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Estratto da III° Symposium Internazionale sul Lisozima di Fleming
MILANO, 3-5 APRILE 1964

COMBINED ACTION OF LYSOZYME WITH ANTIBIOTICS AND CHEMOTHERAPEUTIC AGENTS

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Lysozyme is an enzyme of low molecular weight which is probably responsible, at least in part, of some phenomena of natural immunity (1).

It produces lysis of some bacteria being *Micrococcus lysodeikticus* one of the most susceptible microorganism. According to the investigations of BRUMFITT (2), CALLERIO (3) and RUNTI (4) the lytic effect of lysozyme should be due to the fact that this substance produces the rupture of the glycosidic linkages between muramic acid and N-acetyl glucosamine which are part of the bacteria cellular wall. *M. lysodeikticus* is disrupted quickly in the presence of lysozyme, whereas other bacteria apparently are not affected; however investigations with the electron microscope reveal that in some of them a partial desintegration of the cell membrane takes place.

Investigations both *in-vitro* and with laboratory animals and human patients carried out by MAGRASSI, et al. (5), ROCCHI (6) and many other workers (7, 8) showed that lysozyme has some antibiotic properties and it confers protection against various types of bacterial and viral infections even when the results *in-vitro* are negative.

The present study was carried out with the purpose of establishing whether lysozyme can increase *in-vitro* the antibacterial potency of various antibiotic and chemotherapeutic agents.

MATERIAL AND METHODS

Two experiments were carried out. In the first, the disk method was used and in the second liquid cultures were employed in which the minimum inhibitory concentration (MIC) of each antibiotic and chemotherapeutic agent was determined.

a) *The disk method.* - A solid medium containing Nutrient Agar (2,3 %) and beef extract (1 %) « Difco » was used. The pH was adjusted to 7,4 and the sterilization was made in an autoclave at 118°C for 30 minutes. Then, the medium was cooled and when the temperature was 46°C, there was added 1 ml of the bacterial suspension which corresponded to a 24-hour culture in a liquid medium. This medium was then agitated and 20 ml aliquots were distributed between the sterilized PETRI dishes. Then, it was allowed to solidify. Following this, disks of paper containing the substance under investigation were placed on.

The paper disks used were of 6 mm of diameter, from the Difco (Bactosensitivity disks), the Baltimore Biological Laboratory (sensi-disks-BBL) and others which were made in our laboratory from paper ≠ 740-E from Carl Schleider e Schnell.

For each antibiotic and chemotherapeutic agent and for each dose, to find the appropiate concentration to produce inihibition, 3 disks were used, two containing the agent under study. To one of them, there was added 0.1 ml of saline solution and to the other 0.1 ml of the solutions of lysozyme, which for the *M. lysodeikticus* contained 0.01 mcg and for the other bacteria 10 mcg. The third disk contained only lysozyme in the same amount. Each test was carried out with 6 to 10 replications.

The cultures were incubated at 37°C for 20 hours. At this time, the diameter of the areas of inhibitions were measured and the corresponding averages were established.

b) *Method of serial dilution.* - The liquid medium used had the following composition: peptone 3 %; glucose 0.3 % and red-

fenol 6.2 % from a solution at 0.04 %. The pH was adjusted at 7.4 and the liquid medium was then sterilized at 118°C for 30 minutes. A bacterial suspension in the proportion of 0.1 ml of a 24-hours liquid culture was added to each 100 ml. This preparation was called « inoculum ».

Series of 10 sterile tubes were prepared. There was added 0.5 ml of saline solution to those tubes marked 2 to 10. To each of the tubes 1 and 2, 0.5 ml of solution of the antibacterial agent were added. Then, from tube 2 there was withdrawn 0,5 ml which was transfer to the tube 3 and so forth. In this manner, in each tube there was a concentration which was half of the previous one. To a series, there was added an equal amount of lysozyme always of 0.5 ml per tube. In that tube in which we tested only the antibiotic agent there was added 0.5 ml per tube of saline solution and finally in all of them 1 ml of the « inoculum » per tube was added. The contents of the tubes were shaken and incubated at 38° C for 20 hours. Then, the results were observed. In those tubes in which a bacterial growth developed there was a change in color from red to yellow. As the minimum inhibitory-concentration (MIC) it was considered that which was immediately before to the tube in which a change of color was observed.

After the preliminary tests made to determine the appropiate concentrations, we proceeded to make the definitive tests which were repeated with 5 to 10 replications. These values were then used to calculate the corresponding averages and its standard errors.

The bacteria used were *M. lysodeikticus*, *M. pyogenes var. aureus* and *var. albus* and *Escherichia coli* 0119 B14. The last three were obtained from patients.

The list of drugs used in our work are given in the tables of results. Whenever it was needed, we used the respective soluble salts.

Results

a) *The disk method.* - Lysozyme byself produced the caracteristic halo of inhibition only when it was tested against to *M. lysodeikticus.*

The diameter of the halo of inhibition was of 8.9 mm with a standard deviation of ± 0.4 mm. This was found using a very low quantity of lysozyme, 0.01 mcg per disk. With the other two bacteria no inhibition was found. The results obtained with the antibiotics are given in table I. The addition of lysozyme to the disks containing chloramphenicol, penicillin and rovamycin produced no increase in the halos of inhibition. On the other hand the addition of lysozyme to the disk containing colistin resulted in an increase of 19.8 % in the diameter of the halo of inhibition of *E. coli.* No increase was detected with respect to the gram-positive bacteria. Kanamycin, oxytetracycline and tetracycline in the presence of the lysozyme gave a halo of inhibition larger than that produced for the antibiotic alone. This effect was observed with the gram-positive germs and varied batween 14.5 % and 20.3 %, depending upon the antibiotic and the bacteria. The same antibiotics did not modify the halo of inhibition by the addition of lysozyme when it was tested with *E. coli*

The results obtained with the chemotherapeutic agents are shown in the table II. The addition of lysozyme to cetrimide, furazolidone und thimerosal produced no increase in the halos of inhibition. The sulfonamides by themselves produced no inhibition in 2 species of *Micrococcus* but the addition of sulphonamides to lysozyme, in the case of *M. lysodeikticus* produced an increase of 20 % to 23 % in the halos of inhibition. The 2 drugs neither by themselves nor associated caused no inhibition of *M. pyogenes.* On the other hand, the sulfas produced inhibition of *E. coli.* The effect increased with the presence of lysozyme.

In all cases, when there was an increase in the diameter of the halos of inhibition by the addition of lysozyme, the standard deviation did not reach 10 % and the differences were significant at $P < 0.01$.

b) *Method of serial dilution.* - The results are given in table III. With the exception of kanamycin, all of the results obtained by the disk method were confirmed. Lysozyme did not increase the antibacterial activity of chloramphenicol, penicillin and rovamycin. The MIC of each of these antibiotic agents was the same in the presence or absence of lysozyme.

The potentiation of the antibiotic effect of colistin with respect to *E. coli* and the 2 tetracyclines tested against to the species of Micrococcus, were confirmed.

The MIC of the nitrofuran derivatives were not modified by the addition of lysozyme.

Table I. — Diameter of the area of bacterial inhibition produced by some antibiotics alone and associated with lysozyme.

Antibiotic	Dose/ disk	M. lysodeikticus	M. pyogenes	E. coli
Chloramphenicol	5 mcg	20.5 mm	8.1 mm	23.1 mm
» + lysozyme*		20.6 »	8.3 »	23.3 »
Penicillin	2 U	16.2 »	20.2 »	0 »
» + lysozyme		16.3 »	20.0 »	0 »
Rovamycin	5 mcg	18.4 »	7.1 »	0 »
» + lysozyme		18.3 »	7.2 »	0 »
Colistin	1 mg	23.2 mm	6.0 mm	16.1 mm
» + lysozyme		23.1 »	6.1 »	19.3 »
Kanamycin	5 mcg	17.3 »	13.2 »	14.1 »
» + lysozyme		19.8 »	15.4 »	14.6 »
Oxytetracycline	5 mcg	20.2 »	16.3 »	20.1 »
» + lysozyme		24.1 »	19.4 »	20.0 »
Tetracycline	5 mcg	19.8 »	15.9 »	19.1 »
» + lysozyme		23.2 »	18.1 »	19.2 »

* Lysozyme by itself produced inhibition (8.9 ± 0.4 mm) of the growth only of *M. lysodeikticus*. The standard dose was 0.01 mcg/disk. For *M. pyogenes* and *E. coli* a dose of lysozyme of 10 mcg/disk was used.

Table II. — Diameter of the area of bacterial inhibition produced by some chemotherapeutic agents alone and associated with lysozyme.

Chemotherapic	Dose/ disk	M. lysodeikticus	M. pyogenes var. albus	E. coli
Cetrimide	1 mcg	20.8 mm	11.3 mm	9.4 mm
» + lysozyme*		20.6 »	11.5 »	9.5 »
Furazolidone	100 mcg	21.2 »	9.1 »	15.1 »
» + lysozyme		21.6 »	10.1 »	15.2 »
Thimerosal	1 mcg	22. »	9.0 »	10.2 »
» + lysozyme		23.8 »	10.2 »	10.2 »
Sulfadiazine	250 mcg	0 mm	0 mm	19.1 mm
» + lysozyme		10.6 »	0 »	22.2 »
Sulfamethazine	250 mcg	0 »	0 »	19.2 »
» + lysozyme		11.1 »	0 »	22.6 »
Sulfathiazole	250 mcg	0 »	0 »	18.3 »
» + lysozyme		10.8 »	0 »	21.6 »

* Lysozyme by itself produced inhibition (8.9 ± 0.4 mm) of the growth only of *M. lysodeikticus*. The standard dose was 0.01 mcg/disk. For *M. pyogenes* and *E. coli* a dose of lysozyme of 10 mcg/disk was used.

Table III. — Minimal effective concentration of some antibiotics and chemotherapeutic agents to inhibit completely the bacterial growth.

Antibiotic	M. pyogenes var. albus	M. pyogenes var. aureus	E. coli
Chloramphenicol	6.2 mcg	8.3 U	1.9 mcg
» + lysozyme*	6.1 »	8.1 »	1.8 »
Kanamycin	5.0 »	5.0 »	5.1 »
» + lysozyme	5.0 »	5.0 »	5.0 »
Penicillin	12.5 U	21.6 »	20.0 U
» + lysozyme	12.5 »	20.8 »	20.0 »
Rovamycin	15.2 mcg	10.2 »	15.1 mcg
» + lysozyme	15.0 »	10.6 »	15.1 »
Colistin	410.0 U	400.0 U	106.0 U
» + lysozyme	408.9 »	400.0 »	49.5 »
Streptomycin	122.0 mcg	152.0 mcg	7.5 mcg
» + lysozyme	61.0 »	150.0 »	7.4 »
Oxytetracycline	15.5 »	30.0 »	6.4 »
» + lysozyme	7.2 »	14.8 »	6.2 »
Tetracycline	31.3 »	33.6 »	15.3 »
» + lysozyme	15.4 »	15.2 »	15.2 »
Furazolidone	10.6 mcg	15.8 mcg	10.3 mcg
» + lysozyme	10.4 »	15.6 »	10.0 »
Nitrofurazone	50.0 »	100.0 »	51.2 »
» + lysozyme	50.5 »	108.0 »	50.8 »

* Lysozyme was used in 50 mcg/ml concentration. By itself did not inhibit the growth of these bacteria.

Discussion

The results of this investigation reveal that lysozyme is capable of potentiating the antimicrobial effect of only certain antibiotic agents and only upon certain bacteria.

The studies made by Runti (4) and others show that the gram-positive bacteria have a much greater proportion of linkages of the N-acetyl muramic acid-1-4-N-acetyl-glucosamine than the gram-negative bacteria which might be the selective sites of activity of lysozyme. Therefore, the gram-positive bacteria are more susceptible to the lytic action of this enzyme. In accordance with the results of Hartsell and Caldwell (9), the lytic effect of lysozyme permits in addition to differenciate various species of Streptococcus. It is possible that this lytic effect of the cellular wall might permit selectively to certain antibiotic or chemotherapeutic agents to diffuse more easily into the bacterial cytoplasm with an increase of the antibacterial power. Moreover, the potentiation of the antibiotic effect by lysozyme must also depend on the specific mechanism of action of the antibiotic or chemotherapeutic agents.

Brumfitt and Glynn (10) have found that the destruction of *M. lysodeikticus* intracellulary in the macrophagous should be attributed to a lysis by the lysozyme contained in such leucocytes so that the mutants lysozyme-resistant survive the action of the macrophage.

The administration of lysozyme to animals or human patients could, by various mechanisms, potentiate the effect of certain antibiotic and chemotherapeutic agents. This is in agreement with the results reported by Imbriano and Mazzucco. On the other hand, Losito and Rottini by means

of the disk method, but adding lysozyme to the culture medium but not te the disk with the antibiotic agent, hawe not detected potentiation of the activity neither in the tetracyclines nor in the streptomycin or in the sulfamides.

ERMOLIEVA and coworkers (11) have shown that lysozyme turns more susceptible to strains of *M. pyogenes var. aureus* which are resistant to penicillin and oxytetracyclines to doses which are only 5 times larger than in those of susceptible strains. Furthermore, these authors found with the electron microscope that such bacteria swell, become deformed and change their electronic density by the action of lysozyme.

The disagreement observed in our experiments of the effect associated between kanamycin and lysozyme in the two test procedures can not, at this time, be explained satisfactorily.

RIASSUNTO

Sono state effettuate esperienze « in vitro » per determinare se il lisozima è in grado di aumentare l'attività antibatterica di vari chemioterapici ed antibiotici. Le prove furono effettuate sia in terreno solido, secondo il « metodo su piastra » sia in terreni di coltura liquidi, dove i batteri potevano crescere in presenza di differenti concentrazioni di un agente chemioterapico o di un antibiotico. Nel primo caso l'effetto antibatterico veniva valutato misurando il diametro dell'alone di inibizione e nel secondo caso veniva determinata la concentrazione effettiva minima dell'agente attivo, necessaria per inibire completamente la crescita del germe.

E' stato messo in evidenza che il lisozima potenzia l'azione dei sulfamidici sia sui germi gram-positivi che su quelli gram-negativi. Esso aumenta inoltre l'azione della colistina sui germi gram-negativi, nonchè quella delle tetracicline e della streptomicina sui germi gram-positivi. Il cloroamfenicolo, la penicillina e altri agenti antibatterici non sono stati potenziati in vitro dal lisozima.

SUMMARY

In vitro investigations were made to determine whether lysozyme increases the antibacterial activity of several chemotherapeutic agents and antibiotics. Experiments were done both in solid medium according to the « disk method » and in liquid culture media where the bacteria were allowed to grow in the presence of different concentrations of a chemotherapeutic agent or an antibiotic. In the first case, the antibacterial effect as evaluated by measuring the diameter of the halo of inhibition and in the second case the minimal effective concentration of the active agent necessary to inhibit completely the growth was established.

It was found that lysozyme enhanced the effect of sulphonamides both in the gram-positive and gram-negative bacteria. It also enhanced the effect of colistin on the gram-negative germs as well as that of the group of tetracyclines and streptomycin in the gram-positive germs. Chloramphenicol, penicillin and other antibacterial agents were not potentiated in vitro by lysozyme.

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