In Vitro and in Vivo Cytotoxicity of an Anti-Osteosarcoma Immunotoxin Containing Pokeweed Antiviral Protein

Peter M. Anderson, Dorothea E. Meyers, Diane E. Hasz, Kristin Covalcuc, Daniel Saltzman, Chand Khanna, and Fatih M. Uckun


ABSTRACT

Successful treatment of many patients with osteosarcoma requires more effective chemotherapy. Since new agents are needed, we have developed an immunotoxin using TP-3, an IgG2b mAb which recognizes human and canine osteosarcomas and budding capillaries of tumors. The plant hemotoxin, pokeweed antiviral protein (PAP), was conjugated to TP-3 to produce an immunotoxin highly active against osteosarcoma. After 48 h no viable human OHS osteosarcoma cells were present in cultures containing TP-3-PAP as demonstrated by the absence of [3H]thymidine uptake into DNA. Furthermore, clonogenic assays indicated >3.9 log kill of OHS at 18 h. The IC50 of TP-3-PAP against OHS was 3.5 ± 1.0 (SD) x 10^-12 M. TP-3 mAb without PAP had no effect on OHS proliferation; PAP alone had no effect on OHS growth unless concentrations >1000 µg/ml were used. When TP-3-PAP (1.25 µg-10.0 µg) was given i.p. q.d. on days 3-5 after tumor inoculation, a dose-dependent reduction of the number of lung metastases was observed (P < 0.001). These results indicate that the TP-3-PAP immunotoxin may be useful in the treatment of osteosarcoma and some soft tissue sarcomas.

INTRODUCTION

Adjuvant chemotherapy is a standard treatment approach for many sarcomas including Ewing's sarcoma, rhabdomyosarcoma, and osteosarcoma (1-5). It has been more difficult, however, to demonstrate a significant benefit of chemotherapy in adult soft tissue sarcomas (6) and osteosarcoma with metastatic disease at diagnosis or after the development of lung metastases (7). Since the degree of necrosis of osteosarcoma in the neoadjuvant setting has been shown to be a highly significant predictor of disease-free survival (2-3, 5, 8-9), a need exists to increase the proportion of osteosarcoma patients with favorable initial responses to neoadjuvant chemotherapy, as well as to develop novel treatments for patients with poor responses (2) or metastatic disease (5, 7).

The rarity and heterogeneity of sarcomas (10) has made the development of immunotherapeutic agents against these cancers quite difficult (11). Immunotherapy alone and in combination with chemotherapy after local control has been investigated as a treatment modality for canine osteosarcoma. Recent work by MacEwen et al. with muramyl tripeptide phosphatidylethanolamine liposomes has demonstrated improved survival after surgery alone (12) and synergy when muramyl tripeptide phosphatidylethanolamine liposomes were used with cisplatin (13).

One promising set of mAbs, TP-1 and TP-3, has been shown to react with different epitopes of an Mr 80,000 antigen on human and canine osteosarcoma (14-21). TP-1 and TP-3 also bind a variety of other human sarcomas including hemangioepicytoma, chondrosarcoma, MFH,3 and synovial cell sarcoma (14, 15). Canine osteosarcoma and lung carcinoma were found to react with TP-3 better than TP-1 (16). The antigen recognized by TP-1 and TP-3 mAbs appears to be unique among anti-osteosarcoma antibodies. The distribution of the TP-1/TP-3 antigen on normal tissues is very limited. Negative tissues included fibroblasts, peripheral blood cells, cells in the marrow, fetal skin fibroblasts, fetal lung fibroblasts, amniocytes, fibrous connective tissue, skeletal muscle, cartilage, synovia, peripheral nerve, tonsil, spleen, liver, colon, and lung. Only newly active bone callus, placental endothelial cells, proximal tubule of kidney (weak binding), and occasional cells in the adrenal medulla were positive for TP-1 and TP-3 (14, 15).

Interestingly, although normal endothelium did not bind TP-1 or TP-3 mAbs, budding capillaries of nonsarcomatous origin were also TP-1/TP-3 positive (15). Radioimmunoscintigraphy using 131I-labeled TP-1 or TP-3 mAb preparations demonstrated selective tumor targeting in nude mice (17), dogs with osteosarcoma lung metastases (18), and humans with osteosarcoma metastases (19, 21).

In the case of osteosarcoma, immunotoxin technology may provide a potent means to achieve effective therapeutic use of the TP-3 mAb. Immunotoxins are bifunctional proteins which have been prepared by covalently linking a cell type-specific mAb to one of a variety of catalytic toxins (22-26). A direct comparison of a number of bacterial and plant toxin immunonconjugates recently showed that PAP constructs were among the most potent immunotoxins tested (25). Favorable preclinical and clinical results have been obtained by our group against lymphocytes and lymphoblasts with PAP containing immunotoxins (26-31). We have applied this experience toward the development of a TP-3-PAP immunotoxin for therapy of osteosarcoma in dogs and humans. This report describes high in vitro potency of TP-3-PAP against human osteosarcoma and significant in vivo efficacy against murine sarcoma lung metastases.

MATERIALS AND METHODS

TP-3 mAb Production and Purification. The TP-3 mAb hybridoma was obtained from Dr. R. Zalutsky (Duke University Medical Center) with permission of Dr. Ø. S. Bruland (The Radium Institute, Oslo, Norway). TP-3 hybridoma cells were cultured in DMEM (Celiox, Hopkins, MN) containing 25 µM HEPES, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM nonessential amino acids, 100 mM sodium pyruvate, and 10% FCS (Sigma Chemical Co., St. Louis, MO). BALB/c mice were primed with 0.5 ml pristane (Aldrich Chemical Co., Milwaukee, WI) i.p. 7 days before injection of 2 x 10^6 TP-3 hybridoma cells i.p. Ascites containing TP-3 mAb were collected, centrifuged at 12,000 x g for 20 min, pooled, and filtered through a 0.22-µm filter. TP-3 mAb was further purified using ammonium sulfate precipitation and affinity chromatography with protein A agarose (Immunopure Plus immobilized protein A; Pierce, Rockford, IL). Elution from protein A was accomplished with Immunopure elution buffer (Pierce). TP-3 was dialyzed against PBS and sterile filtered prior to use.

Cell Lines. The OHS line is an adherent human osteosarcoma line with high constitutive expression of the TP-1/TP-3 antigen. OHS was derived by...
Fostad et al. (32) from an adolescent with metastatic osteosarcoma which occurred 13 years after retinoblastoma. For the present studies OHS was obtained from Dr. Deborah Haines (Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada) and passaged in RPMI 1640 with 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FCS. D17 (canine osteosarcoma) was obtained from Dr. Stuart Helfand (University of Wisconsin, Madison, WI); D17 is negative for TP-3 antigen. The human CD19+ ALL cell line, RS4;11, was obtained from Dr. John Kersey (University of Minnesota, Minneapolis, MN) and used as a negative control line for TP-3-PAP studies.

TP-3-PAP Immunotoxin Synthesis. TP-3 antibody was produced and purified using procedures described previously (26). As shown in Fig. 1, TP-3-PAP immunotoxin began to elute approximately 34 min after injection, followed closely by unreacted TP-3 mAb. Free PAP eluted at 56 min and was well separated from the immunotoxin. The HPLC semipurified material still contained some unreacted TP-3 mAb. SDS-PAGE scanning of the dried gel revealed <5% PAP in the final TP-3-PAP immunotoxin preparation which also contained 14% mAb (Mq 150,000), 34% of the Mq 180,000 species consisting of 1 PAP molecule disulfide linked to 1 mAb molecule, 34% of the Mq 210,000 species consisting of 2 PAP molecules linked to 1 mAb molecule, and 18% of the Mq 240,000 species consisting of 3 PAP molecules linked to each mAb molecule (Fig. 1). The absence of significant free PAP contamination in the purified TP-3-PAP immunotoxin was confirmed by Western blot analysis using an anti-PAP antibody, as described previously in detail (26).

TP-3-PAP Immunotoxin Activity against Human OHS Osteosarcoma Cells. Solutions of mAb, toxins, and immunotoxins were tested for effects on OHS cell growth using a [3H]thymidine proliferation assay. Samples were diluted to appropriate concentrations (between 1 mg/ml and 100 ng/ml of protein) in media and added in triplicate to the first row of 96-well flat-bottomed microtiter plates. Samples were then serially diluted 3-fold with the
use of a multichannel pipettor apparatus by adding 50-μl sample from row A to 100-μl media in row B, mixing, and repeating the procedure until the entire plate was serially diluted. OHS cells were grown to confluence and removed by brief treatment with 0.5% trypsin with EDTA (Sigma) for 5 min at 37°C, washed twice in media, and adjusted to a concentration of 8 × 10^5 cells/ml before adding 0.05 ml to each well of the 96-well microtiter plate. Plates were incubated in a 5% CO2 atmosphere at 37°C for 48–96 h. Cell growth was monitored using an inverted microscope.

After incubation of samples and indicator cells for 2–4 days, 25 μl (2 μCi) of [3H]thymidine (DuPont New England Nuclear, Boston, MA) was added to each well, and plates were incubated for 6 h prior to harvesting DNA onto filter paper discs with a PHD cell-harvesting apparatus (Cambridge Technology, Inc., Watertown, MA). After addition of liquid scintillation fluid (Cytoscint; ICN Biochemicals, Costa Mesa, CA), radioactivity was determined using an LKB 1216 liquid scintillation counter. Data was analyzed using an Excel macro routine written by Dr. Bob Jarvis (Department of Computer Sciences, University of Minnesota, Minneapolis, MN) to determine the mean and SD of each triplicate set of samples. Clonogenic assays were done with OHS with the use of methods reported previously (33).

In Vivo Use of TP-3-PAP. Mice were fed and housed by University of Minnesota Research Animal Resources in accordance with NIH guidelines. Procedures and protocols involving live animals were approved by the University of Minnesota Animal Care Committee. The MCA106 sarcoma (34) was obtained from Dr. Jim Mule (National Cancer Institute, Bethesda, MD) and serially passaged in female C57BL/6 mice. Tumors were harvested, minced, and digested by stirring on a magnetic stir plate for 4 h using 0.4 mg/ml hyaluronidase, 0.05 mg/ml DNase, and 4.0 mg/ml collagenase (Sigma) in RPMI 1640 with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were filtered with Cell Strainers (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ), washed 3 times in HBSS without Ca^{2+} or Mg^{2+}, and concentration adjusted to 1 × 10^5 cells/ml. Pulmonary metastases were established by i.v. injection of MCA106 sarcoma cells (0.4 ml containing 40,000 cells/mouse) into the tail vein of 6–8-week-old female C57BL/6 mice. Groups of 10 mice with pulmonary metastases were treated with antibody alone or immunotoxin preparations i.p. The number of metastases was evaluated by direct counting 14 or 17 days after establishment of metastases. After asphyxiation with CO2, India ink (5% with 3 gtt NH4OH/100 ml) was injected asphyxiation with CO2, India ink (5% with 3 gtt NH4OH/100 ml) was injected into the trachea. Lungs and heart were removed en bloc and placed into Fekete’s solution (300 ml 70% ethanol-30 ml 10% formalin-15 ml glacial acetic acid). Lungs were coded and counted by at least two blinded observers. Differences in the number of metastases between treatment groups were evaluated using Student’s unpaired t test; differences in weight of individual mice on days 0 and 7 after tumor inoculation were compared using Student’s paired t test (InStat; GraphPad Software, San Diego, CA).

RESULTS

TP-3-PAP Immunotoxin Efficiently Kills TP-3+ OHS Sarcoma Cells. Fig. 2 shows the effect of TP-3 mAb, TP-3-PAP, PAP alone, and an irrelevant immunotoxin construct which binds CD19 on B cells, B43-PAP, on proliferation of human OHS osteosarcoma cells. The TP-3 mAb alone (i.e., without PAP toxin) had no effect on proliferation; cells incorporated [3H]thymidine into DNA in a manner identical to media with human serum albumin (Fig. 2A). TP-3-PAP, however, completely eliminated uptake of [3H]thymidine in the first 4 wells which had OHS cells; OHS did not survive immunotoxin treatment until TP-3-PAP was diluted to 20 pm or less.

Different lots of TP-3-PAP yielded reproducible and highly efficient killing of OHS. [3H]Thymidine proliferation assays using OHS in 5 separate experiments with lot 1 of TP-3-PAP yielded an IC50 value of 3.1 ± 1.0 pm. Three different experiments using a second lot of TP-3-PAP yielded a mean IC50 of 4.1 ± 0.3 pm. The overall mean IC50 was 3.5 ± 1.0 pm.

Killing of Cells by TP-3-PAP Is Highly Specific for Cells Expressing TP-3 Antigen. PAP alone or B43-PAP, an anti-CD19 immunotoxin, had no effect on OHS proliferation until concentrations were 10,000 pm or more (Fig. 2B). This represents a >3000-fold increase in cytotoxicity if the TP-3 mAb was conjugated to the PAP moiety. If tumors did not express the TP-3 antigen, killing by TP-3-
PAP did not occur at concentrations <10^4 pm (Fig. 3, A and B). B43-PAP, however, was active against the CD19+ cell line RS4;11 (Fig. 3B). Thus, killing by the PAP immunotoxins was conferred by specific mAb binding.

We have used a highly sensitive in vitro serial dilution clonogenic assay system to determine the log kill efficacy of TP-3-PAP immunotoxin against clonogenic OHS human osteosarcoma cells. As shown in Table 1, a 4-h treatment with 100–3000 ng/ml TP-3-PAP at 37°C/5% CO₂ killed clonogenic OHS cells in a dose-dependent fashion with a maximum of 3.9 ± 0.2 logs at 1000 ng/ml (5.6 nm). Notably, this 4-h treatment protocol with TP-3-PAP concentrations ≤100 ng/ml did not significantly inhibit the clonogenic growth of OHS cells (log kill ≤ 0.2 log). By comparison, an 18-h exposure to 1–3000 ng/ml TP-3-PAP killed clonogenic OHS cells in a dose-dependent fashion with 1.2 log kill at 100 ng/ml and >3.9 logs kill at concentrations ≥300 ng/ml (Table 1).

**Table 1** Antitumor activity of TP-3-PAP against clonogenic osteosarcoma cells (OHS human osteosarcoma clonogenic assay)

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>TP-3-PAP concentration (ng/ml)</th>
<th>Colony units (mean ± SEM)</th>
<th>Log kill (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>4588 ± 1556</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>4588 ± 1556</td>
<td>0.00 ± 0.20</td>
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<tr>
<td>4</td>
<td>30</td>
<td>2683 ± 910</td>
<td>0.23 ± 0.20</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>2683 ± 910</td>
<td>0.23 ± 0.20</td>
</tr>
<tr>
<td>4</td>
<td>1000</td>
<td>313 ± 125</td>
<td>1.16 ± 0.23</td>
</tr>
<tr>
<td>4</td>
<td>3000</td>
<td>4 ± 1</td>
<td>3.91 ± 0.23</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>4588 ± 1556</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>2052 ± 550</td>
<td>0.34 ± 0.22</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>917 ± 311</td>
<td>0.69 ± 0.20</td>
</tr>
<tr>
<td>18</td>
<td>30</td>
<td>313 ± 321</td>
<td>0.58 ± 0.18</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
<td>120 ± 135</td>
<td>1.16 ± 0.23</td>
</tr>
<tr>
<td>18</td>
<td>3000</td>
<td>62 ± 27</td>
<td>1.86 ± 0.23</td>
</tr>
<tr>
<td>18</td>
<td>3000</td>
<td>4 ± 1</td>
<td>3.91 ± 0.23</td>
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**DISCUSSION**

PAP, which belongs to the class of plant hemitoxins including gelonin, saporin, and momordica charantia inhibitor, is one of the most active ribosomal inactivating proteins. In a comparison of cytotoxicity of anti-mouse IgG immunotoxins gelonin, ricin A chain, momordin, dianthin 32, saporin, and PAP had IC₅₀ estimates of 1000, 500, 20.0, 10.7, 5.5, and 2.6 pm, respectively (25). TP-3-PAP had an IC₅₀ of 3.5 pm which is similar to the in vitro potency of the PAP anti-mouse IgG immunotoxin tested by Bolognesi et al. (25). Immunotoxins with IC₅₀ values of less than 100 pm are considered excellent. Thus, TP-3-PAP has very high in vitro cytotoxic potency. Since we have seen steady-state concentrations of 500–1000 ng/ml achieved safely in clinical trials of anti-CD19-PAP, the ability of TP-3-PAP to kill 3.9 logs of clonogenic OHS at 300 ng/ml strongly supports a hypothesis that therapeutic exposure levels to TP-3-PAP can be achieved in vivo in dogs or humans with osteosarcoma without excessive toxicity due to the PAP moiety.

The activity of an immunotoxin depends not only on the toxin utilized but also on efficient binding of antibody to antigen, endocytosis, and intracellular release of functional ribosome-inactivating proteins. Since the potency of PAP is such that a few molecules in the cytoplasm are probably sufficient to kill a cell, TP-3 antigen density may be less important than specificity of binding in determining the ultimate usefulness and therapeutic index of this particular immunotoxin. Bruland and Phil (19) recently summarized the current state of knowledge of distribution of the TP-1/TP-3 antigen on normal tissues and mesenchymal tumors. Osteosarcomas stain intensely positive at the surface and TP-1/TP-3 staining of osteosarcomas is homogenous in all regions of the tumors. Significant heterogeneity, however, was seen in the TP-1/TP-3 antigen expression of soft tissue sarcomas. For example, 4 of 11 MFH specimens were strongly positive, 4 of 11 MFH specimens were weakly positive, and 3 of 11 MFH were negative for TP-1/TP-3 binding.

The very limited tissue distribution of the TP-3 antigen makes it an attractive choice for future in vivo immunotoxin studies. The TP-3 antibody recognizes an epitope present on selected dog cancers including osteosarcoma and lung carcinoma. Thus studies of TP-3-PAP in dogs with spontaneous occurring osteosarcomas may be useful in determining in vivo antitumor efficacy in a relevant tumor model and whether unusual toxicities related to the binding of endothelial cells of neovascularity may be a problem. In humans it appears that only placental endothelium and the budding capillaries of tumors may be less important than specificity of binding in determining the activity of an immunotoxin depends not only on the toxin utilized but also on efficient binding of antibody to antigen, endocytosis, and intracellular release of functional ribosome-inactivating proteins.
were seen 24, 48, and 72 h after injection of TP-3-IgG, respectively. Since tumor-blood ratios after TP-3 administration remained more than or equal to 1.0, repeated doses of the TP-3 mAb could possibly accumulate in osteosarcomas. However, since osteosarcoma is a relatively radioresistant cancer, curative therapy using radioconjugates of TP-3 may be difficult to achieve.

Repeated doses or therapeutic courses of immunotoxins could be problematic because of immunogenicity of the toxin moiety. Recent experience with B43-PAP immunotoxin in patients with ALL indicates that this preparation is one of the least immunogenic preparations with a small minority of patients having either serious human anti-mouse antibodies or human anti-PAP antibodies. Whether this is due to the underlying disease (acute lymphoblastic leukemia), the anti-B-cell effect of the B43-PAP immunotoxin, use of cyclophosphamide with the immunotoxin, or low immunogenicity of the PAP protein remains to be determined. Future studies with TP-3-PAP in larger animals such as dogs may determine whether TP-3-PAP also has low immunogenicity.

Good therapeutic results were obtained using TP-3-PAP against a TP-3+ murine soft tissue sarcoma lung metastases in vivo. The efficient decrease of numbers of lung metastases may possibly be accounted for by several different mechanisms: (a) tumor destruction and subsequent induction of an efficient cellular immune response to the sarcoma cells may eliminate some pulmonary metastases; (b) induction of a cellular immune response against the TP-3 ligand could direct the immune response to the tumor; and (c) a direct effect of immunotoxin on the neovasculature of the sarcoma metastases is possible.

It is possible that TP-3-PAP may also act by action on tumor neovasculature. Folkman and others have elegantly reviewed the current state of knowledge of tumor angiogenesis including data indicating dependence of tumors >1 mm in diameter for angiogenesis (35-39). Recent studies, using a murine model with immunotoxin against MHC class II which is on tumor vasculature and another immunotoxin against MHC class I on neuroblastoma tumor cells, demonstrated synergy of the two immunotoxins (40, 41). Thus, tumor vascular targeting by immunotoxins may significantly increase efficacy. Since TP-3 antigen is present on budding capillaries of a wide variety of tumors (15, 16), it is possible that the TP-3-PAP immunotoxin may inhibit the growth of murine lung metastases by selectively destroying tumor vascular endothelium as well as the minority of sarcoma cells which bear the TP-3 antigen.

The limitations of mAb therapy and immunotoxin therapy of cancer are many (42-45). These include low specific uptake by tumor (42), toxicity to "innocent bystander" cells which bind the mAb or toxin, physiological barriers such as the relatively tight endothelium of the lung compared to liver and spleen (43) or increased interstitial pressure within tumors (44), and the production of antibodies to mAb and/or toxins. Although PAP immunotoxins may be less immunogenic than ricin immunotox conjugates, human anti-mouse antibodies and human anti-PAP antibodies have been seen in some patients with ALL. Therefore, strategies to

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Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose of immunotoxin</th>
<th>No. of lung metastases</th>
<th>Student’s t test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS (control)</td>
<td>None</td>
<td>10.20 2.50</td>
<td>NS</td>
</tr>
<tr>
<td>TP-3 MAB</td>
<td>0.0</td>
<td>16.40 2.80</td>
<td>NS</td>
</tr>
<tr>
<td>TP-3-PAP</td>
<td>1.1</td>
<td>5.20 1.70</td>
<td>NS</td>
</tr>
<tr>
<td>TP-3-PAP</td>
<td>3.3</td>
<td>2.20 4.20</td>
<td>0.047</td>
</tr>
<tr>
<td>TP-3-PAP</td>
<td>10.0</td>
<td>0.75 0.75</td>
<td>0.039</td>
</tr>
<tr>
<td>B43-PAP</td>
<td>10.0</td>
<td>6.50 2.80</td>
<td>NS</td>
</tr>
</tbody>
</table>

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Fig. 4. Dose response of TP-3-PAP immunotoxin against MCA106 sarcoma lung metastases. Increasing doses of TP-3-PAP on days 2, 3, and 4 resulted in significantly fewer pulmonary metastases than in control mice 17 days after establishing lung metastases (t test, P = 0.0001 for 5 and 10 μg groups). Columns, mean; bars, SD.

Fig. 5. Effect of TP-3-PAP therapy on weight (wt.) gain of mice. A, total weight of mice (n = 10/group) on days 10 and 12 after therapy with TP-3-PAP on days 3, 4, and 5. B, individual weights of mice in an experiment in which mice in the TP-3-PAP group received 5 μg immunotoxin i.p. on days 3-7. The control had no change in weight (Student’s paired t test, P = 0.85). However, mice receiving TP-3-PAP had a mean loss of 1.7 g (~9% body weight; Student’s paired t test, P = 0.0001).

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Footnotes:

1 F. M. Uckun, unpublished data.
reduce the occurrence of human anti-mouse and anti-PAP antibodies such as “humanization” of the TP-3 antibody, concurrent use of cyclophosphamide, and/or induction of split tolerance (e.g., using this project.

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