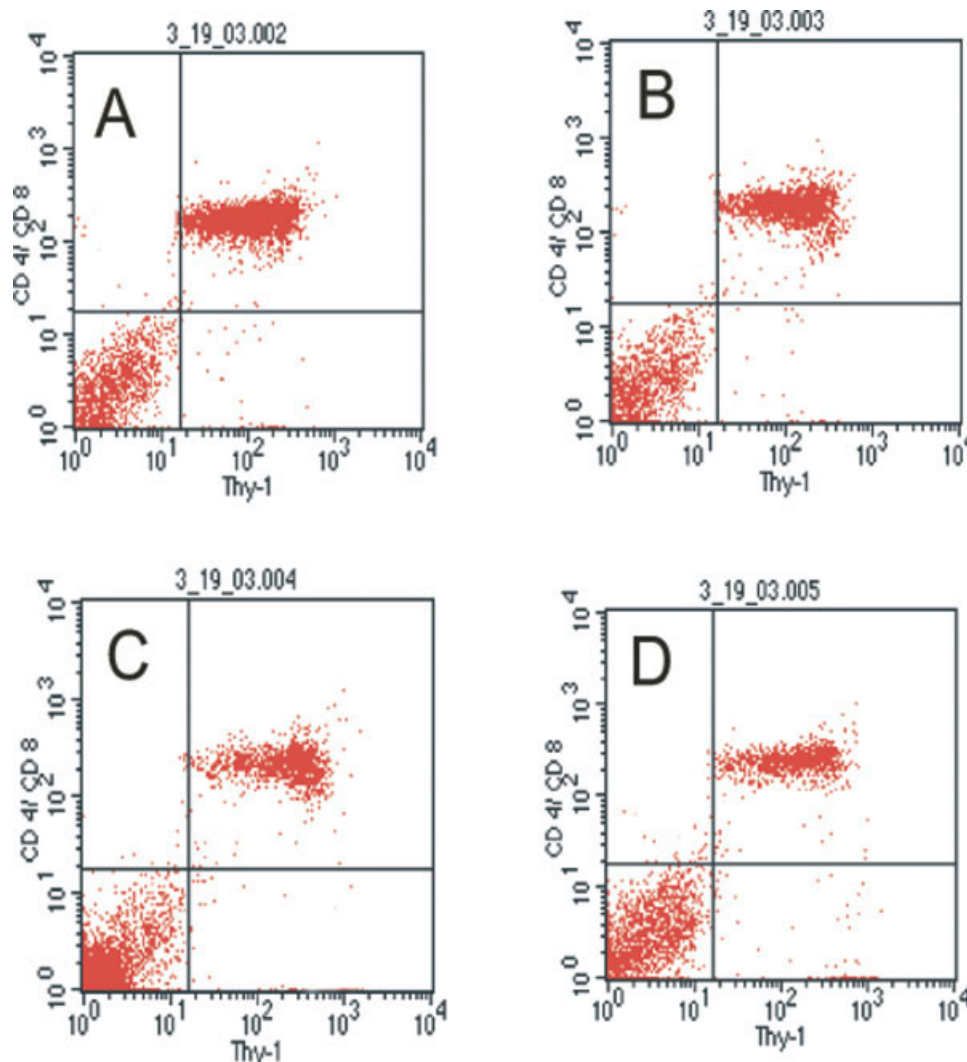
Figure 8. Antibody staining of lymph node cells. A: Negative control (i.e., mice were injected with RPMI-1640 solution only). B: Positive control (i.e., mice were injected with L5178Y cells preincubated with PBS buffer). C: ASNase-treated group (i.e., mice were injected with L5178Y cells preincubated with ASNase. D: TAT-ASNase-treated group (i.e., mice were injected with L5178Y cells preincubated with TAT-ASNase). LN cells were stained with FITC-Thy-1 and PE-CD4/CD8. Tumor phenotype expression was analyzed using flow cytometry. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rather clear in Table I (hematological data), indicating that the tumor progression without treatment at this time point had created detrimental effects on the tumor-bearing animals.

Lastly, metastases of L5178 tumor cells in the livers of the treated animals were also examined. Livers were removed from the mice 8 days after initial tumor implantation, fixed in 10% formalin, and later sectioned and stained by hematoxylin and eosin. As seen in Figure 9, both the positive control and free ASNase-treated mice displayed a diffused infiltration of tumor cells in the liver [Fig. 9(B,C), respectively]. In a sharp contrast, infiltration of L5178Y cells in the TAT-ASNase-treated mice was mostly focal [Fig. 9(D)], similar to the results seen in the negative control group where no tumor cells were injected [Fig. 9(A)]. Based on analysis of these histological results

TABLE I
Number of Peritoneal Cells in the Peritoneum

Group	Number of Peritoneal Cells
Negative control	4.20×10^6
Positive control	4.58×10^8
ASNase	2.94×10^8
TAT-ASNase	1.60×10^8

Eight days after tumor implantation, a mouse from each group was sacrificed and peritoneal lavage was performed according to procedures described in the "Materials and Methods" section. The peritoneal cells were collected and counted using a hemocytometer. The "negative control" represented that mice were injected with RPMI-1640 solution only, whereas the "positive control" represented that mice were injected with L5178Y cells preincubated with PBS buffer.

TABLE II
Hematological Parameters of Various Treatment Groups

Parameters	Negative Control	Positive Control	ASNase	TAT-ASNase
WBC (K/μL)	6.21	9.57	8.44	7.22
RBC (M/μL)	10.53	3.52	6.71	8.63
Hemoglobin (g/dL)	15.3	5	9.5	11.6
Hematocrit (%)	49.5	17.7	31.6	36.2

Approximately 150 μL of blood was removed retro-orbitally from three mice in each treatment group using heparinized capillary tubes according to the procedures described in the "Materials and Methods" section. The blood from each treatment group was pooled together and analyzed at the Hematology Laboratory at the University of Michigan Hospital. The "negative control" represented that mice were injected with RPMI-1640 solution only, whereas the "positive control" represented that mice were injected with L5178Y cells preincubated with PBS buffer.

of the liver sections, it was concluded that tumor growth in the TAT-ASNase-treated mice was significantly slower than the positive control group at day 8 after tumor implantation.

CONCLUSION

This paper provides a rapid and proof-of-concept *in vivo* demonstration of the modified ATTEMPTS approach in delivering protein drugs into tumor cells. Based on findings discovered in this investigation, this modified ATTEMPTS approach could potentially lead to an intracellular capture of ASNase by leukemic cells, causing their self-destruction without systemic depletion of the nutrient ASN from circulation, thereby aborting the toxic effects of ASNase to normal tissues and particularly the high protein production organs such as the liver and pancreas. Because of the lack of an available antibody for targeting during the course of experiments, we adopted a genuine but alternative strategy to mimic the events of tumor targeting and subsequent intracellular ASNase uptake induced by this modified ATTEMPTS system, by injection of ASNase-encapsulated L5178Y cells into DBA/2 mice followed by comparison of the survival data with the control mice administered with L5178Y cells without ASNase encapsulation. The preliminary results revealed that mice inoculated with ASNase-encapsulated L5178Y cells not only displayed a statistically significant prolongation of the survival time over that of the control mice, but also demonstrated a highly improved hematological and liver histological status over the control group. Although an enhancement of ~15% in survival time over the control was observed for the TAT-ASNase-treated mice, this
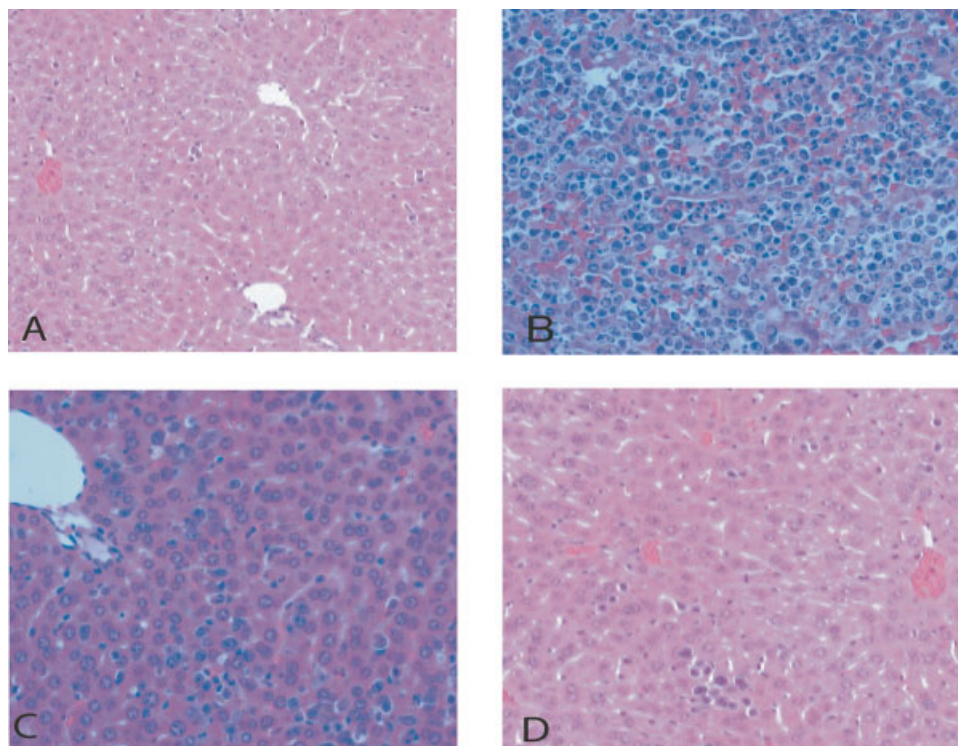

Figure 9. Liver metastases of L5178Y. A: Negative control (i.e., mice were injected with RPMI-1640 solution only). B: Positive control (i.e., mice were injected with L5178Y cells preincubated with PBS buffer). C: ASNase-treated group (i.e., mice were injected with L5178Y cells preincubated with ASNase). D: TAT-ASNase-treated group (i.e., mice were injected with L5178Y cells pre-incubated with TAT-ASNase). Liver cells were fixed in 10% formalin and stained with hematoxylin–eosin. Magnification: ×400. Please refer to the text for detailed information.

improvement was relatively dramatic, considering the fact that the calculated ASNase dose encapsulated in the tumor cells (0.6 IU) was about 14-fold less than that of intravenously administered ASNase dose (8 IU) employed by other investigators in their ASNase therapy study,[4] and yet a comparable enhancement in survival time by their methods (i.e., 16.7%) was achieved. These findings provide a sign of prowess of the proposed delivery system in the treatment of ALL, if ASNase could indeed be intracellularly delivered into the tumor cells by utilizing the ATTEMPTS approach. With an appropriate choice of antibody, a thorough animal evaluation of the entire ATTEMPTS system for targeted tumor therapy is currently in progress in our laboratory.

Lastly, it should be pointed out that the intracellular drug uptake mediated by TAT has been shown to proceed in a receptor- or transporter-independent fashion that directly targets the lipid bilayers.[20] Hence, in theory and practice, all cell types are transducible. Therefore, the proposed modified ATTEMPTS system possesses not only a universal utility in delivering drugs of all types (i.e., small or large), but also a universal applicability to treat cancers of all types (e.g., breast tumor, malignant melanoma, as long as a specific tumor-targeting ligand is available). This generic nature and flexibility in applying ATTEMPTS delivery system could open up the possibility of discovering and/or developing an arena of new drugs that were initially considered improbable for therapeutic uses because of either poor cell uptake or acute toxic side effects. Understandably, such problems have forced biotechnology and pharmaceutical companies to abandon or discard a large number of drugs that have shown promising therapeutic effects *in vitro* but limited functions or severe toxicities *in vivo*. Hence, design of targeted systems such as ATTEMPTS drug delivery system may pave the way to resolve such problems.

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