Morphological Effects of Pasteurella multocida Type-D Dermonecrototoxin on Rat Osteosarcoma Cells in a Nude Mouse Model

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Summary
Of 15 athymic nude mice that received subcutaneous implants of a rat osteosarcoma cell line, two groups of four subsequently received either a short (group 1) or a more prolonged (group 2) course of subcutaneous injections of the dermonecrotic toxin (DNT) of Pasteurella multocida type D. The remaining seven mice (controls) received no DNT. Both groups of DNT-treated mice lost body weight as compared with controls. Tumour weight, expressed as a percentage of body weight, increased in the four group 1 mice. Tumours in this group 1 were consistently larger than those in appropriate controls, indicating that this percentage was not simply a function of decreased body weight. The immunohistochemical labelling of proliferating cell nuclear antigen (PCNA) and morphometric analysis of intratumoral necrosis suggested that the DNT had a mitogenic effect and contributed to the neoplastic growth. The presence of foci of neoplastic osteoblasts in the lungs of some DNT-treated mice suggested that the enhanced tumour growth led to an increased incidence of metastasis.

Introduction
Strains of Pasteurella multocida that produce dermonecrototoxin (DNT) are associated with turbinate atrophy, as seen in atrophic rhinitis of swine (Dominick and Rimler, 1988; Martineau-Doize et al., 1990, 1991a,b; Ghoshal and Niyo, 1993). Experimentally, purified DNT induces marked osteoporosis when applied to the mucosa as an aerosol or injected subcutaneously (Dominick and Rimler, 1988). The mechanism by which DNT induces osteoporosis is unclear. However, DNT may inhibit osteogenesis through a direct toxic effect on osteoblasts, or increase osteoclastic osteolysis (Felix et al., 1992; Foged, 1992; Ackermann et al., 1993; Martineau-Doize et al., 1993; Gwaltney et al., 1997). In-vitro studies have shown that DNT is cytopathogenic to several mesenchymal cell types, such as rat, canine or human osteosarcoma cell lines (Martineau-Doize et al., 1990, Foged, 1992; Pettit et al., 1993a). The toxin has been shown to gain access to target cells by receptor-mediated binding, followed by endocytosis (Idali et al., 1991; Pettit et al., 1993a). Signal transduction studies
indicate that DNT facilitates the coupling of a G protein to phospholipase C, resulting in increased intracytoplasmic calcium concentrations and phosphorylation of a protein kinase C substrate via the 1,4,5 inositol triphosphate/diacylglycerol pathway (Staddon et al., 1990, 1991, 1992; Higgins et al., 1992; Murphy and Rozengurt, 1992). DNT has been sequenced, and the toxA gene identified and cloned in an Escherichia coli vector (Peterson and Foged, 1989; Buys et al., 1990; Lax and Chanter, 1990; Lax et al., 1990; Peterson, 1990). In view of the toxin’s in-vitro effect on different osteosarcoma cell lines, it was felt that a similar cytopathogenic effect might occur in vivo. If so, the toxin could be considered as a possible therapeutic agent for mesenchymal cell cancers such as osteosarcoma. The objective of the study reported here was to examine the effect of DNT on rat osteosarcoma (ROS) cells implanted subcutaneously in nude mice.

Materials and Methods

Toxin Preparation
Toxin from the P. multocida non-encapsulated strain P-45 was purified as previously described (Ackermann et al., 1992). Briefly, bacteria were grown on horse blood agar and lysed with ethylenediaminetetraacetic acid (EDTA), lysozyme, Triton X-100, phenylmethylsulphonyl fluoride, and pepstatin A. The lysate was centrifuged, and crude toxin was precipitated with ammonium sulphate. The precipitate was resuspended in 1 ml of phenylmethylsulphonyl fluoride, centrifuged, and the supernate applied to a 2.6 x 19-cm DEAE-sepharose column (Pharmacia, Piscataway, NJ, USA). The appropriate (second) peak was concentrated and used as a purified toxin. The toxin contained no detectable lipopolysaccharide.

Experimental Design
Sixteen male athymic nude (N:NIH-bg-nu-xid) mice (Charles Rivers Laboratories, Wilmington, MA, USA) were housed in a germ-free isolator unit and fed sterile diet (Charles Rivers). After acclimatization, all mice were given 0.5 ml ROS (Majeska et al., 1978) (3.7 x 10⁷ cells), subcutaneously, over the left flank. This number of cells was chosen to ensure tumour growth. By 14 days post-implantation (PI), tumour growth was apparent in all animals, and mice were treated as follows. Eight of the 16 animals were used as controls, receiving no toxin, but one of these was subsequently excluded from the study because of a suspicion that a significant proportion of the tumour cells had been implanted intramuscularly, instead of subcutaneously. The remaining eight were divided into two treatment groups (1 and 2) of four. Mice in group 1 each received DNT 0.1 μg/kg (determined as the highest sublethal dose) in 0.1 ml diluent, subcutaneously in the dorsal cervical region, on days 16, 20, and 24 PI. This temporal arrangement, determined in pilot studies, ensured survival of the mice. Four days after the final toxin treatment (i.e., on day 28 PI), four control mice, and the four group 1 animals were killed and their tissues harvested. The four group 2 mice received an extended course of treatment with toxin; thus, DNT was injected on days 16, 20, 24, 28, 32 and 36 PI. Four days later (i.e., on day 40 PI), the remaining three control animals and the four group 2 mice were killed and their tissues harvested.

Histopathology
Entire subcutaneous tumours at the injection site were removed, weighed, measured, and placed in 10% neutral buffered formalin with samples of spleen, liver, and lung. Tissues were processed by routine methods, sections being stained with haematoxylin
and eosin and examined by light microscopy. Replicate sections were left unstained and used for immunohistochemical examination.

**Immunohistochemistry**

Sections of neoplastic tissue and selected organs were labelled by an immunohistochemical procedure to detect cells undergoing DNA synthesis, as determined by the presence of proliferating cell nuclear antigen (PCNA). Endogenous peroxidase was eliminated by incubation with hydrogen peroxide 0.5% in absolute methanol. Undiluted goat serum was used as blocking antibody. Primary antibody (anti-PCNA) (Dako, Carpinteria, CA, USA), at a 1 in 15 dilution (Tris buffered saline, pH 7.6), was applied at 37°C for 30 min. Secondary antibody (goat anti-mouse, 1 in 200) was conjugated to streptavidin-peroxidase and incubated with the sections for 30 min, and the chromogen was a 3,3' diaminobenzidinetetrahydrochloride (DAB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA)–levamisole (Vector Laboratories, Burlingame, CA, USA) solution applied for 2 min. Sections were counterstained with Harris’s haematoxylin. Counting of cell nuclei was performed by light microscopy with an intraocular reticle (grid 10 x 10). By means of the reticle, five x 40 fields were randomly examined from each tumour section. Total numbers of negative nuclei per grid were counted, followed by counting of positively labelled nuclei. A single operator performed all the nuclear counts. Percentages of dividing nuclei in each tumour were determined from the data obtained.

**Morphometric Analysis**

Image analysis was carried out by the Iowa State University Image Analysis Facility with a Zeiss SEM-IPS image analysis system (Zeiss-Kontron; IBAS version 2.00). Microscope slides were placed on a copy stand and transilluminated with a ChromaPro 45 lightbox. Images were captured by a Sony DXC-3000A 3 CCD colour video camera, with a 60-mm macro lens. The operator then selected the area to be measured by drawing around the edge of the imaged sample, excluding any extraneous tissue. The neoplastic tissue (dark purple staining) and necrotic tissue (light purple staining) were interactively discriminated from the resulting image. These areas were automatically measured and recorded in the system’s database. The amount of tumour represented as necrotic tissue was determined by calculating the area of necrosis as a percentage value of necrotic tissue plus tumour tissue.

**Statistical Analysis**

Statistical results were obtained with an analysis of variance (ANOVA) based on a completely randomized design. P<0.05 was considered statistically significant.

**Results**

**Macrosopical Appearance of Tumours**

The gross appearance of the neoplasms was consistent throughout the study. After implantation, all mice developed tumours, varying from 0.2 to 2.4 cm in diameter. The tumours were cream to pale grey in colour, the cut surface displaying occasional foci of haemorrhage or necrosis, or both. They were multilobulated, focal to multifocal in distribution, and located subcutaneously. Very occasionally, tumours showed patchy infiltration of adjacent tissues, and
Table 1

Effects of DNT treatment on mice given ROS

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Mouse no.</th>
<th>Mouse weight (g)</th>
<th>Tumour weight (g)</th>
<th>Tumour weight as % of body weight</th>
<th>Necrosis as % of total tumour</th>
<th>Number of dividing cells/×40 field</th>
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<tr>
<td>Controls for group 1</td>
<td>1</td>
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<td>0.61</td>
<td>2.20</td>
<td>15.01</td>
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<td>0.52</td>
<td>2.00</td>
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<td>17.00</td>
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<td></td>
<td>3</td>
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<td>0.31</td>
<td>1.20</td>
<td>1.39</td>
<td>14.00</td>
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<tr>
<td></td>
<td>4</td>
<td>24.85</td>
<td>1.74</td>
<td>7.00</td>
<td>33.07</td>
<td>20.00</td>
</tr>
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<td>1.70</td>
<td>7.50</td>
<td>50.42</td>
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<tr>
<td></td>
<td>2</td>
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<td>1.42</td>
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<td>29.73</td>
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<tr>
<td></td>
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<td>2.23</td>
<td>9.70</td>
<td>25.40</td>
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<tr>
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<td>4</td>
<td>18.62</td>
<td>1.38</td>
<td>7.40</td>
<td>28.56</td>
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<td>24.36</td>
<td>42.50</td>
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<tr>
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<td>1.18</td>
<td>4.60</td>
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<tr>
<td></td>
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<td>N/A*</td>
<td>N/A*</td>
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<td>38.50</td>
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<tr>
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* Control mouse, group 2—intramuscular deposition of ROS precluded accurate tumour extraction and measurement.

one mouse (control for group 2) showed local invasion of the spinal column and retroperitoneum; these findings were believed to have resulted from the accidental intramuscular deposition of some osteosarcoma cells.

Microscopical Appearance of Tumours

Both control and DNT-treated tumours were characterized by a non-encapsulated, well-delineated focus of neoplastic mesenchymal cells, arranged in sheets and haphazard aggregates on a dense fibrous stroma. Neoplastic cells, which were pyriform to cuboidal in shape, with modest amounts of eosinophilic cytoplasm, contained oval to irregular nuclei with dispersed chromatin and one to three distinct nuclei. Intricately branching trabeculae of osteoid were often present between neoplastic osteoblasts. Mitotic figures were common (one to three per ×40 field). Randomly distributed foci of necrosis and multifocal areas of mineralization were also present. These foci, which were entirely intratumoral, were characterized by prominent zones of coagulative necrosis with central areas of mineralization. The margin of each necrotic zone was distinct from the adjacent, viable neoplastic tissue.

Body Weight

The toxin-treated mice (groups 1 and 2) had lower body weights than the controls at the time of killing (\( P=0.0269 \), group 1; \( P=0.0154 \), group 2). The body weights of group 2 mice were slightly but not significantly lower than those of group 1 (Table 1).
**Tumour Weight**

Tumour weights in group 1 mice tended to be higher than those of the control mice for group 1, but this difference was not statistically significant ($P=0.0564$). In contrast, mice from group 2 had significantly reduced tumour weights ($P=0.5059$) (Table 1).

**Tumour Weight/Body Weight (Percentage)**

Toxin-treated mice from group 1 demonstrated a statistically significant higher percentage of body weight as tumour weight than did control mice for group 1 ($P=0.0255$). This trend continued in group 2, but was not statistically significant ($P=0.9294$) (Table 1).

**Necrotic Tissue/Tumour (Percentage)**

The amount of necrosis per tumour was consistently higher, but not significantly so, in the toxin-treated animals from both groups ($P=0.0754$, group 1; $P=0.2405$, group 2) (Table 1).

**Number of Dividing Nuclei**

Immunohistochemical labelling of PCNA showed no significant difference in the numbers of mitotically active cells between toxin-treated and control groups ($P=0.1225$, group 1; $P=0.1535$, group 2). Numbers of dividing cells were somewhat higher for toxin-treated mice, but the trend was mild. Positive labelling, which was restricted to nuclei, varied from intense, diffuse labelling of nucleoplasm, to variable amounts of stippled to margined intranuclear positive reactivity (Fig. 1). Nucleoli were also positive. The pattern of labelling...
Fig 2. Tumour nodule in DNT-treated ROS-implanted mouse. PCNA labelling, × 20. Note distinct line of demarcation (arrowheads) between necrotic (N) and viable (V) tissue. Bar, 40 μm.

was multifocal and randomly distributed throughout all the tumours, and displayed marked variability. However, there was consistent and intense labelling of nuclei in cells immediately adjacent to areas of necrosis (Fig. 2). This feature was found in nearly all tumours examined, giving prominent collars of positively labelled neoplastic cells around zones of coagulative necrosis.

Metastasis

Four out of fifteen (26.6%) mice demonstrated metastasis to the lung. No metastases were observed in the liver or spleen. The metastatic foci varied from randomly-placed microclusters of five to 10 neoplastic osteoblasts to aggregates which occupied larger portions of pulmonary parenchyma. None of the metastatic foci were visible grossly. Three (group 1, one; group 2, two) out of four of these mice had been treated with toxin. Examination of additional sections of lung from all mice failed to reveal the presence of additional metastases.

Discussion

The DNT of *P. multocida* type D causes weight loss and lesions in a number of organs in several animal species (Cheville and Rimler, 1989; Thurston et al., 1989; Williams *et al.*, 1990; Ackermann *et al.*, 1992; Foged, 1992). Pettit *et al.* (1993b) showed that in rats the observed weight loss could be blocked by treatment with toxoid. Toxin-treated, tumour-implanted mice (groups 1
and 2) in the present study showed statistically significant weight loss. The toxin may produce this effect either directly by inducing anorexia and decreased energy intake, or indirectly through the effects of inflammatory cytokines such as interleukin-1 or -6 (IL-1 or IL-6) or tumour necrosis factor (TNF). In addition, the possibility of decreased weight gain as a result of chemical mediators directly secreted by neoplastic cells, or the combined effects of such mediators and inflammatory cytokines, leading to cachexia, cannot be ruled out. The possible effect of DNT on weight gain in this model must be considered, in light of the fact that sublethal doses of toxin were administered, to assess the protein’s possible therapeutic activity. However, DNT appeared to contribute to weight loss.

Tumour weights from the toxin-treated mice of group 1 were consistently, but not significantly, higher than those of the appropriate control mice. Conversely, group 2 toxin-treated mice demonstrated decreased tumour weights. It is possible that the effect of DNT was influenced by the age of the tumours, but the group 1 results require confirmation with a larger number of animals. The suggestion that treatment with toxin increases tumour size is consistent with earlier reports that DNT is mitogenic to some cell lines in vitro, particularly Swiss 3T3 fibroblasts (Rozengurt et al., 1990; Staddon et al., 1990, 1991). Cellular responses to DNT vary widely and some cell lines are resistant to the toxin’s effect (Sterner-Kock et al., 1995). The significantly increased amount of body weight attributable to tumour mass in the toxin-treated mice of group 1 suggests that the effect of the toxin in such mice is at least two-fold. DNT may simultaneously contribute to the significant weight loss, and enhance the mitogenic potential of neoplastic osteoblasts. This observation is supported by the fact that control mice had smaller tumours, and increased body weights. Therefore, there is a suggestion that in group 1 mice the toxin enhanced the proliferative potential of the tumours.

The amount of necrosis observed in each tumour was consistently higher in toxin-treated mice (groups 1 and 2). The mechanism of necrosis production is unclear, but the following possibilities exist: DNT is directly toxic to the neoplastic cells; DNT causes necrosis by inducing a second population of cells to produce humoral factors that kill neoplastic cells; the toxin stimulates neoplastic proliferation at a rate which exceeds the vascular supply, and necrosis due to ischaemia results. It is tempting to speculate that the extent of necrosis is related to the action of the toxin, but there is no direct evidence that such is the case. Indeed, the tumour necrosis might result from (1) outstripping of the blood supply by neoplastic growth, (2) infiltration of the tumour by inflammatory cells (although in the present study minimal inflammation was associated with tumours), or (3) the local release of cytokines. The type of necrosis ranged from coagulative to caseous, and was generally limited to the centre of tumour nodules. Proliferation of tumour cells, in contrast, generally occurred at the periphery of tumour nodules. Proliferation indices were determined by PCNA, a 36-kDa nuclear protein, which increases in concentration during the G1 phase of the cell cycle, peaks during the S phase, and begins to decline in G2 (Trere, 1993). PCNA labelling varied within individual tumours, and between groups. However, as a trend
(which proved not to be statistically significant), toxin-treated mice in both groups 1 and 2 consistently showed increased numbers of positively labelled nuclei. This finding is consistent with the increased tumour weights noted in group 1, and the increased tumour weights as a percentage of body weight seen in groups 1 and 2. These three parameters, taken in concert, suggest that the toxin, rather than causing tumour growth to decrease, stimulated neoplastic proliferation. Previous studies have indicated that DNT binds a cell surface receptor and activates a G protein and phospholipase C; this, in turn, initiates the production of 1,4,5 inositol triphosphate, and diacylglycerol (Staddon et al., 1990, 1991, 1992; Higgins et al., 1992; Murphy and Rozengurt, 1992). 1,4,5 Inositol triphosphate mediates the release of calcium from the endoplasmic reticulum, and increased intracytoplasmic calcium induces several cellular activities, one of which is, in conjunction with diacylglycerol, the activation of protein kinase C (Darnell et al., 1990). Tumour promoters (e.g., phorbol esters) use protein kinase C as a target, which may play an important role in cell proliferation (Darnell et al., 1990). It is reported that increased cytoplasmic calcium concentrations may result in loss of gap-junction permeability, thereby inhibiting intercellular communication; this is also proposed as a mechanism in carcinogenesis (Klaunig and Ruch, 1990). Such metabolic pathways would implicate DNT in the stimulation of cell growth, in particular, neoplastic growth. A distinct pattern of cell proliferation was noted in association with necrotic zones. A prominent rim of cells adjacent to non-viable tissue reacted intensely for PCNA. The change was consistent in both groups, and did not vary with toxin treatment. In tumours in which necrosis was substantial, these proliferative areas often coalesced into larger zones of positively labelled cells. Since areas of necrosis were observed internally in all tumours, the area of most significant proliferation would appear to be just external to dying cells. Therefore, new tumour growth occurred from within, and the neoplasms could be thought of as expanding masses with the foci of most significant proliferation just adjacent to the areas of necrosis.

Evidence of metastasis was seen in four of 15 mice; three of these four were toxin-treated, two belonging to group 2. All metastases were in the lung. The metastatic foci, none of which were grossly visible, varied microscopically from microemboli to prominent aggregates that obliterated large areas of parenchyma. The relationship of metastasis to toxin treatment is unclear, but increased cell proliferation and tumour growth might lead to the selection of a metastatic phenotype. If toxin treatment stimulates such mitogenic activity in osteosarcoma cells, an increased incidence of metastasis might occur as a sequel. An increased sample size would be useful in establishing whether this is a significant possibility. Locally invasive tumour growth was observed in one mouse, but this was felt to be due to intramuscular rather than subcutaneous deposition of ROS. Pulmonary metastasis did not occur in this animal.

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References


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