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Effect of *Lactobacillus plantarum* and *Pseudomonas aeruginosa* culture supernatants on polymorphonuclear damage and inflammatory response

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ABSTRACT

In a previous study we determined that by-products of *Lactobacillus plantarum* inhibited pathogenicity of *Pseudomonas aeruginosa* and is effective in the treatment of infected wounds. This study assesses the cytotoxic activity of acetic acid (AA), supernatants of *L. plantarum* and *P. aeruginosa*, with and without signal acyl-homoserine-lactones (AHL), and mixtures of both bacterial supernatants on human neutrophils. Cytotoxicity was determined through viability using trypan blue, apoptosis by Annexin V, necrosis by propidium iodide and intracellular pH by SNARF-1. We found that supernatants of *L. plantarum* caused less cytotoxicity than AA at the same extracellular pH ($p<0.05$). *P. aeruginosa* induced a remarkable drop in intracellular pH, which was independent of extracellular pH. This intracellular acidity was correlated with a significant decrease in viability and was higher than supernatants of AHL producing *P. aeruginosa* ($p<0.05$). When supernatants were mixed, the quantity of AHL diminished ($p<0.001$) and the cytotoxic effect induced by *P. aeruginosa* was ameliorated by *L. plantarum* supernatant ($p<0.001$ vs $p<0.01$). These results are in agreement with the inflammatory in vivo assays determined by intradermal inoculations in Balb/c mice. Our findings will be useful for the formulation of effective and inexpensive products to resolve infected chronic wounds in our hospitals.

1. Introduction

Multiple factors such as infection, ischemia, advanced age, malnutrition and diabetes have been identified as contributors to impaired wound healing [1]. With regard to infection, pathogenic bacteria delay wound healing through several different mechanisms such as persistent production of inflammatory mediators, metabolic wastes and toxins, or maintenance of necrotic neutrophils, which release cytolytic enzymes and free oxygen radicals. This prolonged inflammatory response contributes to host injury and delays healing. The need for novel antimicrobial agents to combat evolving antibiotic resistance among human bacterial pathogens is clear and imminent [2].

Unlike antibiotics that act selectively on a specific target, antiseptics have multiple targets and a broader activity spectrum. The strongest argument against the application of antiseptics to wounds is that they are cytotoxic to cells such as fibroblasts, keratinocytes and leukocytes that are essential for the wound healing process [3].

One of most frequent pathogens isolated from chronic infections is *Pseudomonas aeruginosa*, a Gram-negative opportunist [4]. Its resistance to antimicrobial agents and numerous virulence factors means that *P. aeruginosa* causes many recalcitrant infections [5]. It has been demonstrated that the expression of certain virulence factors and biofilm is regulated by a cell-density-dependent signaling mechanism known as quorum-sensing signal, chemically recognized as Acyl-homoserine-lactones (AHL) [6]. These AHL produce an enormous inflammatory response: an increase in cellular infiltration and production of COX-2 and several pro-inflammatory cytokines such as IL-6, IL-1 and IL-8 [7]. Chronic infections by *P. aeruginosa* cause alterations in the immune response and inflammation and wound healing processes [8]. Lactobacilli induce a cytokine pattern in peripheral blood cells which is completely different from *P. aeruginosa* [9]. Furthermore, it has been demonstrated *in vitro* that cells and/or the metabolic by-products of lactobacilli have antagonistic effects on pathogens; these results have also been found *in vivo* during trials with urinary and genital infections in humans and mice [10,11] and in wounds infected with *S. aureus* [12]. In previous works we demonstrated that *Lactobacillus plantarum* interferes with the pathogenic capacity of *P. aeruginosa* (quorum sensing, biofilm, virulence factors and growth) [13,14]. Topical treatment with *L. plantarum* cultures is currently being carried out by our medical team with infected burns and chronic venous ulcers in humans [15]

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with encouraging results. The mode of action of *L. plantarum* on infection and wound healing seems to be related to acidity and interference with *P. aeruginosa* quorum-sensing signals.

The aim of this work was to compare *in vitro* polymorphonuclear (PMN) damage caused by *L. plantarum* and *P. aeruginosa* supernatants and mixtures of both as well as acetic acid. Acetic acid is used as an antiseptic agent against *P. aeruginosa* [16] and is frequently used in our hospitals. A second aim was to investigate the *in vivo* inflammatory response induced by these specimens when subcutaneously inoculated in mice. This study enabled us to understand certain aspects of the mechanisms through which *L. plantarum* cultures inhibit a *P. aeruginosa* infection and promote the healing of chronic wounds.

2. Materials and methods

Bacterial strains: Two *P. aeruginosa* strains were used in this study: a standard clinical isolate (PaC) and a qsc mutant (Pa129b). This latter strain, which was generously donated by E. P. Greenberg and K. Lee, University of Iowa, USA, is a non producer of AHL [17]. The *L. plantarum* strain used was ATCC 10241 (Lp).

Supernatants: PaC was grown for 12 h at 37 °C in Luria–Bertani (LB) medium (Gibco, Rockville, MD, USA). Pa129b was grown in LB medium containing 100 μg/ml gentamicin and then for 12 h in LB at 37 °C. *L. plantarum* ATCC 10241 was grown for 12 h in MRS broth (Britania, Buenos Aires, Argentina) at 37 °C. Supernatants of PaC, Pa129b and Lp were recovered after centrifugation and subsequent filtration through 0.22 μm filters and the pH was determined: SPaC (pH 6.33 ± 0.27), S129b (pH 6.99 ± 0.32) and SLp (acid filtrate, pH 5.22 ± 0.43). Aliquots of SLp were neutralized with 8 M NaOH to pH 7 (SLp neutralized: SLpN).

Supernatant mixtures: The following supernatant mixtures (vol:vol) were prepared with corresponding pH: SPaC-SLp (pH 5.54 ± 0.35), SPaC-SLpN (pH 6.71 ± 0.25), S129b-SLp (pH 5.48 ± 0.37) and S129b-SLpN (pH 7.00 ± 0.30).

2.1. In vitro assays

Endotoxins (LPS ELISA for SLpN and MRS medium): The ELISA was performed following standard assay development procedures described elsewhere [18]. LPS from *Escherichia coli* O56:B6 (Sigma, St. Louis Mo, U.S.A.) diluted in PBS pH 7.4 to give a range of concentrations from 1 to 1000 ng ml^{-1}, SLpN and MRS medium undiluted and diluted 1:10 and 1:100 in PBS pH 7.4 were prepared and 100 μl of each of these samples were used to coat 96-well flat-bottomed ELISA plates (NUNC Maxisorp, Denmark) overnight at room temperature. After washing in PBS 0.05% Tween 20, the plates were blocked for 2 h at room temperature in 1% BSA, 0.05% Tween in PBS. After washing as before, 100 μl/well of a 1/100 dilution of rabbit Polyvalent O antisera was added and the plate was incubated for 2 h at room temperature. After washing, 100 μl/well of a 1:1500 dilution of goat anti-rabbit immunoglobulin G specific HRP conjugate (Sigma St. Louis Mo, U.S.A) was added. After washing, 100 μl/well of peroxidase color substrate (OPDA + H_2O_2) was applied to each well and incubated for 5 min and then stopped with a 4 N H_2SO_4 solution. Absorbance values at a wavelength of 490 nm (A_{490}) were obtained for each sample well.

Determination of acyl-homoserine-lactones (AHL): One ml of SPaC was added to 1 ml of SLp, NSLp, MRS broth and LB medium. One part of these mixtures was added to one part of the qsc mutant (Pa129b) strain culture. Following incubation at 37 °C for 1 h, β-galactosidase activity was measured by Miller's reaction [19].

Isolation of PMNs: Heparinized blood samples were collected by venipuncture in healthy individuals of both sexes and 25 to 35 years of age ($n = 10$). Neutrophils were isolated by dextran T-500 (Sigma, St. Louis MO, USA) sedimentation and Ficoll-Hypaque (Sigma, St. Louis MO, USA) gradient centrifugation. Viability of the PMN population measured with Trypan Blue was >96%. Finally, cells were suspended at a concentration of 1×10^6 PMNs/ml in PBS, pH 7.4.

PMN suspensions: Neutrophils (1×10^6 cells) were suspended in each supernatant and mixture. For comparison between acetic acid (AA) and SLp effects, PMNs were suspended in both AA and SLp solutions diluted with PBS with the following pH: 5.22, 6.08 and 6.75. After incubation at 37 °C for 30 min (unless indicated otherwise) viability and apoptosis/necrosis were evaluated.

PMN viability: PMN suspensions were stained with Trypan Blue. The percentage of viable cells was determined by counting 200 cells per sample using a Neubauer's chamber.

Intracellular pH of PMNs (pHi): Determination of pHi was performed using carboxy-SNARF-1-AM as previously described [20]. PMN suspensions were washed twice and suspended in 1 ml of PBS, pH 7.4, loaded with 10 μM carboxy-SNARF-1-AM (Molecular Probes). After 15 min of incubation, cells were washed, re-suspended in PBS and analyzed in a Partec Pas II flow cytometer (Partec, GmbH, Munster, Germany) with excitation at 488 nm and emission at 570 nm (FL2) and 620 nm (FL3). Intracellular pH was estimated from the emission intensity ratio at the two wavelengths and standardized by comparison with the fluorescence intensity ratios of cells whose pHi values were fixed by incubation with 10 μM nigericin in high-potassium buffers. Ten thousand events were collected and data were plotted as forward scatter vs fluorescence ratio (size vs pHi).

Determination of PMN apoptosis/necrosis by annexin V and propidium iodide (PI): PMNs (1×10^6 cells) were suspended in 1 ml of different pH solutions of SLp and acid acetic and then incubated at 37 °C for 15, 30 and 45 min. Doxorrubicin (2 μg/ml) was used as apoptosis control. Cells were washed twice with cold phosphate-buffered saline (PBS) and then with 1× binding buffer (10× binding buffer: 0.1 M HEPES in NaOH, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$). Next, PMNs were suspended in 100 μL of 1× binding buffer supplemented with 5 μL of Annexin V-FITC solution (Pharmingen, San Diego, CA) and 5 μL of PI solution (50 μg PI/ml PBS, pH 7.4) and suspensions were incubated in the dark at room temperature for 15 min. Within 1 h after addition of 400 μL of binding buffer, samples were analyzed in a Partec Pas II flow cytometer with excitation at 488 nm and emission at 540 nm (FL1) for Annexin V-FITC and 570 nm (FL2) for PI. Ten thousand events were collected and data were classified as follows: Annexin V(+)/PI(+): dead cells; Annexin V(−)/PI(−): viable cells and Annexin V(+)/PI(−): early apoptotic cells. Data of each population are given as percentage [21].

2.2. In-vivo assays

Murine inflammatory response: Groups ($n = 5$) of adult Balb/c mice were inoculated subcutaneously with 0.1 ml of each supernatant or mixture mentioned above; a control group was inoculated with a solution of AA (pH 5.22; the same pH as SLp) and PBS. Mice were sacrificed 2 or 24 h after inoculation and blood and skin samples were taken. The skin and connective tissue around the inoculation site were excised, fixed in 10% formalin and embedded in paraffin. Uniformly thin sections (5 μm) were cut and stained with hematoxylin/eosin for light microscopy examination. Inflammation parameters such as vascular congestion, edema, cellular infiltration, necrosis and apoptosis were assessed. Cell infiltration was quantified and classified through microscopy (40×) as follows: Highly Abundant (HA: >100 cells/field); Abundant (A: 50–100 cells/field); Regular (R: 10–50 cells/field) and Weak (W: 1–10 cells/field). Blood collected was used for white blood cell counts using a Neubauer's chamber and Giemsa staining. The protocol used was similar to the one approved by The Animal Care and Use Committee at Texas Tech University Health Sciences Centre.

Statistics: The *t-test* was used for statistical analysis. $P<0.05$ was considered statistically significant.

3. Results

LPS in SLpN and MRS medium: The Polyvalent O antisera reacted strongly with all dilutions of LPS and did not react with SLpN or MRS medium. The mean A_{490} values for LPS ranged from 0.230 ± 0.030 (1 ng) to 0.875 ± 0.042 (100 ng) and for SLpN 0.009 ± 0.003 and MRS medium 0.010 ± 0.004, indicating that SLpN and MRS medium were LPS free. The only source of LPS in SLp and MRS medium might come from the yeast extract, which is produced from baker's yeast by autolysis at 50–55 °C or by plasmolysis in the presence of high concentrations of NaCl. Yeast extract contains amino acids, peptides, water soluble vitamins and carbohydrates without components of cell wall. The LPS is a wall component, hence MRS and SLp should not have LPS as our results indicate.

Determination of AHL: The *L. plantarum* supernatants (SLp and NSLp) inhibited AHL activity in PaC. In this assay, β-galactosidase was significantly less in combinations of SPaC + SLp ($p<0.001$) and SPaC + NSLp ($p<0.01$) than in SPaC alone. MRS (control) also showed less inhibition ($p<0.05$) (Fig. 1).

Effect of extracellular pH (pHo) on pHi and viability of PMN: When PMNs were incubated with SLp and SLpN, their pHi was correlated with the pHo; SLp (acidic) produced a decrease in cell viability when compared to NSLp (neutral) ($p<0.05$). In contrast, incubation of PMNs with *P. aeruginosa* supernatants, SPaC ($pH = 6.33 \pm 0.27$) and S129b ($pH = 6.99 \pm 0.32$), induced a remarkable cytoplasmic acidosis showing a lack of correlation between pHo and pHi with a remarkable decrease in viability ($p<0.001$). The pHi and viability of PMNs incubated with mixture of supernatants (SPaC-SLp, SPaC-SLpN, S129b-SLp and S129b-SLpN) were higher than pHi and viability of PMN incubated with SPaC and SP129b alone ($p<0.01$) (Fig. 2). When comparing the effect produced by SLp and acetic acid on PMN pHi, a correlation between pHi and pHo could be observed. Viability with SLp and AA diminished with decreasing pHo. Incubation with acetic acid produced a higher intracellular acidocis and lesser viability ($p<0.05$) (Fig. 3).

PMN apoptosis/necrosis: As shown in Fig. 4, an increase in cell apoptosis and necrosis was observed, dependent on the incubation time and acidity of both SLp and AA. SLp apoptosis/necrosis values were lower than AA after every incubation time and at each pHo.

Inflammatory response in Balb/c mice (Table 1): Perivascular and interstitial inflammatory infiltrates as well as vasodilatation and edema in epidermis and dermis were observed in all skin samples except for controls. Infiltrated cells were mainly PMNs. In all cases necrosis tissue was evidential. The most pronounced PMN infiltration, edema and vasodilatation were observed 2 h after inoculation with SpaC, S129b and AA, when compared to SLp or SLpN. A lower inflammatory response was observed with a mixture of supernatants (SPaC-SLp, SPaC-SLpN, S129b-SLp and S129b-SLpN) compared to inoculation of pure *P. aeruginosa* supernatants (SPaC and S129). After 24 h of inoculation a decrease in cell infiltration was observed compared to 2 h of inoculation.

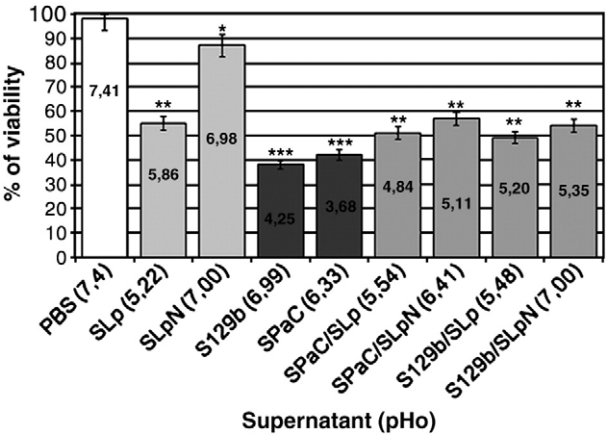

Fig. 2. Effect of supernatants and mixtures of supertnatants on viability and intracellular pH (pHi) of neutrophils. The supernatant pH (outer) is indicated as pHo and the intracellular pH (pHi) is indicated inside each bar. The viability was measured by Trypan Blue and pHi by SNARF-1 stains. Significant difference compared to PBS: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

4. Discussion

In previous works we determined that the topical application of *L. plantarum* cultures on infected wounds promoted debridement, granulation tissue and wound healing [12,15]. We also found that *L. plantarum* culture supernatant *in vitro* inhibited quorum-sensing molecules, biofilm, elastase and growth of *P. aeruginosa*. Acidity was found to be fundamental in this mechanism [13,14].

Acid antiseptics are frequently used in hospitals for the treatment of chronic and infected wounds because they reduce the bacterial load [16]. However, certain authors disapprove the use of antiseptics in open wounds due to their cytotoxic effect on essential cells in the wound healing process, such as fibroblasts, keratinocytes and leukocytes [3]. This cytotoxicity seems to be concentration-dependent. Because of this, several antiseptics are used at low concentrations because they are not cytotoxic and retain their antibacterial activity *in vitro* [16]. Acetic acid, an antiseptic frequently used in our hospital, seems to have this characteristic, but reports about its cytotoxicity are contradictory [3,16]. Ours results showed that cytotoxicity to PMNs caused by SLp and AA depended of the

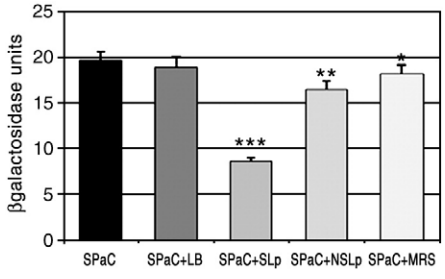

Fig. 1. Effect of Luria–Bertani medium (LB), Supernatants of *L. plantarum* (SLp), Neutralized Supernatants of *L. plantarum* (NSLp) and MRS (De Man, Rogosa and Sharpe medium) on acyl-homoserine-lactones (AHLs) produced by *Pseudomonas aeruginosa* as measured by β-galactosidase activity. Each value is the mean of three samples. AHL production by *P. aeruginosa* grown in Luria–Bertani (LB) medium is indicated in black. Significant difference between SLp, NSLp and MRS compared to LB: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

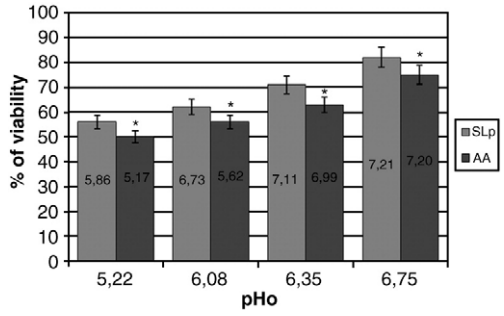

Fig. 3. Effect of SLp and AA solutions with different pH (pHo) on viability (Trypan Blue) and intracellular pH (pHi) of neutrophils. The pHi is indicated inside each bar. Significant difference comparing SLp vs AA: $^{*}p<0.05$.

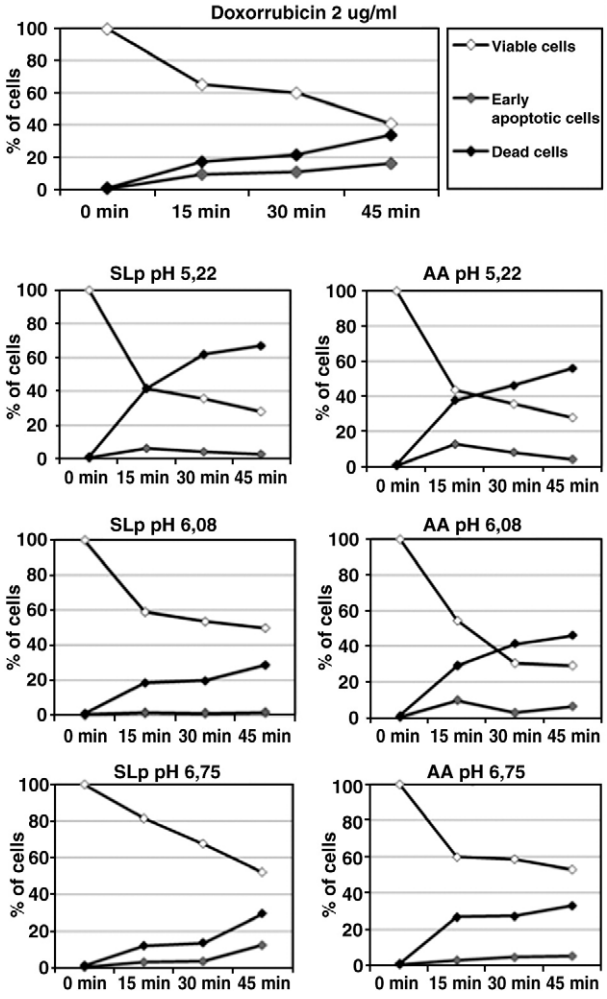

Fig. 4. Comparison of the effect of SLp and AA with different pH (pHo) on apoptosis and necrosis of neutrophils after different incubation times.

incubation time and acidity and that, in all cases, the cytotoxicity of SLp was lower than that of AA.

L. plantarum can produce a mixture of formic, acetic, propionic, butyric and lactic acid. Topical applications to infected wounds of mice and humans lessened the bacterial load and improved the activity of PMNs obtained from lesion tissue by promoting bacterial phagocytosis, modifying production of IL-8 and diminishing their apoptosis/necrosis.

In vitro and *in vivo* apoptosis of PMNs induced by *P. aeruginosa* was inhibited by whole cultures of *L. plantarum* [15]. Our results demonstrate that *P. aeruginosa* supernatants induced PMN cytotoxicity and this induction was higher in the case of a clinically isolated strain when compared to a non-AHL-producer strain. Because of this, AHL could be involved in the pathogenic activity of *P. aeruginosa* [7,22]. This cytotoxicity induced a remarkable drop in pHi, which was independent of the pHo. These results agree with observations by other authors who found that *P. aeruginosa* induced apoptosis and pHi decreased remarkably during the process [22–25]. Our results also show that *L. plantarum* supernatants partially inhibited cytotoxicity produced by *P. aeruginosa*.

The PMN cytotoxic effect induced by bacterial supernatants, mixtures of supernatants and AA was correlated with the inflammatory response in the skin of mice. *P. aeruginosa* supernatants induced a higher inflammatory response, particularly supernatants containing AHL, as reported previously [22]. At the same pH, SLp produced a weaker inflammatory reaction than AA. Neutralization of SLp (SLpN) diminished the inflammation. When *P. aeruginosa* and *L. plantarum* supernatants (acidic and neutralized) were mixed and inoculated, inflammations were less severe than inoculums of pure *P. aeruginosa* supernatants.

In summary, ours studies show that *P. aeruginosa* supernatants promote inflammatory processes partly because they are highly cytotoxic to PMNs and perhaps to other cells involved in tissue repair. Chronic wounds are characterized by a constant influx of PMNs because of the biofilm mode of bacterial infection. PMNs release cytotoxic enzymes, free oxygen radicals and inflammatory cytokines that cause extensive additional damage in the wound tissue. Antibiotic treatment and antimicrobial activity by PMNs, the first defense line of the innate immune system, are inefficient to eliminate biofilm bacterial infection [26]. This is the main reason for using antiseptics in the case of chronic and infected wounds. However, antiseptics are cytotoxic themselves and cause delay in wound healing so they are used with certain reservations [3]. SLp and SLpN were less cytotoxic than AA, and inhibited cytotoxicity and inflammation induced by *P. aeruginosa* supernatants. Previously we reported that *L. plantarum* cultures have a remarkable capacity to inhibit the synthesis of quorum-sensing signals, biofilm and elastase by *P. aeruginosa*. Currently, bacteriotherapy with *L. plantarum* for burns and chronic wounds is being carried out at our hospital [13–15]. Further studies on the mechanisms exerted by *L. plantarum* regarding the tissue repair process are required to improve its performance.

Table 1
Blood cell (leukocytes) counts and cellular skin infiltration in mice after 2 h of inoculations of PBS, MRS medium, LB medium, *L. plantarum* supernatants, *P. aeruginosa* supernatants, mixtures of supernatants, and acetic acid (see text for details).

	Leucocytes/μl	%Neutrophils	Infiltration
PBS	6570–7730	20.6–31.2	W
MRS	6200	35	W
LB	6800	39	R/W
SPaC	11,158	57	HA
SPa129b	7500	48	A
SLp	6516	39.5	R
NSLp	6950	38	R/W
SPaC + SLp	7275	25.5	A
SPa129b + SLp	5125	52.5	R
SPaC + NSLp	5900	44	A/R
S129b + NSLp	6950	41.5	R
AA	7900	39	A

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