EFFECT OF LOW INTRAPHAGOLYSOSOMAL pH ON ANTIMICROBIAL ACTIVITY OF ANTIBIOTICS AGAINST INGESTED STAPHYLOCOCCI

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SUMMARY. The ability of aminoglycoside antibiotics and rifampicin to kill *Staphylococcus aureus* that had been ingested by blood polymorphonuclear leukocytes (PMNs) *in vitro* was investigated. Gentamicin and streptomycin failed to kill intracellular staphylococci, possibly because they could not penetrate PMNs or were inactivated by the low intraphagolysosomal pH. Rifampicin accumulated within the leukocytes in a form that killed staphylococci in a cell-free medium, but the bactericidal activity of intracellular rifampicin against ingested staphylococci was much less than that in a cell-free system. Investigations with granules isolated from PMNs, at various pH-values, revealed that the impairment of rifampicin activity was a result of limitation of the staphylococcal growth rate by a low pH. These observations indicate that the inhibition of intraphagocytic bacterial growth by the low intraphagolysosomal pH and other phagolysosomal bacteristatic factors determines the antimicrobial activity of accumulated antibiotics.

INTRODUCTION

Since the report of Rous and Jones (1916) it has been observed repeatedly that many microbial pathogens are protected from antibiotics to which they are susceptible when inside mammalian cells (Magoffin and Spink, 1951; Shaffer, Kucera and Spink, 1953; Holmes *et al.*, 1966; Solberg and Hellum, 1978; Easmon, 1979; Lam and Mathison, 1982). Little is known about how the intracellular environment protects ingested staphylococci from lethal extracellular antimicrobials. Furthermore, there are conflicting data about the antimicrobial activity of drugs that are concentrated intracellularly. Rifampicin accumulates well in alveolar macrophages (Johnson *et al.*, 1980) and human polymorphonuclear (PMN) leukocytes (Mandell, 1973; Prokesch and Hand, 1982) in a form that kills staphylococci ingested by either normal PMNs (Mandell and Vest, 1972; Solberg and Hellum, 1978; Beam, 1979; Easmon, 1979) or PMNs from patients with chronic granulomatous disease (Ezer and Soothill, 1974)

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and *Escherichia coli* ingested by mouse peritoneal macrophages (Lobo and Mandell, 1973). In contrast, rifampicin does not kill *Mycobacterium tuberculosis* in stimulated rabbit peritoneal macrophages (Clini and Grassi, 1970) or *S. aureus* in mouse resident peritoneal macrophages (Pesanti, 1980).

The low intraphagolysosomal pH after ingestion of a particle by a PMN leukocyte inhibits the growth of staphylococci and the bactericidal activity of penicillin (Lam and Mathison, 1982). The following questions, therefore, arise: (i) are staphylococci in this dormant state more resistant to the action of other antibiotics such as aminoglycosides and rifampicin; and (ii) is there a relationship between the inhibition of staphylococcal growth by the low intracellular pH and sensitivity of the bacteria to intracellular antibiotics? In the present investigation, the activity of gentamicin, streptomycin and rifampicin against intraleukocytic staphylococci was studied. The effect of low pH on the intraleukocytic activity of these agents was studied by simulating the in-vivo micro-environment that is established shortly after ingestion with mixed granule extracts prepared from PMN leukocytes.

MATERIALS AND METHODS

Staphylococcus aureus suspension. *S. aureus* strain C18 from our culture collection was stored in lyophilised form; subcultures were maintained on nutrient-agar slopes. Three colonies were emulsified in 20 ml of Tryptic Soy Broth (TSB, Difco) in a 250-ml conical flask to give c. 1×10^8 cfu/ml; the microbial concentration was determined from a previously constructed graph relating the OD₆₀₀ of bacterial suspensions to viable counts measured as cfu/ml. The cultures were incubated at 37°C on a rotary shaker at 200 rpm until the viable staphylococci increased to c. 1×10^9 cfu/ml. The bacteria were then washed three times in Hanks's balanced salts solution (HBSS). The final suspension was adjusted to 2.5×10^6 cfu/ml in HBSS enriched with pooled rabbit serum 10%. At this bacterial concentration in TSB, the test strain was susceptible to gentamicin 0.24 µg/ml, streptomycin 4.0 µg/ml and rifampicin 0.015 µg/ml.

Preparation of PMN leukocytes. Venous blood from adult New Zealand white rabbits weighing c. 3.5 kg was drawn into siliconized screw-capped bottles containing preservative-free heparin to give a final concentration of 10 units/ml of blood. An almost pure population of PMN leukocytes was isolated from the blood by the method of Böyum (1968); the erythrocytes and polymorphs were passed through Ficoll-hypaque (Pharmacia Ltd, Prince Regent Road, Hounslow, Middlesex TW3 1NE) and the erythrocytes were removed by lysis in Tris-NH₄Cl. The viability of the PMN leukocytes was always >95%, as estimated by the trypan blue dye exclusion test.

Preparation of a mixed granule extract. Granule extracts from 1×10^9 PMN leukocytes isolated from about 160 ml of venous blood were prepared by acetate extraction (0.2 M acetate buffer, pH 4.0) of PMN homogenates prepared in 0.34 M sucrose as described by Rest, Cooney and Spitznagel (1978). The acetate extract was placed in dialysis tubing (mol.-wt cut off 3500) and dialysed against phosphate-buffered saline (pH 7.0; NaCl 7.4 g, KCl 0.285 g, Na₂HPO₄·7H₂O 0.29 mg, and KH₂PO₄ 0.083 g/L of deionised distilled water) and stored in 0.5-ml volumes at -20°C. The total protein content of the extract was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma Ltd, Fancy Road, Poole, Dorset BH17 7NH) as a standard.

Normal rabbit serum was a pool from six healthy adult New Zealand white rabbits weighing c. 3.5 kg; the rabbits were also donors of blood from which PMN leukocytes were isolated (see above). The pooled serum was stored in 1-ml volumes at -20°C and thawed shortly before use.

Preparation of antibiotic solutions. Crystalline gentamicin sulphate (potency approx. 600 µg/mg), streptomycin sulphate and rifampicin (3-[4-methylpiperazinyl-iminomethyl]-rifamycin SV) were obtained from Sigma. Fresh solutions of gentamicin and streptomycin were prepared daily in Eagle's minimum essential medium (E-MEM). The more lipid-soluble rifampicin,

however, was first dissolved in a small amount of dimethyl sulphoxide before E-MEM was added to give the required concentrations.

Assay of the effects of antibiotics on intracellular survival of staphylococci. The phagocytosis mixture had the following composition: 2.5×10^6 PMN leukocytes, 2.5×10^6 cfu staphylococci, rabbit serum 10% in 1.0 ml of HBSS contained in a polypropylene tube. Tubes were incubated at 37°C with end-over-end rotation at *c.* 2 rpm to promote contact between bacteria and phagocytes. After 30 min, lysostaphin was added to give a concentration of 20 µg/ml and the mixtures incubated at 37°C for 20 min to lyse extracellular staphylococci (Easmon, Lanyon and Cole, 1978). The leukocytes containing ingested staphylococci were washed twice in HBSS to remove any bacterial components released during lysostaphin treatment that might influence PMN function. The washed leukocytes were resuspended in 1 ml of E-MEM containing fetal calf serum 10% and lysostaphin 5 µg/ml (to ensure that any bacteria either adhering to the white cells or subsequently released were killed) with or without appropriate concentrations of antibiotics.

Immediately after resuspension and at timed intervals thereafter, duplicate samples were removed and the cells washed three times in HBSS to remove the antibiotic. The leukocytes were lysed in sterile Triton X-100 0.05% to release the surviving staphylococci. Six 20-µl volumes of decimal dilutions of this lysate were plated on to dried nutrient-agar plates and the intraleukocytic killing was estimated from the colony counts after overnight incubation at 37°C. Colony counts of ingested bacteria immediately after washing off the lysostaphin showed consistently that *c.* 80% of the original staphylococci (*c.* 2×10^6 cfu/ml) were intracellular. Therefore, each test was always performed with a control suspension of the staphylococcus containing *c.* 2×10^6 cfu/ml and the antimicrobial agent in E-MEM enriched with fetal calf serum 10%.

Measurement of the influence of antibiotics on the killing of staphylococci by granule extracts. Logarithmic-phase cultures of the staphylococcus containing *c.* 2×10^6 cfu/ml were incubated at 37°C with granule extracts (total protein content 20 µg/ml) in the presence of five times the minimum bactericidal concentration (MBC) of antimicrobial agent in 1.0 ml of assay medium (Tryptone 0.5% plus NaCl 0.5% buffered at either pH 5.0 or pH 7.2). After incubation for 2 h, the surviving bacteria were washed three times in HBSS to remove residual antimicrobials and counted as described above. Appropriate controls contained either antibiotic or granule extract alone.

Measurement of accumulation of antibiotics in PMN leukocytes. Approx. 1×10^9 PMN leukocytes were isolated from about 160 ml of venous blood and allowed to ingest heat-killed staphylococci (*c.* 1×10^9) as described above. The extracellular bacteria were removed by washing the leukocytes three times in HBSS. The cells were resuspended to a concentration of 1×10^9 /ml in E-MEM containing fetal calf serum 10% and the appropriate antimicrobial agent (gentamicin and streptomycin were used at 10 and 100 µg/ml, rifampicin at 5 and 50 µg/ml). After incubation for 2 h, the cells were washed rapidly three times in warm HBSS and the leukocyte pellet was lysed in 0.5 ml of distilled water. Antibiotic activity in the lysate was measured by the agar diffusion method (Garrod, Lambert and O'Grady, 1973). The amount of antibiotic taken up by the leukocytes was expressed as the ratio of antibiotic in the lysate (C) to extracellular antibiotic concentration (E).

RESULTS

Protection of intraleukocytic staphylococci from extra-cellular antibiotics

A large proportion of ingested staphylococci were protected against the bactericidal action of gentamicin, streptomycin and rifampicin (table I). Viable counts of ingested staphylococci during incubation of the leukocytes for 2 h at 37°C in five times the MBC of gentamicin, streptomycin or rifampicin decreased by only 1.14 ± 0.29 , 0.94 ± 0.12 and $1.32 \pm 0.09 \log_{10}$ cfu/ml, but bacterial viable counts in a cell-free system decreased by 2.77 ± 0.47 , 2.32 ± 0.41 and $3.92 \pm 0.08 \log_{10}$ cfu/ml respectively. This

protection was not due to inactivation of antibiotics in the incubation media by PMN leukocytes during the 2 h incubation period; concentrations of antibiotics in the media never fell below the amounts added immediately after removal of lysostaphin. Neither did the protection result from selection of antibiotic-resistant mutants in the ingested bacterial population; organisms recovered at the end of the period of exposure to antibiotics were as sensitive to the three antibiotics as the original strain.

Protection of ingested staphylococci against rifampicin was studied in greater detail. All the bacteria in a cell-free medium were killed by incubation for 24 h with rifampicin 2.5 µg/ml, but only $0.89 \pm 0.21 \log_{10}$ more bacteria (cfu/ml) were killed in PMNs incubated in medium containing the antibiotic than in control PMNs incubated in antibiotic-free medium. The protection afforded ingested staphylococci against extracellular rifampicin during the 24-h incubation period at 37°C was studied in lysates prepared from washed phagocytes containing ingested staphylococci that had been incubated in medium with and without rifampicin. The viable counts of staphylococci in the lysates were measured at zero time and after incubation for 2 h at 37°C. The lysates of control phagocytes failed to inhibit the growth of the staphylococci (the viable count increased by $0.93 \pm 0.23 \log_{10}$ cfu/ml), but the lysates from leukocytes that had been preincubated in medium containing rifampicin killed all the bacteria (the viable count decreased by $> 4 \log_{10}$ cfu/ml).

Accumulation of antibiotics within PMN leukocytes

To determine whether or not the relative inefficiency of gentamicin, streptomycin and rifampicin in killing ingested staphylococci (table I) was because of their failure to penetrate into leukocytes in a microbiologically active form, we studied the uptake of these antibiotics by leukocytes. As shown in table II, only rifampicin accumulated in leukocytes to a concentration greater than that in the surrounding medium; the C:E ratios were 1.04 ± 0.373 and 2.86 ± 0.291 when the extracellular concentrations were 5 and 50 µg/ml. Intracellular concentrations of gentamicin and streptomycin were lower than those in the medium; the C:E ratios were < 1.0 .

TABLE I

Protection of intracellular staphylococci from extracellular antibiotics at $5 \times \text{MBC}$ during a 2-h incubation period

Extracellular medium	Log ₁₀ reduction of viable staphylococci/ml ($\pm$ SD)	
	Intra-leukocytic organisms	Extracellular organisms
E-MEM + FCS 10%	0.82 ± 0.21	$+0.06 \pm 0.01^*$
E-MEM + FCS 10% and gentamicin 2.5 µg/ml	1.14 ± 0.29	2.77 ± 0.47
E-MEM + FCS 10% and streptomycin 20 µg/ml	0.94 ± 0.12	2.32 ± 0.41
E-MEM + FCS 10% and rifampicin 2.5 µg/ml	1.32 ± 0.09	3.92 ± 0.08

* Increase in viable count.
FCS = fetal calf serum.

TABLE II
Penetration of antibiotics into PMN during a 2-h incubation period

Antibiotic	Concentration (µg/ml)	Ratio of intraleukocytic: extracellular antibiotic (C:E)
Gentamicin sulphate	10	<0.36
	100	0.36 ± 0.095
Streptomycin sulphate	10	<0.36
	100	0.36 ± 0.115
Rifampicin	5	1.04 ± 0.373
	50	2.86 ± 0.291

The influence of pH on the interaction between leukocyte granule extracts and antibiotics in killing staphylococci

At the physiological pH of 7.2, all three antibiotics interacted synergistically with the granule extracts in killing staphylococci (table III). At pH 5, however, the activity of gentamicin and streptomycin was markedly reduced (MBCs were 128 µg/ml) and there was no detectable interaction with the leukocyte granule extract. However, rifampicin enhanced significantly the killing of staphylococci by granule extracts at this low pH ($p = < 0.05$), despite a small decrease in its intrinsic activity (MBC = 0.06 µg/ml).

DISCUSSION

Numerous reports indicate that rifampicin enters phagocytes (Mandell, 1973; Johnson *et al.*, 1980; Klempner and Styrt, 1981; Prokesh and Hand, 1982) but there are conflicting data about whether or not intraleukocytic rifampicin can kill ingested staphylococci. The results of the present study showed that rifampicin accumulated in PMN leukocytes (table II) but was much less active against ingested staphylococci than against the same organisms in a cell-free system. Impairment of the antimicrobial activity of rifampicin inside the leukocyte was not due to inactivation of accumulated

TABLE III
Influence of pH on the interaction between leukocyte granule extracts and antibiotics in killing staphylococci during a 2-h incubation period

Antibiotic	Concentration (µg/ml)	Reduction in viable staphylococci (log ₁₀ cfu/ml, ±SD) at			
		pH 5.0		pH 7.2	
		without granules	with granules	without granules	with granules
*Medium (control)		0	0.31 ± 0.09	0.43 ± 0.018	0.18 ± 0.013
Medium + gentamicin	2.5	0	0.36 ± 0.10	0.44 ± 0.023	3.28 ± 0.417
Medium + streptomycin	20.0	0	0.42 ± 0.17	1.87 ± 0.407	3.48 ± 0.315
Medium + rifampicin	2.5	0.92 ± 0.13	2.84 ± 0.48	2.17 ± 0.526	4.72 ± 0.53

* see *Methods*.

antibiotic or the emergence of rifampicin-resistant mutants. These observations suggest that intracellular staphylococci were dormant and, therefore, refractory to the action of rifampicin. Evidence supporting this interpretation comes from the impaired susceptibility of staphylococci to rifampicin in experiments in which the *pH* of the medium was 5.0 (table III). The inhibition of bacterial growth by *pH* and other bacteristatic factors in intact PMNs may influence the enhancement by rifampicin of the natural intraleukocytic killing sequence that occurs *in vivo* within the phagolysosome shortly after ingestion.

There is evidence that aminoglycoside antibiotics either actively taken up by human polymorphs (Easmon, 1979) or passively delivered into the leukocytes in liposomes (Bonventre and Gregoriadis, 1978; Fountain, Dees and Schultz, 1981) kill intraleukocytic staphylococci. The present study failed to confirm these findings. The reason for the discrepancy is not clear. Uptake of aminoglycoside antibiotics by mammalian cells proceeds slowly (Bonventre and Imhoff, 1970; Tulkens and Trouet, 1978). Although we were unable to demonstrate gentamicin and streptomycin in the leukocytes, they could have penetrated into the cells at a concentration less than the minimum detectable by our assay. However, the failure of Vaudaux and Waldvogel (1979 and 1980) to show accumulation of radioactive gentamicin supports the interpretation that the permeability barrier of live leukocytes is one factor that interferes with the bactericidal action of gentamicin in cells.

The results of the present investigation suggest that the low *pH* in phagolysosomes shortly after phagocytosis could neutralise gentamicin and streptomycin and limit their activities, whereas at a physiological *pH*, they interact synergistically with granule enzymes in killing ingested bacteria. This mechanism of impairment of the activity of aminoglycoside antibiotics against intracellular bacteria has been considered repeatedly (Showacre *et al.*, 1961; Vaudaux and Waldvogel, 1980). However, direct experimental evidence for inactivation has been lacking. The present study provides experimental support for this hypothesis.

We have shown here, and elsewhere (Lam and Mathison, 1982) that *pH* has a critical role in the expression of antimicrobial activity in leukocytes; *pH* values inimical to bacterial growth impair the antimicrobial activities of accumulated antibiotics that kill bacteria within leukocytes at *pH* values permitting growth. We strongly advocate that bacteria that do not multiply intracellularly should not be used to assess the intracellular activity of antibiotics.

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REFERENCES

- BEAM, T. R. 1979. Sequestration of staphylococci at an inaccessible focus. *Lancet* **2**, 227-228.
- BONVENTRE, P. F. AND GREGORIADIS, G. 1978. Killing of intraphagocytic *Staphylococcus aureus* by dihydrostreptomycin entrapped within liposomes. *Antimicrobial Agents and Chemotherapy*, **13**, 1049-1051.
- BONVENTRE, P. F. AND IMHOFF, J. G. 1970. Uptake of ³H-Di-hydrostreptomycin by macrophages in culture. *Infection and Immunity*, **2**, 89-95.
- BÖYUM, A. 1968. Separation of leucocytes from blood and bone marrow. *Scandinavian Journal of Clinical and Laboratory Investigation*, **21**, Suppl., 97.

- CLINI, V. AND GRASSI, C. 1970. The action of new antituberculous drugs on intracellular tubercle bacilli. *Antibiotics and Chemotherapy*, **16**, 20–26.
- EASMON, C. S. F. 1979. The effect of antibiotics on the intracellular survival of *Staphylococcus aureus* in vitro. *British Journal of Experimental Pathology*, **60**, 24–28.
- EASMON, C. S. F., LANYON, H. AND COLE, P. J. 1978. Use of lysostaphin to remove cell-adherent staphylococci during in vitro assays of phagocyte function. *British Journal of Experimental Pathology*, **59**, 381–385.
- EZER, G. AND SOOTHILL, J. F. 1974. Intracellular bactericidal effects of rifampicin in both normal and chronic granulomatous disease polymorphs. *Archives of Diseases in Childhood*, **49**, 463–466.
- FOUNTAIN, M. W., DEES, C. AND SCHULTZ, R. D. 1981. Enhanced intracellular killing of *Staphylococcus aureus* by canine monocytes treated with liposomes containing amikacin, gentamicin, kanamycin and tobramycin. *Current Microbiology*, **6**, 373–376.
- GARROD, L. P., LAMBERT, H. P. AND O'GRADY, F. 1973. *Antibiotic and Chemotherapy*, 4th ed. Churchill Livingstone, Edinburgh. pp. 520–522.
- HOLMES, B., QUIE, P. G., WINDHORST, D. B., POLLARA, B. AND GOOD, R. A. 1966. Protection of phagocytized bacteria from the killing action of antibiotics. *Nature*, **210**, 1131–1132.
- JOHNSON, J. D., HAND, W. L., FRANCIS, J. B., KING-THOMPSON, N. AND CORWIN, R. W. 1980. Antibiotic uptake by alveolar macrophages. *Journal of Laboratory and Clinical Medicine*, **95**, 429–439.
- KLEMPNER, M. S. AND STYRT, B. 1981. Clindamycin uptake by human neutrophils. *Journal of Infectious Diseases*, **144**, 472–479.
- LAM, C. AND MATHISON, G. E. 1982. Intraphagocytic protection of staphylococci from extracellular penicillin. *Journal of Medical Microbiology*, **15**, 373–385.
- LOBO, M. C. AND MANDELL, G. L. 1973. The effect of antibiotics on *Escherichia coli* ingested by macrophages. *Proceedings of the Society for Experimental Biology and Medicine*, **142**, 1048–1050.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. AND RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
- MAGOFFIN, R. L. AND SPINK, W. W. 1951. The protection of intracellular brucella against streptomycin alone and in combination with other antibiotics. *Journal of Laboratory and Clinical Medicine*, **37**, 924–930.
- MANDELL, G. L. 1973. Interaction of intraleukocytic bacteria and antibiotics. *The Journal of Clinical Investigation*, **52**, 1673–1679.
- MANDELL, G. L. AND VEST, T. K. 1972. Killing of intra-leukocytic *Staphylococcus aureus* by rifampin: In vitro and in vivo studies. *Journal of Infectious Diseases*, **125**, 486–490.
- PESANTI, E. L. 1980. Protection of staphylococci ingested by macrophages from the bactericidal action of rifampin. *Antimicrobial Agents and Chemotherapy*, **18**, 208–209.
- PROKESCH, R. C. AND HAND, W. L. 1982. Antibiotic entry into human polymorphonuclear leukocytes. *Antimicrobial Agents and Chemotherapy*, **21**, 373–380.
- REST, R. F., COONEY, M. H. AND SPITZNAGEL, J. K. 1978. Bactericidal activity of specific and azurophil granules from human neutrophils: Studies with outer membrane mutants of *Salmonella typhimurium* LT-2. *Infection and Immunity*, **19**, 131–137.
- ROUS, P. AND JONES, F. S. 1916. The protection of pathogenic microorganisms by living tissue cells. *Journal of Experimental Medicine*, **23**, 601–612.
- SHAFFER, J. M., KUCERA, C. J. AND SPINK, W. W. 1953. The protection of intracellular brucella against therapeutic agents and the bactericidal action of serum. *Journal of Experimental Medicine*, **97**, 77–90.
- SHOWACRE, J. L., HOPPS, H. E., DUBUY, H. G. AND SMADEL, J. E. 1961. Effect of antibiotics on intracellular *Salmonella typhosa*. 1. Demonstration by phase microscopy of prompt inhibition of intracellular multiplication. *Journal of Immunology*, **87**, 153–161.
- SOLBERG, C. O. AND HELLMUM, K. B. 1978. Protection of phagocytosed bacteria against antimicrobial agents. *Scandinavia Journal of Infectious Diseases*, Supplement, **14**, 246–250.
- TULKENS, P. AND TROUET, A. 1978. The uptake and intra-cellular accumulation of aminoglyco-

- side antibiotics in lysosomes of cultured rat fibroblasts. *Biochemical Pharmacology*, **27**, 415-424.
- VAUDAUX, P. AND WALDVOGEL, F. A. 1979. Gentamicin antibacterial activity in the presence of human polymorphonuclear leukocytes. *Antimicrobial Agents and Chemotherapy*, **16**, 743-749.
- VAUDAUX, P. AND WALDVOGEL, F. A. 1980. Identification of leukocytic factors interfering with gentamicin bactericidal action in purulent exudates. *Experientia*, **36**, 495.