Inhibition of Antibody Response to *Pseudomonas* Exotoxin and an Immunotoxin Containing *Pseudomonas* Exotoxin by 15-Deoxyspergualin in Mice

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ABSTRACT

Immunotoxins are potent cell-killing agents that may be useful in the treatment of cancer. The early production of neutralizing antibodies to immunotoxins is one of the major limiting factors for their use in humans. 15-Deoxyspergualin (DSG), a derivative of spergualin, which is a metabolite of *Bacillus laterosporus*, has been found to have immunosuppressive activity in rodents, dogs, and primates. We examined the suppressive activity of DSG on the antibody response to *Pseudomonas* exotoxin in mice by enzyme-linked immunosorbent assay. Male BDF1 mice were immunized with a single dose of a nontoxic mutant of *Pseudomonas* exotoxin (40 ng) and then treated with i.p. injections of DSG at a dose of 10 mg/kg for 3 days. Although antibodies to *Pseudomonas* exotoxin were observed within 7 days in the control group, there was complete suppression of antibody production in the DSG-treated group. Immunosuppression has also been observed in animals immunized with multiple doses (10 mg x 7 d) of *Pseudomonas* exotoxin and treated with DSG at a dose of 5 mg/kg for 21 days. Similar immunosuppression was observed in mice given multiple doses of the immunotoxin, anti-Tac-LysPE40. We conclude that the immunosuppressive activity of DSG may be useful in increasing the duration of immunotoxin treatment.

INTRODUCTION

Over the past decade, advances in protein chemistry and hybridoma technology have led to the development of immunotoxins consisting of monoclonal antibodies conjugated to protein toxins made by bacteria and plants: diphtheria toxin, PE2, and ricin have been widely used for this purpose (1-3). These toxin conjugates are potent cell-killing agents, and their possible use in the treatment of cancer is being explored (4, 5). Results from the first clinical trials with these hybrid molecules have shown several problem areas that must be addressed for immunotoxins to become broadly effective therapeutics. One of these problems is the early production of antibodies against immunotoxins, which may neutralize the toxic activity of these conjugates, limiting their therapeutic efficacy (6, 7). The formation of human antibodies against murine monoclonal antibodies is well described and has been shown to alter their pharmacodynamics (8) and pharmacokinetics (9). It is clear that, to improve the efficacy of the immunotoxins, strategies to inhibit the immune response to foreign proteins need to be explored.

Cyclophosphamide, a widely used antitumor alkylating agent, together with prednisone has been shown to inhibit the formation of antibodies against abrin and ricin in mice (7); however, these agents are not without toxic side effects. In this study, we examined the suppressive activity of DSG. This compound is a derivative of spergualin, a metabolite of *B. laterosporus*, and has antitumor activity against murine leukemias (10). It has also been shown to possess immunosuppressive activities in experimental animal models (11, 12). In this study, we assessed the ability of DSG to suppress the antibody response to PE and a PE-containing immunotoxin. The antibody formation against PE was measured either by an ELISA or by the ability of serum from immunized mice to block the cytotoxic action of anti-Tac-LysPE40 in human T-cell lymphoma cells (HUT 102).

MATERIALS AND METHODS

Animals

Male BDF1 mice (20-25 g), 6-8 wk of age, were obtained from the Frederick Cancer Research Facility, Frederick, MD, and kept in an approved animal facility. Animals were fed a standard diet with drinking water *ad libitum*.

Drug

DSG was supplied by Bristol-Myers Squibb Co., Wallingford, CT. It was dissolved in sterile PBS, pH 7.2, just prior to use each day.

Toxins

PE553D. PE553D is a recombinant mutant form of PE expressed in *Escherichia coli* in which the glutamic acid at position 553 is deleted, resulting in loss of ADP-ribosylation activity but not its immunogenicity (13). It was chosen for these experiments because of its lack of toxicity in animals.

Anti-Tac-LysPE40. Construction of this immunotoxin has been previously described (14). LysPE40 is a recombinant form of PE with M, 40,000 that lacks domain I of PE and has very low liver toxicity because it no longer binds to PE receptor present on liver cells. A lysine residue has been included near the amino end, which makes it readily coupled to antibodies. LysPE40 was purified from the culture medium of BL21 (ADE3) cells containing plasmid pJY85L (15). After purification, LysPE40 was chemically coupled to anti-Tac antibody, which binds to the M, 55,000 subunit of human interleukin 2 receptor (16). After the conjugation, 1:1 forms of the immunotoxin were purified on Mono Q and TSK-250 columns. Purified conjugates were found to contain LysPE40 coupled to either the light or the heavy chain of the antibody.

Immunosassay

ELISA was used to determine the presence of mouse anti-PE antibodies in the serum of treated and control animals. Microtiter with plates 96 wells (Dynatech Laboratories, Alexandria, VA) were coated with whole PE or LysPE40 (50 ng/well), incubated overnight at 4°C, and then thoroughly washed. Wells were blocked with PBS-bovine serum albumin 2.5% and incubated for 30 min. Serum samples were first diluted 1:10 in 1% bovine serum albumin in PBS and then serially diluted for titration of binding activity. Samples were added to wells in triplicate and incubated for 1 h at 37°C. Wells were again washed thoroughly and to each well was added 50 μl of peroxidase-conjugated AffiniPure goat anti-mouse IgG+IgM (H + L) or IgM or IgG alone (1:1000 dilution) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). A 30-min incubation was followed by thorough washing with PBS. Substrate [100 μl/well of 1 mg/ml 2,2-azinodi(3-ethylbenzthiazolin)sulfonate) (6) (Boehringer Mannheim) with 1 μl/ml 30% H2O2 in citric acid and sodium phosphate buffer, pH 4.4, was added to each well. Reaction was stopped with 100 μl/well of 10% sodium dodecyl sulfonate in citric acid and sodium phosphate buffer, pH 4.4, was added to each well. Reaction was stopped with 100 μl/well of 10% sodium dodecyl sulfonate in citric acid and sodium phosphate buffer, pH 4.4, was added to each well.

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2. The abbreviations used are: PE, *Pseudomonas* exotoxin; DSG, 15-deoxyspergualin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.
Suppression of Antibody Response to a Single Dose of PE553D

RESULTS

Statistical Analyses

The activity of anti-Tac-LysPE40 after incubation with sera was determined by measuring its effect on protein synthesis on HUT 102 cells. Cells were washed 3 times with appropriate media and plated at a density of 2 x 10⁶ cells/well. Serum (10 µl) incubated with anti-Tac-LysPE40 was added to each well in triplicate and incubated at 37°C for 16-20 h, after which [³H]leucine (5 µCi/ml) was added for 90-120 min. The cells were then harvested using a 96-well Skatron harvester (Skatron, Inc., Sterling, VA). The incorporated radioactivity was determined by scintillation spectroscopy.

Protein Synthesis Assay

The activity of anti-Tac-LysPE40 was determined at different times after the immunization. A control group received sterile PBS. Blood samples were obtained on days 0, 7, 14, and 21, and antibody response to PE was determined at different times after the immunization and treatment by ELISA. Results are reported as absorbance values of serum specimens diluted 1:10. This dilution was selected to provide a standard of comparison among samples within the group. The absorbance numbers reported were obtained after subtraction of the prebleed (background) serum sample.

As shown in Fig. 1, IgM production to a single dose of PE553D was detected by day 4 postimmunization, with a maximal response by day 7. When DSG was administered, there was a significant inhibition of this response. This inhibition was observed on days 4, 7, 14, and 21. The two groups, control and treated, were compared at each time point using the Wilcoxon rank sum test.

Statistical Analyses

Statistical comparisons were made with the Wilcoxon rank sum test.

RESULTS

Suppression of Antibody Response to a Single Dose of PE553D by DSG

To measure the antibody response to a single dose of toxin, groups of 5 mice received a single dose of PE553D (40 µg) i.p. on day 0. The treatment group received DSG, 10 mg/kg, administered i.p. once a day for 3 d starting 24 h after immunization. A control group received sterile PBS. Blood samples were obtained on days 0, 4, 7, 14, and 21, and antibody response to PE was determined at different times after the immunization and treatment by ELISA. Results are reported as absorbance (O.D.) values of serum specimens diluted 1:10. Results demonstrated a significant effect of the DSG (P2 = 0.009). Bars, SD. m, DSG; •PBS.

Suppression of Antibody Response to Anti-Tac-LysPE40 by DSG

Having shown that the antibody response to a single dose of PE553D could be suppressed, DSG was tested for its activity against multiple doses of PE553D. Groups of 5 animals were given daily i.p. doses of PE553D (10 µg/day) diluted in 200 µl of sterile PBS for 7 days. The treatment group received DSG, 5 mg/kg i.p. for 21 days starting on day 1. The other group received only PBS. Blood samples were collected on days 0, 7, 14, 21, and 28. Using this immunization protocol, antibody production was noted by day 7 and continued to rise until day 28 (Fig. 2). The response was almost entirely IgG, with very small amounts of IgM being produced (results not shown). This response was completely suppressed in the DSG-treated group (P2 = 0.001).

Suppression of Antibody Response to Anti-Tac-LysPE40 by DSG

Immunotoxins composed of monoclonal antibodies coupled to Pseudomonas exotoxin are presently being investigated as possible therapeutic agents for cancer therapy. One of these immunotoxins is anti-Tac-LysPE40. Anti-Tac is a murine monoclonal antibody (IgG2a) which binds to the 55-kilodalton subunit of human interleukin 2 receptor. Anti-Tac has been coupled to LysPE40, a recombinant form of PE that is missing the cell-binding domain of PE. It was important to study the immunogenicity of PE40 coupled to a monoclonal antibody

Fig. 1. Histogram of mouse IgM response to PE following a single dose of PE553D. Mice were treated with DSG, 10 mg/kg for 3 days. Antibody response was measured on days 4, 7, 14, and 21 by ELISA. Results are reported as absorbance (O.D.) values of serum specimens diluted 1:10. Results demonstrated a significant effect of the DSG (P2 = 0.009). Bars, SD. m, DSG; •PBS.

Fig. 2. Histogram of mouse IgG response to PE following multiple doses (7) of PE553D. One-half of the mice were treated with DSG, 5 mg/kg for 21 days. Antibody response was measured on days 7, 14, 21, and 28 by ELISA. Results are reported as absorbance (O.D.) values of serum specimens diluted 1:100. Bars, SD. m, DSG; •PBS.
because this is the form in which PE will be used in the clinic.

Anti-Tac-LysPE40 was administered to mice at 20 μg/d for 7 consecutive days by the i.p. route. The treatment group received DSG at 5 mg/kg i.p. for 14 d, starting 1 d after the first dose of immunotoxin. The control group received PBS. The antibody response to PE was determined on days 7, 14, 21, and 28. Fig. 3 shows the IgG and IgM response to PE. IgM was detected by day 7, and IgG was detected by day 14 and continued to rise on day 28. Abolition of the antibody response to PE, IgG, and IgM was observed in the DSG-treated group \( (P^2 = 0.009) \).

Neutralization of PE Activity by the Presence of Anti-PE Antibody

To determine if the antibodies against PE were capable of neutralizing the cytotoxic activity of anti-Tac-LysPE40, sera from animals immunized with multiple (daily × 7) doses of anti-Tac-LysPE40 (four control and four treated with DSG) were diluted 1:1, 1:10, 1:100, and 1:1000 and then incubated with anti-Tac-LysPE40. Samples were then tested for their ability to inhibit protein synthesis in HUT 102 cells (Fig. 4). At 1:1 dilution, sera from animals immunized with anti-Tac-LysPE40 but not treated with DSG showed 100% neutralizing activity to anti-Tac-LysPE40 by day 7 and day 28 (group A); minimal neutralizing activity was observed at 1:1000 dilution. Mice immunized with anti-Tac-LysPE40 and treated with DSG at 5 mg/kg/d × 14 d had no antibodies detectable by ELISA and no significant neutralizing activity (group B). Results are expressed in antibody titer, which is calculated based on the ability of the serum sample to inhibit the cytotoxic effect of anti-Tac-LysPE40 on HUT-102 cells (14).

DISCUSSION

In the present paper we have shown that 15-deoxyspergualin is capable of inhibiting the antibody response to *Pseudomonas* exotoxin, a cytotoxic protein presently being explored for use in immunoconjugates. Antibody response was significantly suppressed in animals given a single dose of toxin, seven daily doses of toxin, or seven daily doses of toxin coupled to a monoclonal antibody. This immunosuppressive property of 15-deoxyspergualin was previously demonstrated using single injections of sheep RBC. Based on that result and the results presented here, DSG would be expected to suppress the antibody response to a wide variety of antigens including ricin and other toxic proteins used to make immunotoxins, allowing an increase in the duration of therapy and making these agents more attractive for use in cancer treatment.

Immunotoxins are a new form of anticancer therapy. Several clinical trials are presently under way using murine monoclonal antibodies coupled with different toxins. We have used a monoclonal antibody coupled to PE in a phase I trial against ovarian cancer. OVB3-PE was given i.p. on days 1 and 4 or 1, 4, and 7, and antibodies against PE were detectable by day 14.3 Monoclonal antibodies alone have been used in cancer diagnosis (17, 18) and therapy (19–21), and the development of antimurine antibody following the administration of unmodified murine monoclonal antibodies has been well documented (22–24). The development of chimeric monoclonal antibodies and human monoclonal antibodies, which are less immunogenic than murine monoclonal antibodies (25, 26), makes their use more attractive, and clinical trials with these constructs are presently being carried out. Protein toxins, however, have strong antigenic determinants. Godal et al. (7) studied antibody formation against abrin and ricin in humans and mice. In mice treated weekly with therapeutic doses of ricin, antibodies were detected after 2–3 wk. Because of the development of antibodies reacting with both the toxin and the mouse antibody, which neutralize the cytotoxic activity of the immunotoxin, the duration of therapy with these agents is presently limited to only a few

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Fig. 3. Histogram of mouse IgG (A) and IgM (B) response to PE following multiple (7) daily doses of anti-Tac-LysPE40 treated with DSG, 5 mg/kg for 14 d. Antibody response was measured on days 7, 14, 21, and 28 by ELISA. Results are reported as absorbance (O.D.) values of serum specimens diluted 1:100. Bars, SD. \( P^2 = 0.001 \). DSG; PBS.
weeks. It is unlikely that highly effective therapy against human tumors can be achieved in such a short period; it is therefore important to develop strategies to inhibit antibody formation.

15-Deoxyspergualin was originally developed as an antitumor agent with documented activity against murine leukemias (10) and has entered phase II trials against human tumors. However, at lower doses than those used to treat leukemia, this agent had immunosuppressive properties in animal models (27–30). The exact immunopharmacological mode of action remains unclear. Dickneite et al. reported that DSG suppressed certain macrophage/monocyte functions such as chemiluminescence, secretion of hydrolytic enzymes, and major histocompatibility complex class II expression and that the secretion of interleukin 1 by macrophages was decreased (31), which would lead to an early blocking in the immune response, suggesting that DSG may act at the stage of the antigen presenting cell.

In our experiment, both the antigen and DSG were given i.p., but previous studies showed that DSG has a pronounced inhibitory effect on the antibody response to various kinds of antigens when given by i.v., i.m., or i.p. routes (27–30), thus providing evidence for a systemic rather than a local effect of this agent. Most antigens, such as heterologous serum protein or sheep RBC, require T-cell help for B-cells to initiate antibody production and are T-dependent antigens. Bacterial toxins, such as Pseudomonas exotoxin, are also T-dependent antigens. Other antigens, such as trinitrophenylated lipopolysaccharide and trinitrophenylated Ficoll, appeared not to require T-cell help and are called T-independent antigens. Makino et al. (31) showed that DSG significantly suppressed the antibody response to both T-dependent and T-independent antigens in C57BL/6 mice, suggesting that DSG acts on both B- and T-cells.

In this study we have described the immunogenicity of Pseudomonas exotoxin in mice and the suppression of antibody production to PE by DSG. Mice given single or multiple doses of PE553D or an immunotoxin containing PE developed antibody against PE within 7 d of the immunization. The antibody was capable of neutralizing the cytotoxic activity of an immunotoxin containing PE. The finding that deoxyspergualin is capable of suppressing the antibody response to the toxin in mice is encouraging because this may allow one to administer immunotoxins for a longer period, increasing the time for effective immunotoxin therapy.

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