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J Clin Invest. 1987;79(2):399-403. https://doi.org/10.1172/JCI112825.

Research Article

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Indomethacin In Vivo Increases the Sensitivity to *Listeria* Infection in Mice

A Possible Role for Macrophage Thromboxane A_2 Synthesis

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Abstract

This paper demonstrates that in the presence of indomethacin, a cyclooxygenase inhibitor, 100% of the mice died when infected with live *Listeria*, whereas none of the animals died in the absence of the drug. The death of the animals correlated with the numbers of bacteria found extraperitoneally in the spleen and not with the Ia expression of the peritoneal macrophages. Increases in the spleen bacterial numbers between mice treated with either indomethacin or a specific thromboxane synthase inhibitor, OKY1581, and those not receiving either drug, were found as early as 2–4 h after infection. The differences in the initial increased bacterial spleen counts in the presence of indomethacin were reversed by administration of a stable thromboxane A_2 analog or another potent vasoconstrictor, phenylephrine. Because thromboxane A_2 does not regulate macrophage or T cell functions directly (Tripp, C. S., A. Wyche, E. R. Unanue, and P. Needleman, 1986, *J. Immunol.*, In press; and Ceuppens, J. S., S. Vertessen, H. Deckmyn, and J. Vermylen, 1985, *Cell Immunol.*, 90:458–463), but is probably generated at the site of an infection (Tripp, C. S., K. M. Leahy, and P. Needleman, 1985, *J. Clin. Invest.*, 76:898–901), these data suggest an important role for the vasoconstrictive properties of thromboxane A_2 in the regulation of immunity to *Listeria* infection.

Introduction

The ability of peritoneal macrophages to synthesize thromboxane (Tx)[1] A_2 remains unaltered during a local *Listeria monocytogenes* (LM) infection (1) whereas, in contrast, their ability to produce prostaglandin (PG) E_2 and PGI_2 as well as leukotriene (LT) C_4 are dramatically decreased (1–3). The mechanism by which macrophage Tx synthesis is selectively conserved during in vivo activation with LM appears to be the result of the migration of blood monocytes to the site of infection, carrying a distinct profile of arachidonate metabolic enzyme activities compared with resident cells (4). Whereas resident murine peritoneal macrophages produce large amounts of PGI_2, PGE_2, and LTC_4 compared with TxA_2 (1–3) when they are stimulated with an agonist, both murine and human blood monocytes produce predominantly TxA_2 compared with PGE_2, PGI_2, and LTC_4 (4, 5). Thus, the rapid migration of blood monocytes to the peritoneal cavity during infection not only increases the number of phagocytic cells capable of taking up the bacteria but also ensures the rapid, decreased synthesis of the potent inhibitory immunomodulators, PGI_2 and PGE_2 (6, 7–9) while maintaining TxA_2 synthesis. However the physiological role for the conservation of macrophage TxA_2 synthesis is not known because this arachidonate metabolite does not appear to regulate any cellular immune function directly (6, 10).

All of these arachidonate products have potent effects on the vasculature during the immune response (reviewed in references 11 and 12). PGE_2 and PGI_2 are primary inducers of increased blood flow (13, 14) and can synergize with C5a to increase vascular permeability (15, 16). LTC_4 can also cause a direct increase in vascular permeability. Furthermore resident peritoneal cells increase the synthesis of these products in response to phagocytic stimuli in vivo (17). An increased blood flow and vascular permeability would be advantageous at the onset of an infection by promoting an influx of immune cells, including lymphocytes, polymorphonuclear leukocytes, and monocytes to the site of an infection.

In contrast, TxA_2, a potent vasoconstrictor, would be expected to decrease blood flow. Because the monocytes migrating to the site of infection can produce large amounts of TxA_2 in response to various immunological stimuli including immune complexes and bacteria (4, 5), a decrease in blood flow to the site of infection may be expected with time after infection. This may be advantageous if a decreased blood flow helped to confine the bacteria locally and thus allow the cellular effector systems of the immune response to eliminate it more effectively.

In this paper we present data that support the hypothesis that a TxA_2-mediated vasoconstriction may decrease the numbers of bacteria leaving the initial site of infection. Furthermore, we propose that this may be an important mechanism by which TxA_2 may regulate the immune response in vivo and hence increase the survival rate of the animal.

Methods

Infection and quantitation of bacterial loads. Adult B10.A/Sg Sn J (Jackson Laboratories, Bar Harbor, ME) were infected with 10^4–10^6 live LM intraperitoneally in 0.25 ml. The numbers of bacteria in the spleen were quantitated, after infection, by mincing the spleens in phosphate-buffered saline (PBS) containing 0.05% Triton X-100. The spleens were then aseptically passed through a sieve in 0.05% Triton X-100/PBS and then homogenized in a dounce homogenizer. The homogenates were serially diluted and plated on brain–heart infusion agar. Homogeneous bacterial colonies could be seen in 24 h at 37°C and were counted.

Drug administration. All drugs were given intraperitoneally in pyrogen-free PBS as follows: indomethacin (Merck, Sharp and Dohme Div.,

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Received for publication 11 July 1986 and in revised form 12 September 1986.

1. *Abbreviations used in this paper:* AA, arachidonic acid; LM, *Listeria monocytogenes;* LT, leukotriene; PEC, peritoneal exudate cells; Tx, thromboxane.

J. Clin. Invest.

0021-9738/87/02/0399/05 $1.00
Volume 79, February 1987, 399–403

West Point, PA) was initially dissolved in 0.1 M Tris buffer at pH 8.1, diluted (50-fold) into PBS, and given at 2 mg/kg in 0.25 ml; OKY1581 (Ono Pharmaceuticals, Osaka, Japan) was given at 20 mg/kg in 0.1 ml of PBS; the stable TxA_2 analog (Ono Industries Inc.) was initially dissolved in ethanol, diluted (10^5-fold) and given at 1 μg/kg in 0.1 ml; phenylephrine was given at 30 μg/kg in 0.1 ml of PBS. PBS was administered to control animals.

Macrophage isolation and Ia expression. Macrophages were obtained from the peritoneal cavity of adult B10.A/SgSnJ mice (Jackson Laboratories) by lavaging with PBS. $1-2 \times 10^5$ peritoneal cells estimated by hemocytometer counts were allowed to adhere to 7-mm-diameter tissue culture dishes for 2 h (37°C, 5% CO_2) in α-minimal essential medium (Gibco, Grand Island, NY), 5% fetal calf serum, and 100 μg penicillin/streptomycin. The nonadherent cells were removed by washing with PBS. Surface Ia expression was determined using a radioimmunoassay. Hybridoma cells (10.2-16) secreting anti-Ia antibody (18) were grown in liter quantities and the antibody was isolated from the media by protein A-Sepharose 4B column chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) (19). The antibody was eluted from the column at pH 3.0 and then dialyzed against PBS at pH 7.2. Antibody isolated by this method was 99% pure as judged by comparing the protein concentration with the antibody concentration determined by a solid-phase enzyme-linked immunosorbent assay. Antibody (400 μg) was iodinated with 10 mCi Na ^{125}I (Amersham Corp., Arlington Heights, IL) and 0.75 mg chloramine-T in 0.75 ml PBS (20). The reaction was stopped with 0.75 mg of sodium metabisulfite after 30 min on ice. A Sephadex G-25 column (Pharmacia Fine Chemicals) was used to separate the antibody from the unreacted ^{125}I. 99% of the radioactivity was associated with the 10% TCA-precipitated protein. The specific activity of the antibody iodinated by this means ranged between 5 and 10 μCi/μg protein.

To determine Ia expression, the cultured macrophages were washed with PBS and allowed to cool to 0°C for 15 min. For maximum binding, 3 μg/ml ^{125}I anti-Ia antibody was added to the macrophages in 50 μl of Hanks' balanced salt solution containing 10% bovine serum albumin and 10% normal mouse serum (pH 7.2). The cells were incubated at 0°C for 2 h (maximum binding occurred by 90 min). At the end of this incubation the unbound antibody was removed and the cells were washed quickly three times with PBS at 0°C. The cells were removed from the plate by solubilization with 0.62 N NaOH. The cell-associated radioactivity was determined using a gamma counter and the cellular protein determined using the fluorescamine assay. The nonspecific binding determined using negative haplotype macrophages, B10.D2, was negligible.

Bioassay. The conversion of arachidonic acid (AA) by peritoneal exudate cells (PEC) into PGE_2 and TxA_2 was determined by the contraction of bioassay tissues, rat stomach, and rabbit thoracic aorta, respectively. Oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution at 37°C was directed over the bioassay tissues. For PGE_2 production, freshly isolated PEC (10^5) were incubated with 10 μM AA in 0.1 ml PBS for 5 min at 37°C and the cell suspension was pipetted directly over the bioassay tissues. For TxA_2 production, freshly isolated PEC (10^6) were centrifuged for 2 min at 150 *g*, resuspended in 0.1 ml PBS, incubated with 10 μM AA for 1 min, and directed over the bioassay tissues. When indomethacin and OKY1581 were used in vitro, the cells were incubated for 5 min at 37°C before the addition of AA.

Results

Our results will show that the administration of the cyclooxygenase inhibitor, indomethacin, profoundly affected the resistence of mice to LM. First, mice infected intraperitoneally with varying doses of live LM showed an increase in the numbers of cells in the peritoneal cavity, increased numbers of activated macrophages (exemplified by Ia expression), and an increase in the numbers of bacteria found in the spleen (Table I) and liver (data not shown). Note, that there was no dose-dependent relationship between the numbers of bacteria injected (between 10^4 and 10^5) and the increased numbers of PEC or the activation of peritoneal macrophages exemplified as increased Ia expression, although increasing the numbers of bacteria injected intraperitoneally resulted in more bacteria in the spleen. Increasing the bacterial dose to 3×10^5 resulted in the death of all of the mice < 1 wk. With the increase in bacterial growth we also found a reduction in macrophage Ia expression, the mechanism of which has not been studied.

Table I. Listeria Monocytogenes LD_{50} in B10.A Mice

Bacteria dose*	PEC mouse	Ia‡	Bacteria No.§	
			Spleen	Death
		ng Ab/μg protein		
0	$1-2 \times 10^6$	0.38±0.02	0	0/6
10^4	5×10^6	1.65±0.06	1.0×10^4	0/6
3×10^4	5×10^6	1.61±0.08	2.0×10^4	0/6
10^5	5×10^6	1.42±0.07	3.2×10^5	0/6
3×10^5	5×10^6	0.19±0.01	9.9×10^5	6/6 (4–5 d)
10^6	N.D.	N.D.	N.D.	6/6 (2–3 d)

N.D., not determined.

* B10.A mice were injected intraperitoneally with varying numbers of LM or PBS. Some mice were sacrificed after 3 d and the PEC and spleens were isolated.

‡ Peritoneal macrophages were purified by adherence in culture and the quantity of Ia was determined by ^{125}I anti-Ia antibody (Ab) binding. The data represent the mean±S.E. of one of two representative experiments.

§ Dilutions of spleen homogenates (three spleen per homogenate) were plated on brain-heart infusion agar and individual bacterial colonies were counted 24 h later. The data is a representative of one of two experiments.

The arachidonate metabolic enzyme inhibitors, indomethacin and OKY1581, can be used to analyze the regulation of the immune response to LM by arachidonate metabolites in vivo. Indomethacin inhibits cyclooxygenase, decreasing the synthesis of PGE_2, PGI_2 and TxA_2, whereas OKY1581 inhibits thromboxane synthase selectively. To verify the duration of inhibition of these drugs given in vivo on PEC, the rat stomach, which contrasts to PGE_2, and rabbit thoracic aorta, which contracts to PGH_2 and TxA_2, were used as bioassay tissues (Fig. 1). Freshly isolated mouse PEC produced PGE_2 (Fig. 1 *A*) and TxA_2 (Fig. 1 *B*) in response to exogenous AA. Note that neither AA nor PEC alone had any effect on the bioassay tissues. Furthermore, the production of PGE_2 was blocked by indomethacin pretreatment in vitro while TxA_2 synthesis was inhibited by both indomethacin and OKY1581 treatment in vitro. When indomethacin was administered to mice intraperitoneally at 2 mg/kg, the drug inhibited the production of PGE_2 from isolated PEC for ~ 24 h. Thus the cyclooxygenase activity of these cells was inhibited for 1 d after indomethacin treatment. The thromboxane synthase inhibitor, OKY1581, only inhibited TxA_2 production from PEC for ~ 30 min after intraperitoneal administration at 20 mg/kg.

Because indomethacin inhibits peritoneal cell cyclooxygenase activity for 24 h, the long term effects of the inhibition of prostaglandin and Tx synthesis on the ability of the animals to control an LM infection intraperitoneally could be examined. LM are phagocytized by macrophages, stimulating the synthesis of PGI_2,

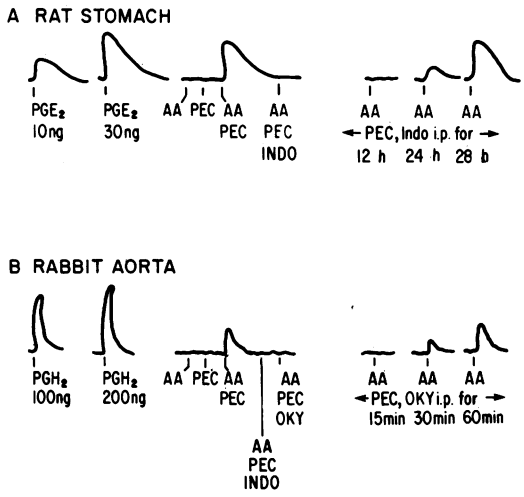

Figure 1. The duration of inhibition of indomethacin and OKY1581 on peritoneal exudate cells' arachidonate metabolic enzymes. Contraction of rat stomach (*A*) and rabbit thoracic aorta (*B*) was calibrated with exogenous PGE_2 and PGH_2, respectively. A PEC suspension incubated with 10 μM AA was allowed to flow over the bioassay tissues. Indomethacin (INDO) and OKY1581 (OKY) were used to inhibit arachidonate metabolite production both in vitro and in vivo.

PGE_2, LTC_4, and TxA_2 in vitro (6). Infection with LM in the presence of indomethacin resulted in a significant increase in both the numbers of bacterial spleen counts and the death rate, which indicated an increased susceptibility to the bacteria (Table II). Infection with LM in the presence of indomethacin neither changed macrophage Ia expression, nor had a significant effect on either PEC numbers (Table II) or on the cellular composition of the exudate (data not shown). Thus the animals were not able to control the infection as well in the presence of indomethacin even though the potent inhibitors of the immune response, PGE_2 and PGI_2, had been removed. The increase in spleen bacterial load indicated that perhaps the LM were not being confined to the peritoneal cavity as effectively in the presence of the drug. Therefore the appearance of bacteria were monitored with time after the initial intraperitoneal infection.

Table II. Indomethacin In Vivo Increases the Sensitivity to LM Infection

Treatment*	PEC/mouse	Ia‡	Bacteria No.§ Spleen	Death
		ng Ab/μg protein		
No treatment	$1-2\times10^6$	0.48±0.04	0	0/6
10^5 LM	$5-10\times10^6$	2.01±0.07	$3.7\pm1.6\times10^5$	0/6
Indomethacin	$3-5\times10^6$	0.61±0.01	0	0/6
10^5 LM + indomethacin	$5-10\times10^6$	2.22±0.11	$4.1\pm1.1\times10^7$	6/6 (3–4 d)

* Indomethacin (2 mg/kg) or PBS was administered intraperitoneally 30 min before and 24 h after intraperitoneal injection of 10^5 live LM or PBS. The animals were sacrificed 3 d after infection.

‡ PEC were obtained by lavage and macrophages were isolated in culture by adherence. The quantity of Ia was determined by ^{125}I anti-Ia antibody (Ab) binding. The data represents the mean±S.E. of four cultures in a representative experiment. The experimental protocol was repeated three times.

§ Spleens from three animals were homogenized and dilutions of the homogenates were plated on brain–heart infusion agar. Individual bacterial colonies were counted 24 h after plating. The data represents the mean±S.E. of three separate experiments.

Mice infected with 10^5 live LM had bacteria in the spleen within 1 h (Table III A) and their levels remained constant up to 8 h. Furthermore, the dose of bacteria in the spleen within 3 h was proportional to the infective dose (Table III B). Intraperitoneal treatment with indomethacin or the Tx synthase inhibitor, OKY1581, resulted in an increase in bacterial spleen counts by 3 h (Tables III and IV). This increase in spleen bacterial numbers in response to indomethacin was not found if either of two drugs were injected simultaneously, a stable TxA_2 analog or another vasoconstrictor, phenylephrine (Table IV). However administering the stable TxA_2 analog alone over a dose range of 10^4 to 10^6 LM only decreased the spleen bacteria counts by 50% at high concentrations of bacteria (data not shown), indicating that vasoconstriction may be an important secondary defense mechanism enabling the primary cellular effector systems to remove the pathogen more effectively.

Discussion

We found that when indomethacin is administered simultaneously with LM intraperitoneally, there is an increased sensi-

Table III. Short-term Effects of Indomethacin on LM Infection

Treatments	Bacteria No. per spleen§ − Indomethacin	+ Indomethacin
Time course*		
Experiment 1:		
1 h	4.4×10^2	4.3×10^3
2 h	2.2×10^2	2.1×10^3
4 h	3.3×10^2	1.4×10^3
8 h	1.6×10^3	1.0×10^4
Experiment 2:		
1 h	3.1×10^2	2.6×10^2
2 h	2.9×10^2	9.3×10^2
4 h	4.2×10^2	6.6×10^3
LM dose‡		
Experiment 1:		
3×10^4	3.8×10^1	
10^5	1.8×10^2	
3×10^5	2.2×10^3	
10^6	4.8×10^4	
Experiment 2:		
3×10^4	5.0×10^1	
10^5	2.1×10^2	
3×10^5	3.1×10^3	
10^6	4.8×10^4	

* LM (10^5) were injected intraperitoneally and the spleens were removed at the indicated times and homogenized.

‡ Varying doses of live LM were injected intraperitoneally and the spleens were removed 3 h later and homogenized.

§ Spleen homogenates (three spleens per homogenate) were plated on brain–heart infusion agar and individual colonies were counted 24 h later.

Table IV. Production of TxA_2 Modulates LM Infection

Treatment*	Bacteria No. per spleen‡ $\times 10^{-2}$	
		n
10^5 LM	1.5±0.2	6
10^5 LM + indomethacin	8.2±1.1	5
10^5 LM + OKY1581	8.8±0.5	3
10^5 LM + indomethacin + TxA_2 analog	2.2±0.4	3
10^5 LM + indomethacin + phenylephrine	2.5±0.6	3

* The various enzyme inhibitors and/or analogs were administered intraperitoneally as follows: indomethacin; 2 mg/kg, one injection 30 min before intraperitoneal LM challenge; OKY1581, 20 mg/kg, injections every half hour starting 30 min before LM challenge; TxA_2 analog, 1 μg/kg and phenylephrine, 30 μg/kg, injections every half-hour starting simultaneously with LM challenge.

‡ 3 h after the LM were injected, spleens from three animals were isolated and homogenized. The homogenates were plated on brain–heart infusion agar and individual colonies were counted 24 h later. The data represent the mean±S.E. of the indicated number of experiments.

tivity to the infection. This was surprising because indomethacin has been shown to potentiate both T cell proliferation and macrophage activation in vitro (7, 8, 21, 22). Furthermore, indomethacin potentiates the protective effects of muramyl dipeptide, causing an increased survival to a subsequent bacterial infection in vivo (23). The published data indicate the inhibitory roles of PGI_2 and PGE_2 on immune functions in vitro and in vivo (8–10) and do not favor the notion that TxA_2 is a direct stimulator of immune cells. Thus, if PGE_2 and PGI_2 are the only cyclooxygenase metabolites regulating the immune response in vivo, an increased resistance to bacterial infections would be expected in the presence of cyclooxygenase inhibitors.

However, arachidonate metabolites also have dramatic effects on the vasculature and these effects may explain our results. These products may contribute to the regulation of the immune response in vivo by modulating blood flow and vascular permeability at the site of infection (11–16). For example, resident macrophages synthesize large amounts of PGI_2, PGE_2, and LTC_4, which increase blood flow via vasodilation and promote edema via increased vascular permeability. These initial effects on the vasculature certainly facilitate the rapid migration of the effector cells of the immune response to the site of infection (reviewed in references 11 and 12). However, within hours after intraperitoneal injection of LM, monocytes increase in numbers compromising 50% of the cells in the peritoneal cavity and their predominant arachidonate product is TxA_2, a potent vasoconstrictor (4). Decreasing the blood flow to the site of infection should isolate the infected tissue to some degree, thus aiding the cellular immune system in its removal of the invading pathogen. If the infection were not confined, it may be more difficult for the immune system to efficiently eliminate the organism.

The possibility that inhibiting macrophage TxA_2 synthesis with indomethacin favors the dissemination of bacteria, resulting in the increased death rate of the infected mice, is supported by several lines of evidence. First, the effects of indomethacin are not due to the inhibition of macrophage activation in vivo because Ia expression was unaffected by the drug treatment. Second, treatment with indomethacin did not affect the increase in cell numbers at the site of infection nor did it change the composition of the PEC occurring during the immune response to LM. Third, the increased numbers of bacteria found in the spleen in the presence of indomethacin was mimicked by a specific Tx synthetase inhibitor, OKY1581, and was counteracted by a stable analog of TxA_2. These data pinpoint these effects of indomethacin to the endogenous synthesis of TxA_2 and not the other cyclooxygenase metabolites. Lastly, because the increased bacterial spleen counts resulting from indomethacin treatment was also counteracted by another vasoconstrictor, phenylephrine, the data support the proposal that TxA_2 aids in the control of the infection in vivo via its vasoconstrictive properties. Of course these data do not eliminate other possible effects of these drugs on the ability to control bacterial infection, namely enhanced multiplication of bacteria and/or reduced bacteria killing. Yet the hypothesis explains two striking observations, that of the increased susceptibility to infection with indomethacin reported here, and that of TxA_2 being synthesized by activated macrophages throughout the immune response (1, 4).

Acknowledgments

We thank Dr. Herbert W. Virgin IV for discussing with us the initial observation that indomethacin increased the sensitivity to *Listeria* infection. Also, thanks go to Dr. John Russell for his invaluable consultations and to Angela Wyche for her technical assistance.

This work was supported by National Institutes of Health grants HL-07275, HL-20787, and A-122609.

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