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Induction of dog IL-1 by free and liposomal encapsulated doxorubicin

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Liposomal encapsulated doxorubicin, free doxorubicin and a mixture of free doxorubicin plus empty liposomes were observed to induce the production and release of IL-1 from dog peripheral blood lymphocytes (PBLs) *in vitro* whereas empty liposomes were observed not to induce IL-1 when evaluated with the EL-4/CTLL-1 biological assay. Tumor necrosis factor was not induced as measured by the L-929 biological assay. Maximum IL-1 induction occurred at doxorubicin concentrations of 0.3–0.8 μg/ml. Dog PBLs can be classified as low, moderate and high responders according to their response to doxorubicin induced IL-1 release. Agarose immobilized doxorubicin does not induce IL-1 indicating that internalization of doxorubicin is required for IL-1 release. Limulus amebocyte lysate testing of doxorubicin indicates that endotoxin contamination is not responsible for this observed release of IL-1.

Key words: Beagle dog, doxorubicin, IL-1, liposomes, pyrexia.

Introduction

The anthracycline antibiotic doxorubicin has a wide range of anti-tumor activity being useful in treating both hematologic malignancies and solid tumors.[1] Its mechanism of action is multi-fold and includes binding and intercalation into DNA,[2] DNA strand damage,[3] inhibition of topoisomerase II,[4] and damage to cell membranes.[5] Toxicities include bone marrow depression, GI ulceration, alopecia and an irreversible, therapy limiting cardiomyopathy which limits the total cumulative dose of doxorubicin that can be safely given to 550 mg/m^2.[6] Liposomal encapsulation of doxorubicin has been proposed as a method to retain or enhance anti-tumor efficacy yet reduce the severity of doxorubicin associated toxicities allowing the use of doxorubicin in excess of 550 mg/m^2 cumulative dose in patients with responsive tumors. Kanter *et al.*[7] reported the results of a comprehensive multispecies preclinical toxicologic evaluation of liposomal encapsulated doxorubicin in comparison with free doxorubicin in dogs and observed a 50% increase in the maximally tolerated dose for liposomal encapsulated doxorubicin as compared to free doxorubicin with an overall reduction the the severity of organ specific toxicities. A unique toxicity of liposomal encapsulated doxorubicin observed in 50% of the dogs was pyrexia occurring 12–18 h post-treatment which was independent of dose. This report elucidates the mechanism of this delayed pyrexia by evaluating the response of dog peripheral blood lymphocytes (PBLs) treated *in vitro* with liposomal encapsulated doxorubicin and its components.

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Materials and methods

Preparation of free doxorubicin

A 10 mg vial of Adriamycin RDG (Adria Laboratories, Columbus, OH) was reconstituted using 5 ml of 0.9% sodium chloride solution.

Preparation of liposome encapsulated doxorubicin

Liposomal encapsulation was accomplished as described previously.[7]

Preparation of agarose immobilized doxorubicin

Agarose immobilized doxorubicin was synthesized using Reacti-gel 6X (Pierce Chemical, Rockford, IL) as described by Tritton.[5] [^{14}C]Doxorubicin hydrochloride labeled at the carbon 14 position (17.0 mCi/mmol, 29 μCi/mg; SRI International, Menlo Park, CA) was included in order to quantitate doxorubicin substitution of the agarose bead and to follow stability of the agarose-immobilized doxorubicin. The drug–agarose mixture was washed with saline until background levels of radioactivity were obtained and sterilized with 70% ethanol/water for 24 h.

Dog PBL isolation

Whole blood was collected by venipuncture from beagle dogs obtained from the dog colony of this Institute. Sodium citrate (3.8%) was added as anticoagulant to a final concentration of 5% (v/v), the whole blood layered onto Histopaque 1077 (Sigma, St Louis, MO) and centrifuged at 400 *g* for 30 min at 18°C. The PBLs were removed with a Pasteur pipet and washed 4 times with phosphate buffered saline (PBS). The cells were resuspended to a concentration of 1×10^6 cells/ml in 5% heat inactivated FCS in RPMI 1640 medium supplemented with 200 μM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY).

In vitro induction of IL-1

Doxorubicin preparations were diluted in 5% heat inactivated FCS in RPMI 1640 medium and distributed to the wells of a 24-well plate. Gamma irradiated lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma) was included as a positive control. Dog PBLs (1 ml) were added to each well and the plate incubated for 48 h at 37°C (5% CO_2 atmosphere). The plates were centrifuged at 400 g for 10 min, the conditioned medium aspirated and evaluated for cytokine activity as described below.

IL-1 quantitation

The two-stage IL-1 assay developed by Simon *et al.*[8] was modified by the use of XTT[9] for the final cell quantitation. The EL-4 and CTLL-2 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Briefly, 100 μl test material was serially, 2-fold diluted in a 96-well plate using 2% heat inactivated FCS in RPMI 1640 medium. Log phase EL-4 cells were washed twice with PBS, resuspended at a concentration of 1×10^6 cells/ml in 2% heat inactivated FCS in RPMI 1640 medium and 100 μl of this cell suspension added to the wells of 96-well plate. Stock solutions of A23187 (Sigma, St Louis, MO) in dimethyl sulfoxide were diluted with 2% FCS/RPMI 1640 to 5×10^{-7} M with 50 μl being added to each well of the 96-well plate. The 96-well plates were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. The next day the plates were centrifuged (400 *g*, 10 min, 4°C) followed by the transfer of 100 μl of cell-free conditioned medium from each well to a second 96-well plate. CTLL-2 cells, maintained in the presence of rIL-2, were washed 4 times with rIL-2-free RPMI 1640, resuspended to a concentration of 2×10^5 cells/ml in 10% heat inactivated FCS in RPMI 1640 medium with 100 μl of the cell suspension being added to the wells of the second 96-well plate. The 96-well plates were incubated for 48 h at 37°C followed by the addition of 50 μl of 1 mg/ml XTT (Polysciences, Warrington, PA) plus 0.025 mM phenazine methosulfate (PMS; Sigma) in RPMI 1640 medium to each well. After an additional 18 h incubation, the metabolism of XTT was determined by measuring the absorbance at 450 nm with a BIO-TEK plate reader. Human rIL-1 β (Genzyme, Cambridge, MA) was included as a positive control.

IL-2 quantitation

Serially 2-fold diluted dog PBL conditioned medium was mixed with an equal volume of IL-2-free CTLL-2 cells (2×10^5 cells/ml) followed by incubation at 37°C for 48 h. Cell proliferation was quantitated using the XTT assay as described above.

Tumor necrosis factor (TNF) quantitation

TNF was quantitated by the L-929 mouse fibroblast lytic assay as described by Aggarwal *et al.*[10] L-929 mouse fibroblasts and TNF were kindly provided by Dr W Dembinski of this Institute.

Pyrogen determination

Pyrogen determination was performed using the Limulus amebocyte lysate E-TOXATE kit (Sigma).

Results

Treatment of dog PBLs with liposomal encapsulated doxorubicin and its components

Table 1 lists the results of the *in vitro* treatment of dog peripheral blood lymphocytes with various preparations of liposomal and free doxorubicin. LPS stimulation produces a dramatic increase in IL-1 as measured by the EL-4/CTLL-2 assay and a concomitant rise in TNF release as measured by the L-929 biologic assay. Treatment of dog PBLs with 0.1 μg/ml doxorubicin, 0.1 μg/ml liposomal encapsulated doxorubicin, 0.1 μg/ml free doxorubicin

Table 1. Treatment of dog PBLs with doxorubicin

	IL-1	TNF
Control	0.132 U/ml	0.039 U/ml
Lipopolysaccharide	629.75 U/ml	0.274 U/ml
Free doxorubicin		
0.1 μg/ml	0.098 U/ml	0.042 U/ml
1.0 μg/ml	11.567 U/ml (87.6-fold)[c]	0.041 U/ml
Lipo-doxorubicin[a]		
0.1 μg/ml	0.111 U/ml	0.043 U/ml
1.0 μg/ml	8.415 U/ml (63.75-fold)[c]	0.046 U/ml
Free liposome[b]		
0.1 μg/ml	0.122 U/ml	0.043U/ml
1.0 μg/ml	0.105 U/ml (0.79-fold)[c]	0.038 U/ml
Free liposome[b] + free doxorubicin		
0.1 μg/ml[a]	0.145 U/ml	0.039 U/ml
1.0 μg/ml[a]	6.558 U/ml (49.68-fold)[c]	0.036 U/ml

[a] Doxorubicin concentration.
[b] Concentration of free liposome equivalent to liposome encapsulated doxorubicin.
[c] Stimulation index = treated/control.

plus empty liposomes or empty liposomes did not induce significant measurable IL-1 or TNF. Treatment with 1.0 μg/ml free doxorubicin, 1.0 μg/ml liposomal encapsulated doxorubicin or the mixture of 1.0 μg/ml free doxorubicin plus empty liposomes resulted in a 49- to 88-fold increase in the release of measurable IL-1. No significant change in TNF or IL-2 levels were observed. Empty liposomes did not increase IL-1, IL-2 or TNF from negative control levels.

Titration of dog PBLs with doxorubicin

PBLs from seven dogs were treated with increasing concentrations of doxorubicin and the results presented in Figure 1. PBLs from all animals demonstrated an increase in IL-1 release with increasing doxorubicin concentrations. It is apparent that there are three levels of response to doxorubicin treatment: low responders with maximum IL-1 release of 27–62 U/ml, moderate responders with maximum IL-1 release of 150–170 U/ml and high responders with maximum release of 869–1459 U/ml. Table 2 lists the control IL-1 levels, the doxorubicin concentrations resulting in maximal IL-1 release and the corresponding stimulation index. Maximum IL-1 production was observed to occur with doxorubicin concentrations ranging from 0.3 to 0.8 μg/ml with four of seven dogs demonstrating maximal IL-1 release at 0.6 μg/ml.

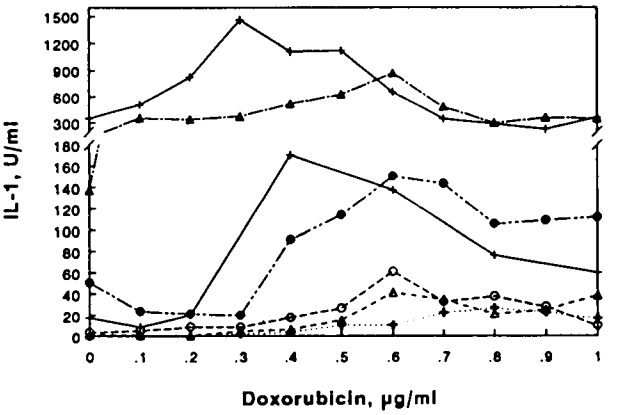

Figure 1. Induction of IL-1 by doxorubicin. PBLs from seven dogs were treated with increasing concentrations of doxorubicin for 48 h. The conditioned medium was quantitated for IL-1 using the EL-4/CTLL-2 bioassay.

Titration of dog PBLs with agarose immobilized doxorubicin

Treatment of dog PBLs with agarose immobilized doxorubicin (Figure 2) demonstrated an increase in IL-1 release of 26.2 U/ml at 0.8 μg/ml immobilized doxorubicin. The medium from the agarose immobilized doxorubicin treated PBLs was evaluated for [^{14}C]doxorubicin activity and is listed in Figure 3. At 0.8 μg/ml immobilized doxorubicin, 0.314 μg/ml soluble doxorubicin was quantitated in the growth medium suggesting that the observed IL-1 activity in the immobilized doxorubicin group is induced by soluble doxorubicin and not due to immobilized doxorubicin. Treatment with free doxorubicin resulted in a peak IL-1 release of 64.7 U/ml at 0.4 μg/ml doxorubicin. Free agarose did not significantly increase IL-1 levels from control values. Treatment with a mixture of free doxorubicin and free agarose resulted in peak IL-1 release of 32.1 U/ml at 0.4 μg doxorubicin.

Endotoxin testing

Endotoxin testing of doxorubicin using the limulus amebocyte lysate (LAL) clot assay was conducted to ensure that endotoxin contamination was not responsible for the observed IL-1 release (Table 3). The level of sensitivity of the E-TOXATE test kit is 0.125 EU/ml when performed according to the manufacturers instruction. Doxorubicin does not demonstrate any endotoxin contamination at the level of sensitivity of 0.125 U/ml of the E-TOXATE

Table 2. Doxorubicin concentrations inducing maximum IL-1 release

Dog	0 μg/ml doxorubicin	Maximum IL-1	[Doxorubicin]	Stimulation index[a]
A	346.20 U/ml	1458.70 U/ml	@ 0.3 μg/ml	4.21
B	17.43 U/ml	170.45 U/ml	@ 0.4 μg/ml	9.78
C	1.38 U/ml	41.65 U/ml	@ 0.6 μg/ml	30.18
D	3.80 U/ml	61.32 U/ml	@ 0.6 μg/ml	16.14
E	137.90 U/ml	869.30 U/ml	@ 0.6 μg/ml	6.30
F	50.90 U/ml	150.45 U/ml	@ 0.6 μg/ml	2.96
G	0.00 U/ml	27.05 U/ml	@ 0.8 μg/ml	—

[a] Stimulation index = doxorubicin treated/control.

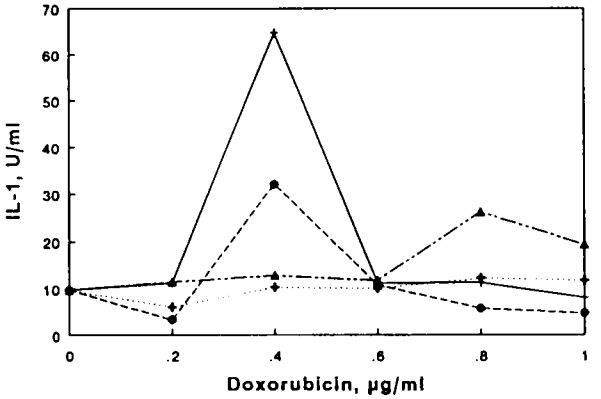

Figure 2. Dog PBLs were treated with agarose immoliblized doxorubicin (▲), free doxorubicin (+), free agarose (■) or a mixture of free doxorubicin and free agarose (●) for 48 h with IL-1 activity being quantitated as described in Materials and methods.

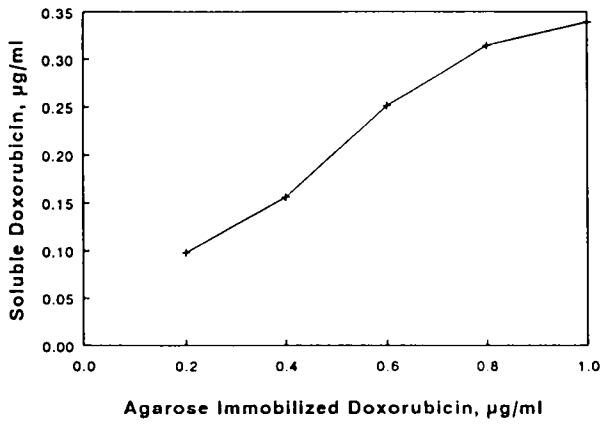

Figure 3. Release of doxorubicin from agarose immobilized doxorubicin. [^{14}C]Doxorubicin was used to synthesize agarose immobilized doxorubicin. Dog PBLs were treated with agarose immobilized doxorubicin for 48 h. Soluble doxorubicin was quantitated by liquid scintillation counting of conditioned medium.

Table 3. Endotoxin testing of doxorubicin

		clot formation
A	doxorubicin	–
B	doxorubicin + endotoxin	+
C	negative control	–
D	0.5 EU/ml	+
E	0.25 EU/ml	+
F	0.125 EU/ml	+
G	0.0625 EU/ml	–
H	0.031 EU/ml	–
I	0.0156 EU/ml	–

test kit. When endotoxin and doxorubicin were mixed, clot formation proceeded normally indicating that inhibitory substances are not present in the doxorubicin sample which would prevent the detection of endotoxin contamination.

Discussion

The purpose of this study was to identify the etiology of a delayed pyrexia phenomenon (12–18 h post-treatment) in dogs observed during the preclinical toxicologic evaluation of intravenously administered liposomal encapsulated doxorubicin.[7] Liposome encapsulation of doxorubicin results in a greater uptake by the phagocytic cells of the reticuloendothelial system[12,13] suggesting that soluble mediators of inflammation, such as IL-1 and TNF, may be responsible for the observed pyrexia.

Doxorubicin (free, liposome encapsulated or mixed with empty liposomes) was observed to induce IL-1 in dog PBL cultures. The EL-4/CTLL-2 bioassay demonstrated that 1.0 μg/ml free doxorubicin, 1.0 μg/ml liposomal encapsulated doxorubicin, and the mixture of 1.0 μg/ml free doxorubicin

and free liposomes were capable of inducing IL-1 in 48 h dog PBL cultures. No IL-1 was induced by free liposomes indicating that the doxorubicin component was responsible for the observed IL-1 release. TNF and IL-2 were not induced by doxorubicin treatment.

Doxorubicin treatment of PBL cultures from seven dogs demonstrated the individual variation of both control and inducible levels of IL-1 requiring the establishment of baseline values for each animal (Figure 1 and Table 2). The 48 h untreated control values for IL-1 ranged from 0 to 346.2 U/ml. All animals responded to doxorubicin treatment with maximum IL-1 release of 27.05–1458.70 U/ml occurring after 0.3–0.8 μg/ml doxorubicin treatment. Four of the dogs demonstrated peak IL-1 release at 0.6 μg/ml doxorubicin. In addition, three categories of IL-1 response to doxorubicin were observed. Low responder dogs demonstrated maximum IL-1 release of 61 U/ml or less, moderate responder dogs demonstrated maximum IL-1 release of 170 U/ml and high responder dogs demonstrated maximum IL-1 release of 1460 U/ml. This may explain why approximately 50% of the liposomal encapsulated doxorubicin treated dogs were observed to develop an increase in body temperature during the preclinical toxicology study.[7] PBLs from four of seven (57%) of the test animals sampled appear to be moderate or high responders and may be the dog population susceptible to IL-1 mediated pyrexia upon liposomal encapsulated doxorubicin treatment.

Tritton[5] had reported that the membrane of mammalian cells may be a likely target for doxorubicin mediated cytotoxicity. In order to determine whether the observed IL-1 release is mediated by doxorubicin–membrane interactions or requires doxorubicin internalization, dog PBLs were treated with agarose-immobilized doxorubicin (Figure 2). Agarose-immobilized doxorubicin treatment resulted in a 2.7-fold increase in IL-1 of 26.2 U/ml at 0.8 μg/ml immobilized doxorubicin as compared with control levels. Evaluation of the conditioned medium for soluble [^{14}C]Doxorubicin (Figure 3) confirmed that 39.2% of the 0.8 μg/ml immobilized doxorubicin had been released to the medium over the 48 h culture period and is likely responsible for the observed IL-1 release. This observed instability of doxorubicin bound to agarose is consistent with other observations in which leakage rates of up to 1% bound drug per hour have been obtained.[14] Treatment with free doxorubicin resulted in a 6.66-fold increase in IL-1 release of 64.7 U/ml at 0.4 μg/ml while the treatment combination of free doxorubicin and free liposomes resulted in the 3.30-fold increase in IL-1 release of 32.1 U/ml at 0.4 μg/ml. These results indicate that doxorubicin must enter the cell in order to elicit the observed IL-1 release. Stimulation of the cell membrane by direct contact with agarose-immobilized doxorubicin or by free agarose beads does not stimulate the release of IL-1.

The doxorubicin component of liposomal encapsulated doxorubicin appears to be the etiologic agent for the induction of IL-1. Since it is derived from the fungus *Streptomyces peucetius* var. *caesius*, the potential exists that pyrogen contamination may be responsible for the observed IL-1 effect. The injection of pyrogen results in a rapid increase in body temperature 1–3 h post-injection which is inconsistent with the observed delayed pyrexia (12–18 h post-injection).[7] However, liposomal encapsulation may delay the release of pyrogen contaminants resulting in the observed increase in body temperature within 24 h. LAL testing indicated the lack of measurable pyrogen contamination of doxorubicin and the inability of doxorubicin to directly inhibit the LAL clot forming assay.

These results indicate that internalization of doxorubicin, either free or liposome encapsulated, results in the release of IL-1 by dog PBLs *in vitro*. Intravenous administration of free doxorubicin results in initial peak plasma concentrations[11] comparable with those doxorubicin concentrations observed to induce IL-1 *in vitro*. Patients treated with 60 mg/m^2 doxorubicin attain 60 min post-infusion doxorubicin plasma levels of 200–600 ng/ml which rapidly decline to 60 ng/ml at 5 h post-infusion.[11] This rapid decline, due to drug metabolism and redistribution of the free drug from plasma to tissue, results in concentrations incapable of inducing IL-1 *in vitro* and explains why dogs treated with the free drug do not develop pyrexia.[7] Liposome encapsulation modifies the tissue distribution of doxorubicin resulting in the accumulation of the drug by phagocytic cells.[12,13] This results in the prolonged exposure of these cells to doxorubicin concentrations capable of inducing IL-1 release, giving rise to the observed delayed pyrexia phenomenon.[7]

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References

1. Adriamycin production information. *The physicians desk reference*. Oradell: Edward R Barnhardt publisher, Medical Economics Inc. 1986: 557.
2. Bachur NR. Mechanisms of action of the anthracycline antibiotics. In: Methe G, Maral M, DeJager R, eds. *Anthracyclines: current status and development*. New York: Masson 1981: 75–8.
3. Schwartz HS. DNA breaks in P288 tumor cells in mice after treatment with daunomycin and adriamycin. *Res Commun Chem Path Pharm* 1975; **10**: 51–64.
4. Tewey KM, Rowe TC, Yang L, *et al*. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 1984; **226**: 466–8.
5. Tritton T, Yee G. The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science* 1982; **217**: 248–50.
6. Saltiel E, McGuire W. Doxorubicin (adriamycin) cardiomyopathy: a critical review. *West J Med* 1983; **139**: 332–41.
7. Kanter PM, Bullard GA, Pilkwewicz FG, *et al*. Preclinical toxicology study of liposome encapsulated doxorubicin (TLC-99): comparison with doxorubicin and empty liposomes in mice and dogs. *In Vivo* 1993; **7**: 85–96.
8. Simon PL, Laydon JT, Lee JC. A modified assay for interleukin-1 (IL-1). *J Immunol Methods* 1985; **84**: 85–94.
9. Scudiero DA, Shoemaker RH, Paull KD, *et al*. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* 1988; **48**: 4827–33.
10. Aggarwal B, Kohr W, Hass P, *et al*. Human tumor necrosis factor: production, purification and characterization. *J Biol Chem* 1985; **260**: 2345–54.
11. Watson E, Chan KK. Rapid analytic method for adriamycin and metabolites in human thin-film fluorescence scanner. *Cancer Treat Rep* 1976; **60**: 1611–18.
12. Hisano G, Fidler I. Systemic activation of macrophages by liposome-entrapped muramyl tripeptide in mice pretreated with chemotherapeutic agent adriamycin. *Cancer Immunol Immunother* 1982; **14**: 61–6.
13. Paeker R, Hartman K, Sieber S. Lymphatic absorption and tissue disposition of liposome-entrapped ^{14}C-adriamycin following intraperitoneal administration to rats. *Cancer Res* 1981; **41**: 1311–7.
14. Vanqulelin G, Lacombe ML, Hanoune J, *et al*. Stability of isoproterenol bound to cyanogen bromide activated agarose. *Biochem Biophy Res Commun* 1975; **64**: 1076–82.

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