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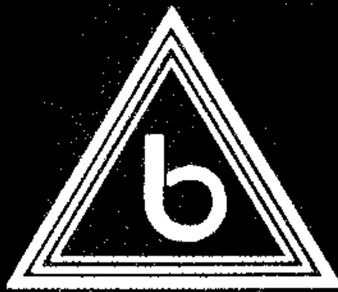

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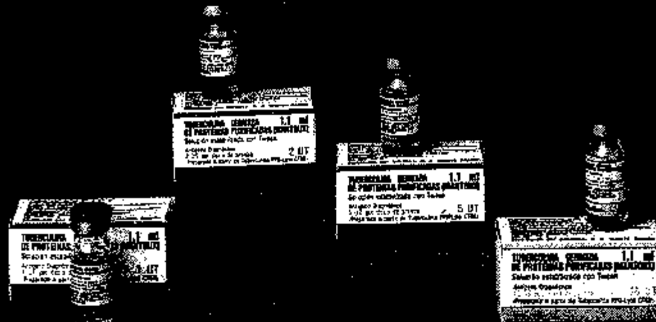

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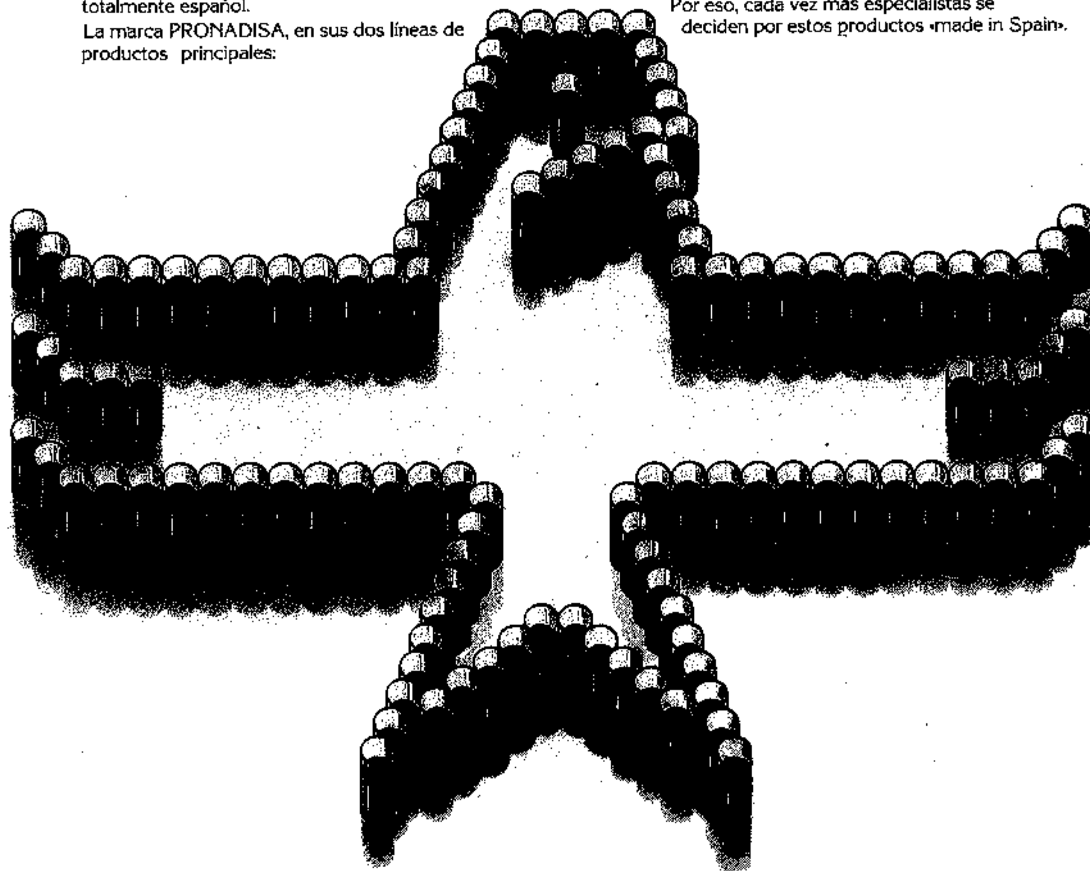

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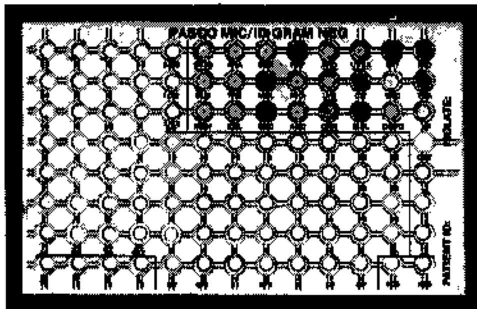

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(*) Corresponding author.

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MINIREVIEW

Proteins covalently linked to nucleic acids

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Summary

The existence of specific proteins covalently linked at the 5' ends of nucleic acids, either DNA or RNA, has been reported recently. In several of these cases it has been shown that the terminal proteins (TP) become associated with the nucleic acid in the process of the initiation of replication by a protein-priming mechanism. In the presence of the terminal protein-DNA template, a free molecule of the terminal protein forms a covalent complex with the dNMP at the 5' end in a reaction catalyzed by a specific DNA polymerase that further elongates the TP-dNMP initiation complex formed. The protein-priming mechanism is a new way to initiate replication in linear nucleic acids which contain a terminal protein at their 5' ends.

Key words: Bacteriophager, replication, protein-priming; DNA pylomerase.

Resumen

La existencia de proteínas específicas unidas covalentemente a los extremos 5' de ácidos nucleicos, ya sean DNA o RNA, ha sido descrita recientemente. En varios de estos casos se ha demostrado que las proteínas terminales (TP) llegan a estar asociadas al ácido nucléico en el proceso de iniciación de la replicación a través de un mecanismo de proteína-cebadora («protein-priming»). En presencia del molde proteína terminal-DNA, una molécula libre de la TP forma un complejo covalente con el dNMP en el extremo 5' en una reacción catalizada por una DNA polimerasa específica que elonga el complejo de iniciación TP-dNMP ya formado. El mecanismo de «protein-priming» es una nueva vía de iniciar la replicación en ácidos nucléicos lineales que contienen proteína terminal en sus extremos 5'.

During the last eight years, the existence of a new class of proteins covalently linked to nucleic acids, either DNA or RNA, has been reported. However, the first indication on the existence uf such proteins was shown in 1971 With the finding that the DNA of the Bacillus *subtilis* phage ∅29 could be obtained from the phage particles in a circular form that was converted into linear DNA of the same length upon treatment with proteolytic enzymes (31). Two years later, the same finding was reported for adenovirus DNA (36). These

results suggested that a protein was bound to ∅29 and adenovirus DNAs and was involved in the formation of circular DNA molecules.

Later on, the protein linked to adenovirus DNA was characterized as a 55 kilodaltons (kd) protein (35) and the one linked to ∅29 DNA was shown to be the product of the viral gene 3, p3, with a molecular weight of 27 kd 938). In both cases, it was shown that the protein was covalently linked to the two 5' ends of the DNA. In addition, a model was proposed in which the terminal protein could act as a primer for the initiation of replication by reaction with the nucleotide at the 5' ends of the DNA and formation of a covalent complex that would provide the 3'0H group needed form elongation by the DNA polymerase (35). Indeed, the replication of both adenovirus (24) and ∅29 (12,19) was shown to start at either end of the DNA and the replicating molecules obtained *in vivo* were consistent with the model proposed (21, 40).

In vitro formation of a terminal protein-dNMP covalent complex

The confirmation of the protein-priming model was obtained by developing *in vitro* systems for ∅29 and adenovirus DNA replication (37, 41). By using extracts from adenovirus-infected cells a covalent complex between a precursor of the adenovirus terminal protein (pTP) and dCMP was found (4). Similarly, by using extracts from ∅29-infected *B. subtilis,* a covalent complex between the ∅29 TP and dAMP was obtained (31).

More recently, both the adenovirus and ∅29 replication proteins have been highly purified. In the case of the adenovirus system, three proteins are needed for the formation of the pTP-dCMP covalent complex, the pTP, the adenovirus-coded DNA polymerase and nuclear factor I, purified from uninfected cells (29). For the elongation of the pTP-dCMP complex, the adenovirus DNA-binding protein (DBP) is required (19). In addition, to obtain full-length adenovirus DNA, the addition of the nuclear factor II, shown to be a type I topoisomerase, is needed (30). In the case of the ∅29 system, two proteins are required for the formation of the TP-dAMP covalent complex, the terminal protein p3 and the ∅29-encoded DNA polymerase p2 (3). In the presence of these two highly purified proteins, the p3-dAMP initiation complex formed can be elongated to produce full-length ∅29 DNA (3). The results obtained with both systems indicate that the viral coded DNA polymerases are the only ones involved in ∅29 and adenovirus DNA replication. It is interesting to note the fact that both DNA polymerases are able to catalyze, not only the covalent linkage of a dNMP to the 3'OH group of a dNMP as any other DNA polymerase, but also they are able to catalyze the covalent linkage of a dNMP to the OH group of a specific serine group in the terminal protein (14, 39). Both, the ∅29 (2) and the adenovirus (9) DNA polymerases have 3'→5' exonuclease activity on single-stranded DNA that could act as a proofreading mechanism.

In the ∅29 system, in addition to the TP and the DNA polymerase, another viral coded protein, p6, has been highly purified and shown to stimulate the initiation reaction (33) as well as the elongation of the DNA chain (Blanco, L., Gutiérrez, J., Lázaro, J.M., Bernad, A. and Salas, M., submitted for publication).

Although, both in the adenovirus and the ∅29 systems, the natural template is the TP-DNA complex, it is possible to obtain activity with protein-free DNA. Indeed, the replication origins of adenovirus and ∅29 DNA have been cloned and the recombinant

plasmids, when cut to release the replication origins at the DNA ends, were active as templates for replication (41; Gutiérrez, J., García, J.A., Blanco, L. and Salas, M., submitted for publication). With the use of the origin-containing recombinant plasmids it has been shown that nuclear factor I binds specifically to an adenovirus DNA sequence from nucleotide 25 to nucleotide 40 from the DNA ends (25). Deletion mutants of the ∅29 replication origins are being prepared to define the minimal origin sequence needed for ∅29 DNA replication.

Other linear nucleic acids with a terminal protein

In addition to phage ∅29 and adenovirus, two other phages contain a TP covalently linked at the two 5' ends. They are phage PRD1 that infects gram-negative hosts as *Escherichia coli* and *Salmonella typhimurinum* (1) and phage Cp-1 from *Streptococcus pneumoniae* (10). The linkage between the TP and the DNA is a phosphodiester bond between tyrosine and dGMP for PRD1 (1) and between threonine and dAMP for Cp-1 (11). In the case of phage ∅29 and adenovirus, the linkage is between serine and dAMP (15) and dCMP (6), respectively.

Other linear DNAs with TP are plasmid pSLA2 from *Streptomyces* (17), the killer plasmids pGKL1 and pGKL2 from yeast (23) and S1 and S2 mitochondrial DNA from maize (22).

It is interesting to note that, in all cases studied in which a linear DNA contains a TP, there is an inverted terminal repetition (ITR). The ITRs are 6 bp in phage ∅29 (8, 47), 103-162 bp in adenovirus (45), 236 bp in phage Cp-1 (7), 614 bp in plasmid pSLA2 (16), 202 bp in plasmid pGKL1 and 184 bp in pGKL2 (18) and 208 bp in the S1 and S2 mitochondrial DNA from maize (26, 32). Although the role of the ITR is not yet clear, it has been implicated in the mechanism of replication of the displaced strand in the case of adenovirus (24, 42).

A different case in which a TP exists linked at the 5' ends of one of the two DNA strands of a circular DNA molecule is hepatitis B virus (12). The TP has been also implicated in replication since protein-containing replicative intermediates have been found (27). However, the replication of hepatitis B virus seems to be quite different to that of the linear DNAs since it proceeds through a reverse transcription step (43).

In addition, TPs are also present at the 5' ends of the RNA of animal viruses such as polio, foot and mouth, encephalomyocarditis, vesicular exanthema and infectious pancreatic necrosis, or plant viruses such as cowpea mosaic, tobacco ringspot, southern bean mosaic, tobacco etch, tobacco leafroll and pea enation (5).

Evidence for the formation of a covalent complex *in vitro* between the TP and the 5' terminal nucleotide has been obtained, in addition to ∅29 and adenovirus, in phages PRD1 (1) and Cp-1 (11) as well as in the RNA-containing viruses polio (28) and encephalomyocarditis (46). Therefore, the protein-priming mechanism seems to be a new way to initiate replication in linear nucleic acids which contain a TP at their 5' ends.

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In vitro sensitivity of herpes simplex virus types 1 and 2 isolates against 9-β-D-arabinofuranosyladenine, 9-((2-hydroxy-1-(hydroxymethyl) ethoxy) methyl) guanine and 9-(2-hydroxyethoxymethyl) guanine

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Summary

The sensitivity of 62 clinical isolates of herpes simplex virus, types 1 and 2, against Ara A, DHPG and acyclovir, was evaluated in an «in vitro» assay. Virus were obtained from patients not treated with antivirals. In most cases type 1 isolates showed higher sensitivity to the three antivirals, althoug a clear cut difference between types 1 and 2 could not be found.

Variation in the sensitivity to the three different drugs was observed among isolates of the same type. However in no case the ID_{50} of a given isolate was 4 times higher than the value corresponding to the average of its type.

The in vitro efficacy of ACV and DHPG was of the same order of magnitude, both being far superior to Ara A.

Key words: Ara A, acyclovir, DHPG, herpes simplex virus.

Resumen

Se ha valorado mediante un ensayo in vitro, la sensibilidad de 62 aislados clínicos de virus herpes simplex de los tipos 1 y 2, frente a Ara A, DHPG y acyclovir. Los virus se habían aislado de pacientes no tratados con antivirales. Los virus del tipo 1, en la mayor parte de los casos, tenían una mayor sensibilidad a los tres antivirales, sin embargo, no se encontró una clara separación de los tipos 1 y 2.

Se observaron variaciones en la sensibilidad de los aislados a los tres antivirales. Sin embargo, en ningún caso, la DI_{50} de uno de ellos, llegó a ser cuatro veces mayor al valor medio encontrado para los aislados del mismo tipo.

La eficacia in vitro de acyclovir y DHPG fue similar, y en ambos casos, claramente superior a la encontrada para Ara A.

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Introduction

In the last years several chemiotherapeutical agents against herpes simplex virus types 1 and 2, have been developed. These antivirals include the classical ones, as 9-β-D-arabinofuranosyladenine (Ara A) (15) and other more specific of the «new generation», as 9-(2 hydroxyethoxymethyl) guanine (acyclovir, ACV or ACG) (10), (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) (8), 1-(2'-fluoro-β-D-arabinofuranosyl)-5-iodo-cytosine (FIAC) (13), 9-((2-hydroxy-1-hydroxymethyl) ethoxy) menthyl) guanine (DHPG, 2'NDG or BIOLF-62) (1) and 1-(2'deoxy-2'fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) (18).

Ara A is phosphorylated by cellular enzymes and then incorporated into DNA. Consequently it inhibits both cellular and viral DNA synthesis, the latter being more sensitive.

Antiviral agents of the «new generation» are more potent and selective inhibitors of herpes simplex virus replication. The selectivity of DHPG and acyclovir is primarily based upon specific phosphorylation of these compounds by the virus specified timidine kinase. Following this initial phosphorylation, the monophosphate forms are then further phosphorylated to ACG-5'-triphosphate and DHPG-5'-triphosphate by cellular kinases.

Numerous studies have been carried out by different groups to measure the sensitivity of herpes simplex virus, types 1 and 2, against several of these antiviral agents (9, 11, 16).

Although titrations have been done with a small number of isolates (1, 6, 17), an extensive study of the relative potencies of the DHPG and acyclovir, in the same in vitro system, has not been reported.

Materials and Methods

Virus

Virus isolates were kindly given to us by Dr. Pilar Pérez Breña (Servicio de Virologia. Centro Nacional de Microbiología, Virología e Inmunología Sanitarias). They had been typed by immunofluorescence using specific antisera. In our laboratory they were further classified by their sensitivity to BVDU. All the isolates were obtained from patients with different clinical syndromes who had not been treated with antivirals.

Virus stocks were prepared in Vero cells. After 3-4 days of infection at 37 °C, when the cells were coming off the plates, culture supernatans were centrifuged at 2000 rpm. Cell pellets were sonicated and cellular debris eliminated by centrifugation at low speed. Finally, virus was resuspended in culture medium and kept at −70 °C until used.

Virus was titrated by inoculation of serial 10 fold dilutions into Vero cells grown in microtiter plates (96 well, flat-bottom culture plates). The titer was determinated by the Reed-Muench method (14) and expressed as tissue culture infectious dose ($TCID_{50}$).

Antiviral drugs

Ara A was purchased from Sigma Chemical Co. and the other drugs were supplied as gils: ACV from Dr. Sandra Nusinoff lehrman, Burroughs Wellcome Co., Research Triangle Park and DHPG from Dr. Arthur K. Field, Merk Sharp & Dohme, Research

Laboratories, West Point. One mg/ml solutions of acyclovir and DHPG, in distiled water, were prepared and stored at −70 °C.

Virus inhibition test

The sensitivity of herpes simplex virus to the inhibition by DHPG, Ara A and acyclovir was analyzed in a cytopathic reduction test done in microtiter plates (2). The same amount of virus was mixed with eight 2 fold dilutions of the drugs. Each dilution was inoculated into 4 wells. After 3-4 days in culture, supernatanst were discarded. Monolayers were fixed with 10 % formaldehyde and, after washing twice with saline, stained with 10 % Giemsa. A positive cytopathic effect was considered when more than 50 % of the monolayer had been destroyed. The ID_{50} for each drug was calculated by the Kärber method (12). Due to variations from assay to assay, virus was titrated in each cytopathic reduction test in the absence of drugs; only those assays in which the titre was between 31.6 and 316 $TCID_{50}$/0.1 ml of the dilution used were considered significative. One of the isolates was always included in the reduction test as an internal standard.

Results

We wanted to make an extensive evaluation of the sensitivity of herpes simplex virus isolates to antiviral agents. We selected two of the most commonly used drugs. (Ara A and acyclovir) to compared with the newly developed DHPG. All these drugs were tested in the cytopathic reduction assay described under Materials and Methods. Figure 1 shows the ID_{50} values obtained for each of the isolates against acyclovir. A wide range of values can be observed but, in general, type 1 isolates showed higher sensitivity to the drug than isolates from type 2; nevertheless a clear cut difference between type 1 and type 2

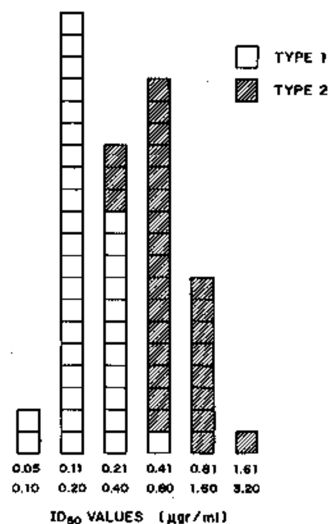

Fig. 1. Sensitivities of herpes simplex virus types 1 and 2 isolates to acyclovir.

virus could not be assigned. Similarly, figure 2 shows the results obtained when assaying the viral isolates against Ara A. As in the first case, type 1 virus were more sensitive than type 2 whith some overlaping between the two types. When the virus were tested against DHPG, type 1 isolates showed again higher sensitivity to the drug than type 2 virus (Fig. 3).

All these are gathered in Table 1 where the mean ID_{50} against the three antivirals and the minimun and maximun values are shown.

ACV inhibited type 1 virus at ID_{50} of 0.05-0.74 μg/ml (mean, 0.19). DHPG inhibited the same isolates at ID_{50} values of 0.05-0.44 μg/ml (mean, 0.16) which are very similar to those of acyclovir. On the contrary, Ara A was less active than the other antivirals, having an ID_{50} of 2.2-14.9 μg/ml (mean, 7.36).

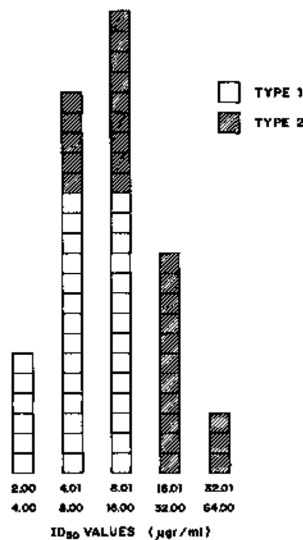

Fig. 2. Sensitivities of herpes simplex virus types 1 and 2 isolates to ara A.

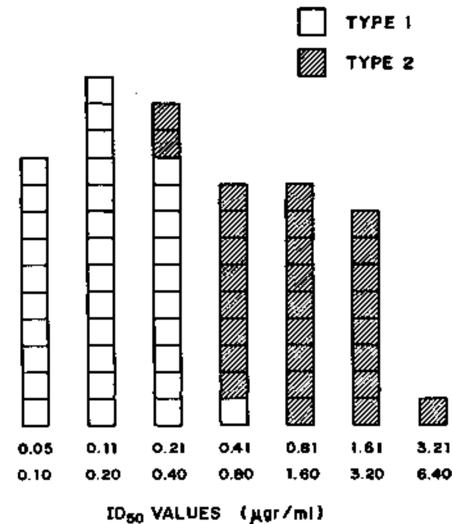

Fig. 3. Sensitivities of herpes simplex virus types 1 and 2 isolates to DHPG.

As with type 1 isolates, only slight differences were observed between the antiviral activities of DHPG (ID_{50} 0.26-3.55 μg/ml, mean 1.29) and acyclovir (ID_{50} 0.26-2.51 μg/ml, mean 0.80) in the *in vitro* test, against the 28 herpes simplex virys tope 2. Ara A was again less active than, the other two drugs (ID_{50} 4.5-35.7 μg/ml, mean 17.88). The mean ID_{50} whith acyclovir for the 34 type 1 isolates was 4 fold higher than the mean ID_{50} for the 28 type 2 isolates. A higher difference was observed between type 1 and type 2 isolates wit DHPG. However only slight differences were observed with Ara A.

Although the mean sensitivity values of types 1 and 2 isolates, for the three drugs, were clearly different, the variation among isolates of the same type was so high as to preclude a total differentiation between them.

TABLE 1

IN VITRO SENSITIVITY OF 62 HERPES SIMPLEX VIRUS ISOLATES AGAINST ACYCLOVIR, DHPG AND ARA A

Isolates	Number	Acyclovir	DHPG	Ara A
Type 1	34	0.19 (0.05-0.74)	0.16 (0.05-0.44)	7.36 (2.2-14.9)
Type 2	28	0.80 (0.26-2.51)	1.29 (0.26-3.55)	17.88 (4.5-35.7)

The mean sensitivity value of type 1 and 2 isolates to the three antivirals is given as ID_{50} in μg/ml. The minimun and maximun values are given in brakes.

Discussion

Numerous factors affect the antiviral sensitivity in the «in vitro» test, specially the cells where the test is done and the actual method to carry out the assay. Preliminary results (4) indicated a similar sensitivity of herpes simplex virus, types 1 and 2, to acyclovir. However, later studies (7) showed that the sensitivity of herpes simplex type 1 isolates was 10 times higher than those of type 2. The different sensitivity that we find (the isolates of herpes simplex virus type 1 being approximately 4 times more sensitive than the isolates of type 2) is similar to what it has been described previously by others (5).

The sensitivity of herpes simplex virus, types 1 and 2, to DHPG, has not been investigated in detail. Although initial studies suggested that both types were equally sensitive (1), later results showed that the strains of herpes simplex virus type 1 have higher sensitivity than type 2 (6, 17). Our results show an even higher difference in the mean sensitivity to DHPG between type 1 and type 2 isolates than against acyclovir.

Slight differences in the sensitivity between type 1 and type 2 isolates against Ara A have been described (3), although other reports showed no significative differences (19). Our results show a two times higher sensitivity of type 1 isolates to Ara A than type 2.

The comparative results between ACV and DHPG show similar sensitivities of the isolates against both drugs, DHPG being slightly more potent with type 1 isolates and acyclovir with those of type 2.

Resistance to antiviral drugs is not a clinical problem at the moment. However in view of the rapidly increasing clinical use of antiherpes compounds in our country, and the possibility of developing resistance to the drugs, the in vitro cytopathic reduction assay that we have developed will be of interest to test clinical isolates from patients treated with those antiviral agents.

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Cell wall polysaccharides of *Penicillium chrysogenum*

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Summary

The amount of cell wall produced by *Penicillium chrysogenum* increased during the growth phase up to a maximum and then remained constant. The mycelial dry weight increased and then decreased as a consequence of cytoplasm autolysis. Minor changes were observed in the composition of the cell walls obtained from mycelium of different ages. The protein content decreased from 12 % (3 days) to 5 % (30 days). The content of chitin increased slightly with the age of the cell wall preparation. Galactose and mannose increased slightly with age, while glucose increased from 27 % (3 days) to 42 % (30 days).

The following fractions were obtained from the cell walls: F1, an α-glucan soluble in 1 M NaOH at 20° amounting to 20 % F2, a β glucan-chitin complex solubilized with 1 M NaOH at 20° C, from the previous residue left overnight at −25° C, amounting from 3 to 5 %; F3, a β-glucan rich in xylose soluble in 1 M NaOH at 70° C, amounting from 3 to 5 %; and F4 the insoluble residue, a β-glucan-chitin complex similar to F2, amounting to 50 % of the cell wall.

Key words: Penicillium chrysogenum, cell wall, polysaccharides.

Resumen

La cantidad de pared celular producida por *Penicillium chrysogenum* aumentó durante la fase de crecimiento hasta un máximo y después permaneció constante. El peso seco de micelio aumentó y a continuación decreció como consecuencia de la autólisis del citoplasma. En la composición de las paredes celulares obtenidas de micelio de diferentes edades se observaron pequeños cambios. El contenido de proteína disminuyó del 12 % (3 días) al 5 % (30 días), mientras que la cantidad de quitina aumentó ligeramente con la edad de la pared celular. La galactosa y la manosa incrementaron ligeramente con la edad de la preparación mientras que la glucosa lo hizo del 27 % (3 días) al 42 % (30 días).

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De la pared celular se obtuvieron las siguientes fracciones: F1 (20 %), un α-glucano soluble en NaOH 1M a 20° C; F2 (3-5 %), un complejo β-glucano-quinina solubilizado con NaOH 1M a 20° C del residuo de la extracción anterior congelado durante la noche a −*25° C; F3 (3-5 %), un* β-glucano rico en xilosa, soluble en NaOH 1M a 70° C; y F-4 (50 %), residuo insoluble formado por un complejo β-glucano-quinina similar a F2.

Introduction

Variations in overall composition of fungal cell walls tend to follow phylogenetic lines and the corresponding data have been of help in taxonomy (6). The considerable information on fungal cell walls and cell polysaccharides has been reviewed (3, 6, 11, 16, 30). Work on the composition of the cell walls of species of *Penicillium* has been mainly concerned with the analysis of their hydrolysates (1, 2, 12, 13, 17, 18, 26, 29, 31, 32). Chemical changes in the cell wall of conidia of *Penicillium chrysogenum* (25) during germination and in the composition of wall fractions of *P. allahabadense* (15) during aging have been reported. Because the composition of cell walls and the kinds of polymers present could vary in species from different taxa, cell wall analysis is increasingly used in fungal classification (33, 34, 35, 36, 37). Since changes in cell wall composition may occur at different stages of the life cycle of a single organism or as a consequence of the culture conditions (6, 7) information is required on the effect of these factors in cell wall composition.

The purpose of this work was the study of the changes in composition of *P. chrysogenum* cell wall during aging and the characterization of wall polysaccharide fractions that could be relevant for chemotaxonomic studies of the genus.

Materials and methods

Organism and growth conditions. P. chrysogenum Thom. strain 1398 was obtained from Dr. C. Ramírez Collection (Instituto Jaime Ferrán, CSIC Madrid, Spain). It was maintained on slants of Bacto potato dextrose agar (Difco). Suspensions of conidia (1 ml) from 10 day-old agar slants in 10 ml of sterile water were used as inocula. The basal medium for mycelial production contained: KH_2PO_4, 0.5 g; $MgSO_4$ $7H_2O$, 0.5 g; $FeSO_4$ $7H_2O$, 0.5 mg; $ZnSO_4$ $7H_2O$, 0.5 mg; $CuSO_4$ $5H_2O$, 0.02 mg; $MnCl_2$, $4H_2O$, 0.02 mg; Na_2MoO_4 $2H_2O$, 0.02 mg; thiamine hydrochloride, 1 mg; D-glucose, 15 g; casamino acids (Difco), 3 g; yeast extract (Difco), 1 g; and water up to one litre. The media were distributed in 1 l portions into 2 l Erlenmeyer flasks. The media were adjusted to pH 6.5 before autoclaving at 120 °C for 15 min. The cultures were incubated during 5 days at 25 °C ± 1 and 150 rpm in a Gallenkamp IH-465 orbital incubator.

Cell wall preparation. At different incubation periods, two flasks were harvested. The mycelium was collected by filtration, washed with distilled water and freeze-dried. The dried mycelium was triturated in a Sorvall omnimixer at full speed, suspended in cold distilled water and sonicated (MSE model MK2, 150W) for periods of 2 min until the cells were broken. The cell walls were washed with distilled water by centrifugation

until they appeared empty when observed through a phase-contrast-microscope and did not stain with Coomasie Blue. The cell wall preparation was then washed with a series of alcohol 50, 75 and 100 % and acetone, and dried at 60 °C.

Cell wall extraction procedures. For the isolation of cell wall monomers and polymers, a modification of the method of Mahadevan and Tatum (23) was used. This procedure is detailed in (Fig. 1).

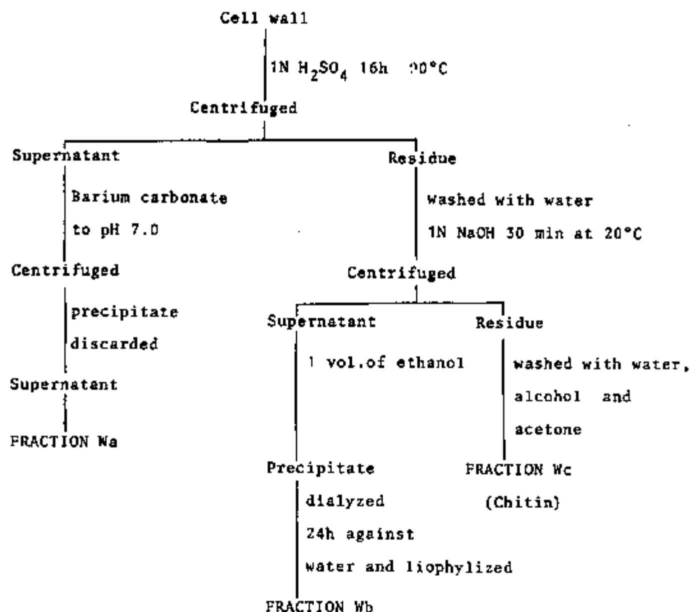

Fig. 1. Fractionation procedure used to obtain three major components from the cell wall of *P. chrysogenum.*

In other procedure a stepwise extraction with 1M-NaOH at different temperatures was used. Dry cell wall material (2 g) was treated with 1M-NaOH (100 ml) at 20 °C (Fraction 1). The residue from the previous treatment was left overnight at −25 °C and extracted with 1M-NaOH (100 ml) at 20 °C (Fraction 2). The residue from Fraction 2 was extracted with 100 ml of 1M-NaOH at 70 °C (Fraction 3). The cell wall residue, insoluble in alkali, constituted Fraction 4.

Fraction 4 (300 mg) was subjected to the Mahadevan and Tatum modified method, obtaining Fraction 4a (acid-soluble), Fraction 4b (alkali-soluble) and Fraction 4c (acid and alkali-insoluble).

All the extractions described above were continued until no precipitate was formed when ethanol was added to the supernatant. The precipitates were pooled together, washed with 50 % ethanol until the supernatant was free from alkali, and then with acetone. Fraction 4b was dialyzed against water, and freeze-dried. Fraction 4 and 4c were washed with distilled water until the supernatant was free from alkali and with ethanol and acetone, and stored desiccated.

Chemical analysis. Neutral hexosans were determined by the anthrone procedure (10) with glucose as standard. The cell wall and its polysaccharides were hydrolyzed with H_2SO_4 of different concentrations at 100 °C and for different periods of time, and neutralized with barium carbonate. The neutral sugars, converted into the corresponding alditol acetates (21) were identified and quantified by gas-liquid chromatography (GLC) on 3 % SP-2340 on 100-120 Supelcoport. A 2 m x 2 mm glass column was used at 200 to 230° with a temperature rise of 10°/min, a 3 min initial hold, and a final temperature time of 10 min. The N_2 flow rate was 30 ml/min. A flame ionization detector, sensitivity 10^{-10} sample size 3 μl, was used in a Perkin-Elmer 10 and Sigma 3 chromatograph. Peaks were identified on the basis of sample coincidence with the relative retention times of standard. Inositol was used as internal standard. Amino sugars were identified and quantified in 6N HC1 hydrolysates by a Biotronik amino acid analyzer LC 7000 and by the method of Chen and Johnson (8). Total protein was measured by the method of Lowry et al., (22) with bovine serum albumin as standard. Volatile matter was determined by heating 100 mg of cell wall at 100° C until constant weight. For ash determination 100 mg of the wall material was ashed to constant weight.

Infrared (I.R.) spectra were obtained by the KBr technique on a Perkin-Elmer 457 infrared spectrophotometer. An X-ray diagram was obtained with a PW 1065 Philips recording diffractometer. Nickel-filtered Cuk X radiation was employed. The X-ray tube was operated at 36 KV and 20 mA. The periodate oxidation was performed according to Aspinall and Ferrier (4). The formic acid formed was determined by the method of Kabat and Mayer (20). The oxidized polysaccharide fractions were subjected to a Smith degradation (14). The procedure was somewhat modified: the polyalcohol obtained after periodate oxidation-borohydrid reduction was hydrolyzed with 1N H_2SO_4 for 16h. The hydrolysates were reduced, acetylated and analyzed by GLC chromatography as described above.

Elecron microscopy. Cell walls were successively treated with 1N NaOH at 20° C for 30 minutes (five times), 1N N_2SO_4 at 100° C for 16h and 1N NaOH at 20° C for 30 minutes (twice). A portion of the cell wall (CW) and the insoluble residue of each step of the extraction (R1, R2 and R3) were water-washed until alkali or acid free. Drops of a diluted suspension of these materials were transferred to specimen grids bearing carbon films. The grids were then dried in a desiccator over anhydrous $CaSO_4$, prior to shadowing with platinum. Shadowed specimens were examined in a JEOL JEM-100B electron microscope.

Results

Mycelium and cell wall production

The changes in the culture medium and the production of mycelium and cell wall during the incubation period are shown in Fig. 2. The mycelial dry weight increased up to the 6th day and then decreased, while the dry weight corresponding to cell walls increased in parallel with that of the mycelium until it reached a maximun and then remained constant up to the end of the experiment, when it represented about 80 % of the mycelium dry weight.

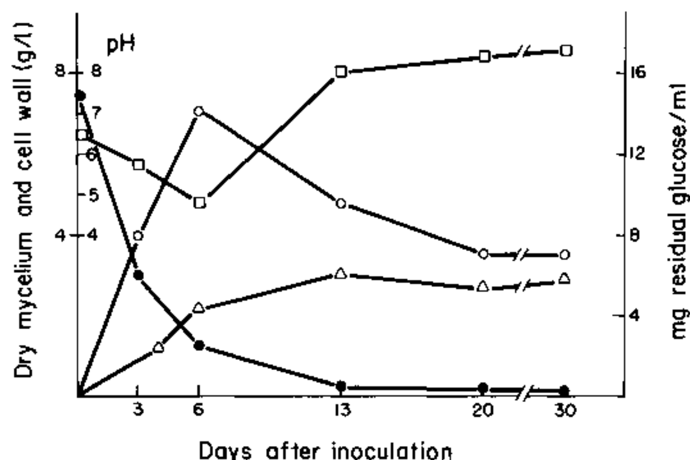

Fig. 2. Dry weight of mycelia (○) and cell walls (△), pH (□) and residual glucose (●) obtained from cultures of *P. chrysogenum* of 3, 6, 13, 20 and 30 days after inoculation.

Changes in cell wall composition with age

The main components of cell walls obtained after different incubation periods are presented in Table 1. Cell wall from 3 to 6 days has a higher content of protein. The content of chitin decreased at 6 and 13 days and the increased slightly towards the end of the experiment.

TABLE 1

CHEMICAL COMPOSITION (%) OF *P. CHRYSOGENUM* CELL WALLS OF DIFFERENT AGES

	Days					
Components	3	4	6	13	20	30
Neutral sugars	40.0	44.6	48.2	52.3	51.8	52.6
Protein	12.2	11.5	10.3	6.7	4.5	4.7
Amino sugars	13.4	12.6	11.7	12.2	13.4	14.1
Volatile matter	9.0	9.5	9.4	9.6	9.3	9.0
Ash	0.8	0.7	1.0	1.2	1.3	1.5

All the values are average of triplicate determinations and expressed as percent of cell wall dry weight.

The neutral sugars liberated by 4N H_2SO_4, 5h hydrolysis of the cell walls (Fraction Wa) of different ages were: mannose (4.8 %), galactose (6.9 %) and glucose (43.1 %). The concentration of these sugars remained constant in walls of 13, 20 and 30 days. The content of glucose was lower (27 to 34 %) in walls of 3, 4 and 6 days. The acid-resistant alkali-soluble glucan fraction (Wb), amounted to about 7 %, increasing slightly along the incubation period. The insoluble residue (Fraction Wc), identified as chitin, represented 12.7-15 %.

The yield of the fractions obtained from cell walls by different treatments are shown in Table 2. Fractions F1 (20 %) and F4 (50 %) were the most abundant.

TABLE 2

CELL WALL FRACTIONS (%) OBTAINED FROM *P. CHRYSOGENUM* CELL WALLS OF DIFFERENT AGES AFTER SUCCESSIVE TREATMENTS

Fraction	Treatment	Days			
		4	13	20	30
F1	1M-NaOH 20 °C	16.8	20.1	20.9	19.2
F2	1M-NaOH −25 °C	3.2	3.7	4.5	2.9
F3	1M-NaOH 70 °C	2.0	3.2	3.5	4.0
F4	Insoluble residue	46.5	54.4	57.2	53.6
$F4_a$	1N-H_2SO_4 90 °C 16h	32.7	32.3	20.7	35.7
$F4_b$	1M-NaOH 20 °C	10.7	14.9	20.3	18.9
$F4_c$	Chitin	27.7	21.3	22.9	22.9

2g of cell walls were extracted successively until fraction F4, Fraction F4a, b and c were obtained from 0.3 g of fraction F-4. All values are averages of duplicate determinations and expressed as percent of fraction dry weight.

The gas-chromatograms of the neutral sugars liberated by hydrolysis with 1N H_2SO_4 from 4 day-old cell wall fractions are shown in Fig. 3, and the amount of released sugars in Table 3. All fractions contained mainly glucose and in lesser amount mannose and galactose. Fraction 3 contained also arabinose and a large proportion of xylose (34 %). Fractions F2 and F4 were formed by a βglucan-chitin complex. Glucosamine was identified in the 6N HCl hydrolysates by amino acid analyzer. In the hydrolysis of F4, glucose, galactose and mannose (F4a) were released and an insoluble material remained containing an alkali soluble β-glucan (F4b) and chitin (F4c). The presence of β-glucan after hydrolysis of fraction F4 indicates that this glucan is resistant to hydrolysis with 1N and 4N H_2SO_4.

TABLE 3

NEUTRAL SUGARS RELEASED (%) OF *P. CHRYSOGENUM* CELL WALL FRACTIONS (4 DAY-OLD) HYDROLYSED WITH 1N H_2SO_4 FOR 16H AT 100 °C, DETERMINED AS ALDITOL ACETATES BY GLC

	Neutral sugar (%)					
Fraction	Arabinose	Xylose	Mannose	Galactose	Glucose	Recovery (%)
F1	0.0	0.0	2.0	0.6	81.2	83.8
F2	0.0	0.0	4.5	4.8	54.7	64.0
F3	5.6	33.8	3.3	1.9	24.3	69.9
F4	0.0	0.0	4.4	3.4	24.2	32.0
$F4_a$	0.0	0.0	4.5	3.7	24.5	32.7
$F4_b$	0.0	0.0	2.9	0.0	48.9	51.8
$F4_c$	0.7	0.5	0.3	0.4	1.7	3.6

All the values are averages of two determinations.

Characterization of the fractions

The I.R. spectra of the different fractions are shown in Fig. 4. The spectrum of F1 shows a band at 850 cm^{-1} characteristic of α-linked glucans. The other fractions show an absorption band at 890 cm^{-1} and lack the 850 cm^{-1} band, which is characteristic of β-linked polysaccharides (5). The spectra of F2 and F4 were very similar, with absorption bands at 1550 and 1650 cm^{-1} characteristic of the -CO-NH- linkage of chitin. The X-ray power diffraction diagram of F1 was characteristic of S-glucan (38).

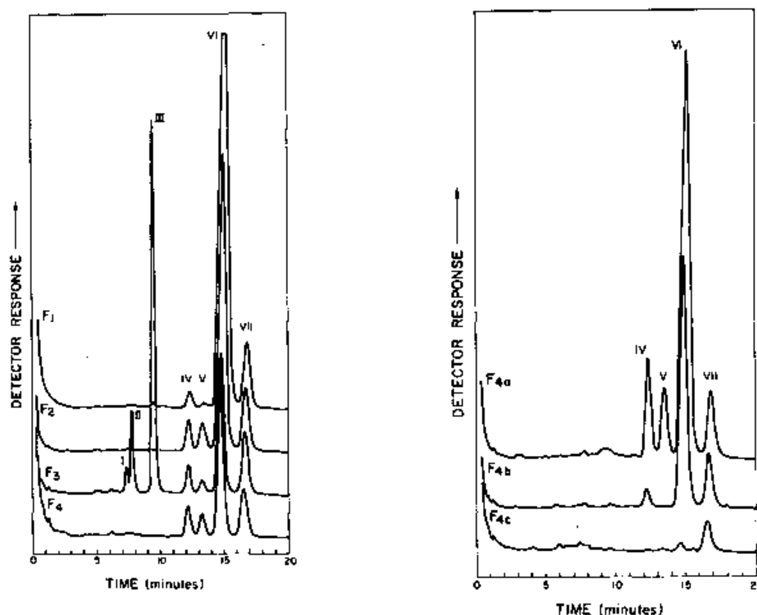

Fig. 3. Gas chromatograms of the acetyl derivatives of 4 day-old *P. chrysogenum* cell wall fractions after 1N H_2SO_4 hydrolysis for 16h. I, ribose; II, arabinose; III, xylose; IV, mannose; V, galactose; VI, glucose; VII, inositol (internal standard).

Periodate oxidation

Periodate oxidation was performed in the polysaccharide fractions suspended in 0.15M $NaIO_4$ (0.5 mg/ml). Absorbance was measured daily until constant values were obtained. Consumption of $NaIO_4$ (mol/mol hexose residue) amounted in F1 to 0.13, in F3 to 0.26 and in F4b to 0.18. The formic acid produced in this treatment represented in F1 and F4b 0.02 and in F3 0.08 (mol/mol hexose residue). The polysaccharide fractions, after treatment with $NaIO_4$, were reduced and hydrolysed with 1N H_2SO_4 for 16h. Glucose did not decrease as a result of the treatment in any fraction, indicating that it was 1—3 linked.

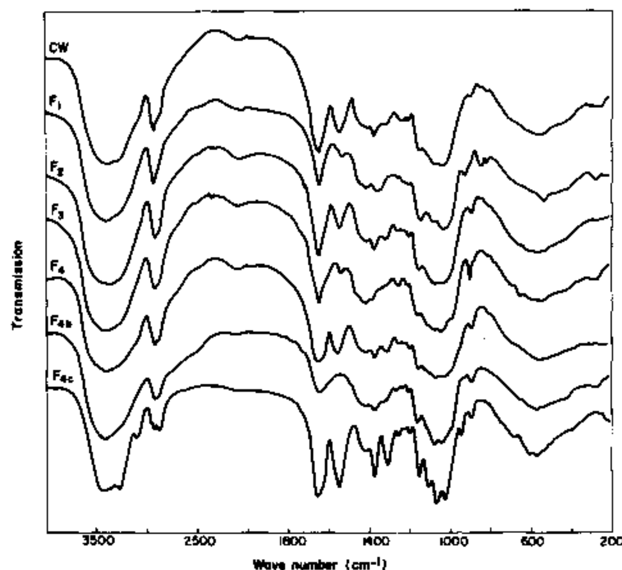

Fig. 4. Infrared spectra of 4 day-old *P. chrysogenum* cell wall (CW) and its fractions obtained by 1M NaOH treatment: (F1) at 20 °C; (F2) at −25 °C; (F3) at 70 °C. From the insoluble residue (F4) were obtained: (F4b) acid resistant-alkali soluble; and (F4c) acid and alkali resistant.

Electron microscopy of the cell wall and their residues

Electron microscopy of a shadowed preparation of the cell wall revealed the microfibrilar architecture of its surface (Fig. 5, CW). When the α-glucan was extracted by 1M NaOH the surface of the insoluble residue had a smoother texture (Fig. 5, R1). When this residue was treated with 1N H_2SO_4 at 100 °C and most of the β-glucan was hydrolysed the fibrilar architecture of chitin appeared (Fig. 5, R2). At this stage the shape and size of the cell wall were maintained. After extractions of R2 with 1 M NaOH, which released a small proportion of β-glucan resistant to the acid treatment, the chitin residue (Fig. 5. R3) was obtained. The chitin microfibrilar net work appeare more tightly packed and damaged.

Discussion

During the early growth phase of *P. chrysogenum* the cell wall represented 33 % of the mycelium dry weight. In older mycelium the proportion was higher, up to 80 %, due to the autolysis of the cytoplasm while the cell wall was not degraded. In contrast, *P. oxalicum* cell walls are degraded about 90 % during autolysis (27).

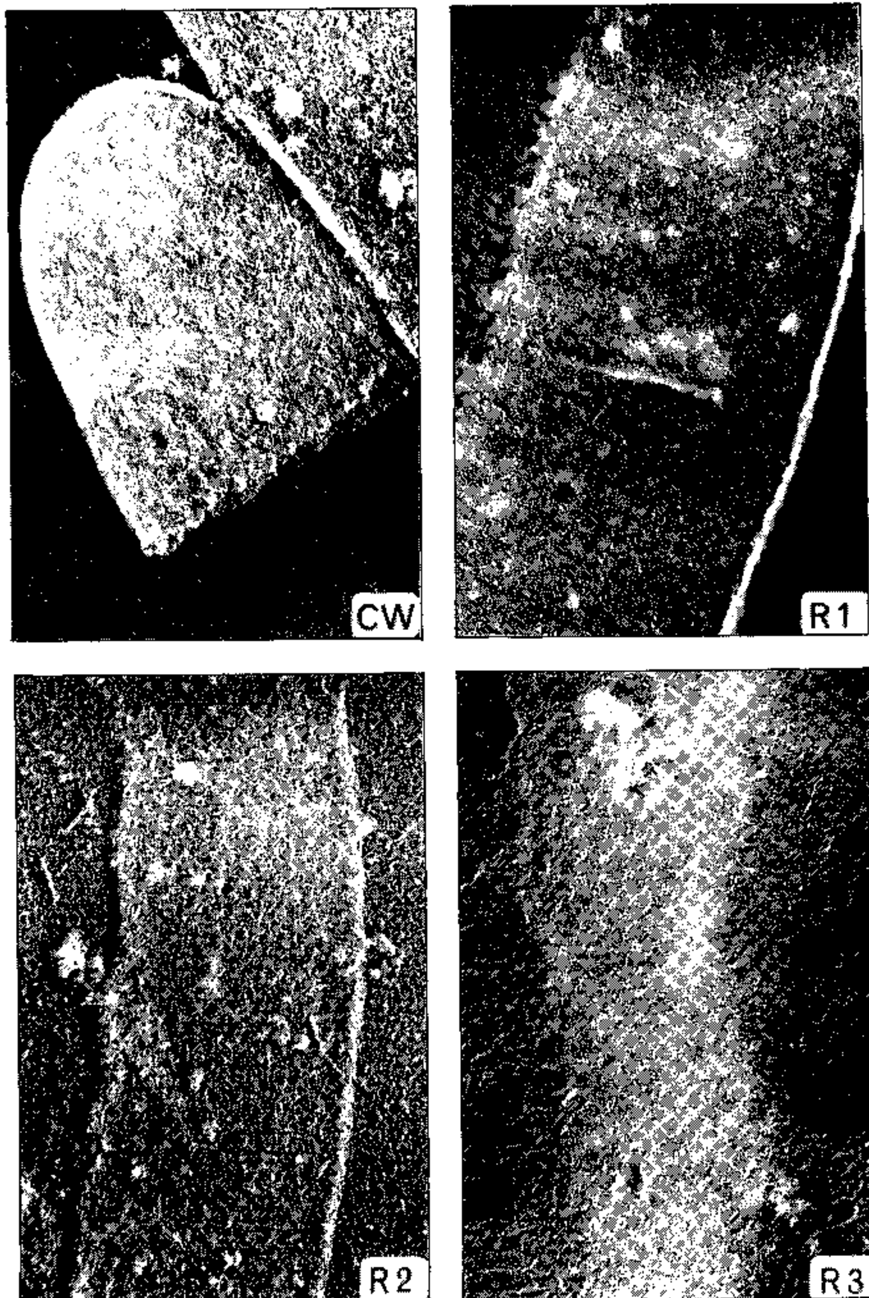

Fig. 5. Electron micrographs of *P. chrysogenum* cell wall (CW) and residues from succesive extractions (R1, R2, R3), as describeb under Materials and Methods, shadowed with gold-palladium (x 16,188).

In general, the main cell wall constituents found in our strain of *P. chrysogenum* were close to those reported by Applegarth (1). We have found that protein decreased in the cell wall with age during the growth phase, while the content of neutral polysaccharides increased. Chitin remained rather constant during the whole period of incubation. The proportion of neutral sugars remained constant after 13 days, the corresponding values being 4.5 % mannose, 6.5 % galactose and 40.7 % glucose. Similar composition has been reported for the walls of other species of *Penicillium* but *P. allahabadense* cell wall contains less glucose (20 %) and more galactose (19 %) (15). The neutral polysaccharides amounted to about 55 % of the cell wall from mycelium obtained after 13 days of incubation, but the values were lower in sample collected earlier. The yield of fractions obtained by NaOH treatment of cell wall of different ages also confirmed that cell walls from young mycelium contained less neutral polysaccharides, nevertheless the cromatographic patterns obtained when analyzing walls of different ages were not greatly affected. Freaction 1 was about 20 % of the cell wall, which agrees with the value found by Applegarth (1). The chemical and physical analysis of this fraction were similat to the S-glucan found in *Schizophillum* (38). Nevertheless *P. allahabadense* cell walls do not contain α-glucan and the material extracted with 1 M NaOH at 20° C is a β galacto-glucan (15). Since *P. chrysogenum* and *P. allahabadense* belong to different sections of the genus *Penicillium* (28) there is a possibility that differences in the polysaccharide fractions might be found among the species of *Penicillium* belonging to other sections. Fraction 2 was similar to F4, both are β-glucan-chitin complexes. Glucosamine was confirmed in both fractions by aminoacid analyzer and their I.R. spectra had the 1560 and 1650 cm^{-1} bands characteristic of the -NH-CO-group of chitin. We do not know whether F2 is solubilized from the primary glucan-chitin complex or it forms a secondary layer with similar composition. Fraction 3, which represented 3-5 % of the cell wall was of interest due to its content in xylose (33 %), and arabinose, in addition to glucose, mannose and galactose, with β-type linkages. Since this fraction represents less than 5 % of the cell wall, the concentration of xylose in whole cell wall hydrolysates is too low to be detected. Xylose (2.1 %) has been reported in the cell walls of *P. chrysogenum* (18) and trace amounts in *P. digitatum* and *P. italicum* (17). The presence of xylose in cell walls has been more often reported in Basidiomycetes (6, 35) but its presence in other taxonomical groups can not be ruled out.

The cromatographic patterns obtained from cell wall hydrolysates are useful in chemotaxonomy (33). For related species, the amount of the polysaccharide fractions, the patterns obtained from their hydrolysates and the physical and chemical characterization of the fractions should be of great help, since a few simple sugars can be linked and combined to form different polysaccharides, which would not be detected by analysis of whole walls hydrolysates.

In the application of wall composition to chemotaxonomy, culture conditions and age should be considered. Cultural conditions play a fundamental role in the dimorphism of *Mucor* and the two types of cell walls show chemical and structural differences (7). Cell wall carbohydrate composition might change with age as in *Paecilomyces persinus* (24) or remain stable as in *Aspergillus clavatus* (9). Research on the stability of cell wall composition along the incubation period and in different environmental conditions is required for a better understanding of this structure and for better utilization in chemotaxonomy.

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Characterization of β-lactamases produced by commensal *Neisseria sicca* and *Branhamella catarrhalis*

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Summary

The β-lactamases produced by two representative strains isolated from routine throat cultures, namely *Neisseria sicca* 739 and *Branhamella catarrhalis* 740, have been characterized. The former produced a plasmid-coded enzyme with properties very similar to those of the well known TEM-type β-lactamase from *Escherichia coli* coded by plasmid R6K. The enzyme was quite active on benzylpenicillin, ampicillin and cephaloridin but not on carbenicillin. p-Chloromercurybenzoate efficiently blocked the activity of both *N. sicca* and *E. coli* β-lactamases as shown in experiments carried out with the chromogenic substrate nitrocephin. On the other hand, *B. catarrhalis* β-lactamase, which was chromosomally coded, behaved essentially as a penicillinase with very low activity on cephaloridin and high on carbenicillin. Despite these differences, the three enzymes were of the same estimated molecular weights (approximately 24,300 daltons) and inhibited by cloxacillin.

Key words: Penicillinase, plasmids, transposon.

Resumen

Se ha llevado a cabo la caracterización de las β-lactamasas producidas por dos cepas representativas de las aisladas de cultivos de frotis faríngeos, *Neisseria sicca* 739 y *Branhamella catarrhalis* 740. La enzima producida por la primera cepa, codificada por un plásmido, tenía propiedades muy similares a la β-lactamasa tipo TEM de *Escherichia coli* que determina el plásmido R6K. Esta enzima resultó activa sobre bencilpenicilina, ampicilina y cefaloridina, pero no sobre carbenicilina. Además, en ensayos realizados con el sustrato cromogénico nitrocefín se produjo una fuerte inhibición por p-cloromercuri-benzoato. Por otro lado, la β-lactamasa de *B. catarrhalis*, de codificación cromosómica,

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se comportó más bien como una penicilinasa con actividad baja sobre cefalosporina y mucho más alta sobre carbenicilina. A pesar de estas diferencias el peso molecular estimado para las tres enzimas fue el mismo (aproximadamente 24.300 Dalton) siendo también las tres muy sensibles a inhibición por cloxacilina.

Introduction

The production of enzymes capable of hydrolyzing the β-lactam ring of penicillins and cephalosporins represents the biochemical basis for resistance of many bacterial strains to these antibiotics. Among these enzymes (β-lactamases), the group designated as TEM-type (Class III of Richmond and Sykes, 1973) are specially significant due to their ubiquity (6) and wide range of activity against β-lactams. The introduction of plasmid-borne β-lactamase determinants in *Neisseria* has apparently taken place recently; plasmids of 3.2 and 4.8 megadaltons (4) were detected in β-lactamase producing gonococcal isolates (1). And the recent finding of a β-lactamase plasmid in strains of *N. meningitidis* (2) confirmed the possibility of extending the problem of β-lactamase production to the other pathogen of the genus *Neisseria*. On the other hand, isolates of *Branhamella catarrhalis* have also been shown to produce β-lactamase, although of a different type and coded by chromosomal determinants (7, 12).

We have contended that the population of commensal *Neisseria* sp. and *B. catarrhalis*, which represents a significant part of the human bacterial flora, might be specially relevant for the manteinance of R plasmids and their eventual transmission to meningococci and gonococci. In connection to this, we have shown that penicillin resistant strains of commensal *Neisseria* and *B. catarrhalis* can be easily isolated from routine throat cultures (13) and we have described a 6.0 MDal-plasmid harbored by several *Neisseria* sp. isolates, whereas plasmid DNA was not detected in *B. catarrhalis* strains which produced β-lactamase.

In view of these results, it seemed interesting to characterize the resistant strains. This communication describes the properties of β-lactamases produced by *N. sicca* 739 and *B. catarrhalis* 740, two of the comensal strains isolated in our laboratory.

Material and methods

Bacterial strains and culture media

N. sicca 739 (a carrier of the R plasmid pFM739) and *B. catarrhalis* 740, were isolated as described (13). Both strains produced β-lactamase. *Escherichia coli* W3110 (which carrier plasmid R6K) was kindly supplied by J. M. Ortiz, and was used as reference strain producing TEM-type β-lactamase (18). *N. sicca* and *B. catarrhallis* were grown in Brain Heart Infusion (BBL) broth and *E. coli* in Luria-Bertani Broth (LB) (0.5 % yeast extract, 1 % tryptone and 1 % NaCl). Cells were usually grown at 37° C, by shaking in an orbital incubator (Gallemkamp).

Enzyme preparations

Cells from a 18 hours culture were centrifuged, washed and resuspended in 0.01M phosphate buffer pH 7.0. Cell suspensions were sonicated in a MSE Ultrasonic Power Desintegrator; sonications were carried out under refrigeration in an ice bath for 20 periods of 15 seconds for *N. sicca* and *B. catarrhalis* and for 12 periods of the same duration for *E. coli*. The treatment resulted in almost 100 % cell breakage. Cell debris were sedimented by centrifugation at 48.000 x g and 4° C for 30 min in a Beckman J2-21 centrifuge, and the supernatant fluids dialyzed against the same buffer used to suspend the cell. β-lactamase was partially purified from dialyzed supernatant fluids by a procedure whit consisted of the following steps: i) DEAE-Sephadex A25 column cromatograpy (bed dimensions, 20 x 1.8 cm) of the preparations in 0.01 M phosphate buffer pH 7.0. Under these conditions the β-lactamase was not ratained by the ion-exchange. ii) Ammonium sulfate precipitation; the protein fractions precipitating between 20 and 70 % saturation in the case of *N. sicca*, and between 0 and 30 % in the case of *B. catarrhalis*, contained most of the activity and were dissolved and dialyzed against 10 mM Tris-HCl buffer pH 8.0. In the case of *E. coli* this step was omitted. iii) The precipitated protein solutions were again subjected to DEAE-Sephadex A-25 column cromatography (20 x 1.8 cm) in this last buffer. Under these conditions β-lactamases were retained by the ion-exchanger and could be eluted with a NaCl gradient (O to 0,4)M) in the same buffer; the active fractions were pooled, concentrated by liophylization and dialyzed against the same buffer. iv) The *b*-lactamase preparations were finally chromatographed by gel filtration in a Sephadex G-75 column (70 x 1.6 cm).

Enzyme assays

Nitrocephin (kindly supplied by Glaxo) is a very convenient substrate for the assay of β-lactamase, which can be carried out by the colorimetric method of O'Callagham *et al.* (11). Assays with this substrate, at a final concentration of 0.1 mM were started by adding 0,1 ml of the enzyme preparation to 3 ml of the substrate solution, followed by measuring the increase in absorbance at 482 nm, in a Beckman 35 spectrophotometer with a thermostatic cuvette, set at 37° C. Absorbance was monitored for a short period (1 to 5 min, depending on the enzyme contraction) of linear increase.

The enzymes were also assayed with other substrates as described by Samuni (16) and O'Callagham *et al.* (10). Conditions were essentially the same with the only difference that the assay temperature was 30° C and that the reaction was followed by measuring the decrease in absorbance at 240 nm with benzylpenicillin or carbenicillin, at 235 nm with ampicillin, and at 255 with cephaloridine. In all cases one unit of enzyme was defined as the amount of enzyme that hydrolyzed 1 μmole of substrate per minute under the conditions of the reaction.

The kinetic constants, Vm and Km, were calculated from double reciprocal (Lineweaver-Burk) plots. Inhibition by cloxacillin and p-chloromercurybenzoate (pCMB) was determined as described by Jack and Richmond (8), by assaying the enzyme as indicated avove. The effect of L-cysteine on p-CMB inhibition was studied by adding this aminoacid at a final concentration of 0.5 mM. Protein was determined by the Lowry method with bovine albumin as a standard.

Estimation of the molecular weight of β-lactamases

The enzyme preparations were subjected to electrophoresis in SDS-polyacrylamide (10 %) slab gels, according to Weber and Osborn (18). After running, the gel was stained with nitrocephin at a concentration of 0.5 mg/ml in 0.05 M phosphate buffer, pH 7.0, in order to visualize the protein bands with enzymatic activity. Molecular weights of the active polypeptides were estimated by comparison with a set of protein standards (Sigma Chemical Co.) between 66,000 and 14,300 daltons, which were stained with Coomasie blue R-2500.

Results

Based on its substrate profile, β-lactamase produced by *N. sicca* 739 has been tentatively identified as a TEM-type β-lactamase (15). The comparison of kinetic constants, for several substrates, of this enzyme and a well kown TEM-type β-lactamase (coded by plasmid R6K) confirmed the similarity of both enzymes (Table I). Both β-lactamases hydrolyzed benzylpenicillin, ampicillin and cephaloridin, and their activities against carbenicillin were very reduced. By contrast, β-lactamase produced by *B. catarrhalis* 740 differed significantly specially due to its low activity against cephaloridin and a much higher activity against carbenicillin (Table 1).

TABLE 1

KINETIC CONSTANTS (Km AND Vmax) OF β-LACTAMASES FROM *N. SICCA* 739, *B. CATARRHALIS* 740 AND *E. COLI* W3110/R6K

	N. sicca 739		*B. catarrhalis* 740		*E. coli* W3110/R6K	
	Km (μM)	Vmax (μmol/min)	Km (μM)	Vmax (μmol/min)	Km (μM)	Vmax (μmol/min)
Benzylpenicillin	34	60 (100)	35	5 (100)	24	72 (100)
Ampicillin	91	101 (168)	101	6 (120)	70	127 (176)
Carbenicillin	33	5 (8)	13	4 (80)	18	6 (8)
Cephaloridin	521	483 (805)	29	1 (20)	366	487 (676)

Figures in parentheses represent relatives values after asigning 100 to the Vmax for benzylpenidillin.

The molecular weights of the three enzymes were estimated by SDS gel electrophoresis, under conditions in which the active polypeptides retained part of the activity, that allowed staining of the gels by hidrolysis of the substrate. Despite the differences in affinity for substrates that have been mentioned, the estimated molecular weights for the three stained βlactamase bands were the same, aproximately 24,300 daltons (Fig. 1).

Another interesting aspect for the characterization of β-lactamases is their inhibition by certain agents (17). In this work, we have determined pCMB and cloxacillin inhibition with two substrates, that required very different amounts of enzyme in the reaction mixtures, due to the differences in affinity (Table 2). A concentration of 0.5 mM pCMB was required to block the activity of *E. coli* and *N. sicca* β-lactamase against nitrocephin,

inhibition by 0.05 mM pCMB being almost negligible. On the other hand, *B. catarrhalis* β-lactamase was comparatively much less affected by this inhibitor. The addition of 0.5 mM cysteine to reaction mixtures prevented pCMB inhibition, thus suggesting that the inhibitor acted by blocking $-SH$ groups in the corresponding polypeptides.

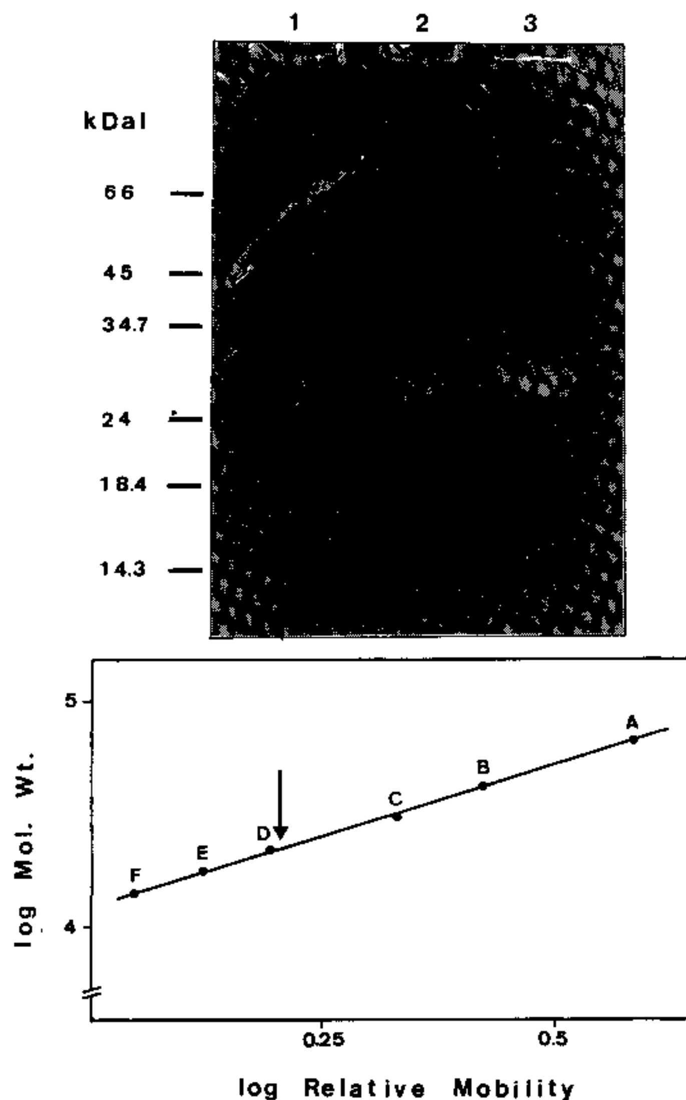

Fig. 1. SDS-poliacrylamide gel electrophoresis of partially purified β-lactamase preparations from (1) *E. coli* W3110/R6K, (2) *N. sicca* 739, and (3) *B. catarrhalis* 740. The gel was stained with nitrocephin for detection of β-lactamase bands. Molecular weight standards were run in the other part of this gel (not shown in the picture) that was stained with Coomasie brilliant blue. The positions of the standards are indicated, and relative mobilities plotted versus molecular weights. Arrow indicates mobility of β-lactamase bands. (A) Albumin, bovine (66,000). (B) Albumin, egg (45,000). (C) Pepsin (34,700). (D) Trypsinogen (24,000). (E) Lactoglobulin (18,400). (F) Lysozyme (14,300).

The assay of β-lactamases with cephaloridin required much higher amounts of enzyme in the reaction mixtures than with nitrocephin (Table 2, experiment 3). Under these conditions the inhibition by 0.5 mM pCMB was negligible. Finally cloxacillin effectively blocked the activity of the three enzymes against nitrocephin and cephaloridin. Even the much higher amounts of enzyme requiered for the assay with the later were clearly inhibited.

TABLE 2

CLOXACILLIN AND P-CHLOROMERCURYBENZOATE INHIBITION OF β-LACTAMASES FROM *N. SICCA* 739, *B. CATARRHALIS* 740 AND *E. COLI* W3110/R6K

Experiment no.	Substrate* (0.1 mM concentration)	Inhibitor	Units of β-lactamase in the preparation from *E. coli* W3110/R6K	*N. sicca* 739	*B. catarrhalis* 740
1	Nitrocephin	none	1.0	1.0	1.0
	"	p-CMB (0.05 mM)	0.9 (10*)	0.9 (10)	0.8 (20)
	"	p-CMB (0.5 mM)	0 (100)	0 (100)	0.6 (40)
2	Nitrocephin	none	0.9	0.9	0.9
	"	Cloxacillin (0.1 mM)	0.2 (78)	0.2 (78)	0 (100)
3	Cephaloridin	none	2.1	3.0	3.2
	"	p-CMB (0.5 mM)	1.8 (14)	2.5 (17)	3.2 (0)
	Nitrocephin	none	8.4	7.2	40.8
4	Cephaloridin	none	3.5	3.0	3.4
	"	Cloxacillin	0.2 (94)	0.3 (90)	0.5 (85)

* Values in parentheses represent % inhibition.

Discussion

The determination of the substrate profile of β-lactamases is particularly useful for their characterization. The development of spectrophotometric methods for the assay of penicillins (16) and cephalosporins (10) hydrolysis enables one to use a similar method to determine β-lactamase activity with both types of substrates, but requires the use of, at least, partially purified preparations to avoid interfering substances. On the other hand, it is more appropiate to determine kinetic constants (Vm and Km) rather a single value of reaction velocity for each substrate (9).

β-lactamase produced by *N. sicca* 739 had been tentatively identified as a TEM-type β-lactamase (15). This was confirmed by the results presented here judging from the similarity of kinetic constants and inhibition properties as well as the identity of the molecular weight of this enzyme and that of the well known TEM-type β-lactamase coded by plasmid R6K. The results are consistent with our observations (R. Rotger and C. Nombela, umpublished), indicating that a fragment of a Tn-*3*-like transposon, which includes the *bla*, gene is inserted in plasmid pFM739, the extrachromosomal element responsible for penicillin resistance of *N. sicca* 739 (13). Vm values of both enzymes for cephaloridin are higher than reported for other TEM enzymes (9, 14) probably due to the use of a different method of assay. Our results also indicate that the interpretation of results on β-lactamase inhibition by different agents should be made with caution. In

contrast to what has been described for other TEM-type β-lactamases, p-CMB effectively inhibited *N. sicca* and *E. coli* (R6K) β-lactamases when assayed with nitrocephin as substrate. However, the much higher amounts of enzymes in reaction mixtures, that were needed to assay these enzymes with cephaloridine were not affected by the inbitor. In any case, it can be concluded that both enzymes are p-CMB sensitive.

β-lactamase produced by *B. catarrhalis* 740 behaved as a typical penicillinase with a very low activity on cephaloridin and much higher activity on carbenicillin, that the TEM enzymes. There are a few reports in the literature on properties of *B. catarrhalis* β-lactamases (3, 5, 7, 12); the enzyme showed properties similar to those of some strains and different from others confirming the diversity among this group of β-lactamases which are coded by chromosomal genes.

An unexpected observation was that the molecular weights of the three enzymes characterized in this work were the same. However the lack of data on molecular weights of *B. catarrhalis* β-lactamases makes it impossible to compare our results with others in this regard.

Acknowledgement

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SEM

Estudio de la resistencia al cloranfenicol en cepas hospitalarias de bacilos gram-negativos

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Summary

From a total of 948 Gram-negative strains resistant to choramphenicol and other aminoglycosides, isolated at the University Hospital of Zaragoza over a 8-year period (1976-1984) 60 were chosen to study their mechanism of resistance to chloramphenicol. In 60 % of the strains this resistance was found to be plasmid-mediated and due to the production of the drug-inactivating enzyme chloramphenicol acetyltransferase (CAT) whereas in the remaining 40 % this resistance would be caused by a barrier of the cellular membrane to the permeability of the drug into the cell. The chloramphenicol resistance of 59 % of isolates —in which CAT activity was not detected— was found to be specified by two kind of plasmids transferable to *Escherichia coli* K-12 by conjugation whereas no extrachromosomal DNA was found in the remaining 41 %.

The study of the enzymatic CAT activity in the enzyme producing strains showed that this reached the highest level at the stationary phase. *In vitro* The optimun pH was 7.8 and the maximun thermal stability of the enzyme corresponded to 40 °C. The calcium and magnesium ions induced the acetylating activity but it was inhibited by EDTAA and zinc ion.

The quantitative evaluation of the strains isolated at the University Hospital revealed an increase of plasmid-mediated chloramphenicol resistance during the period April 1980-March 1981. The ulterior decrease could be partially related to the controlled use of the antibiotic.

Key words: Aminoglycosides, conjugation, plasmids.

Resumen

De un total de 946 cepas de bacilos gram-negativos resistentes al cloranfenicol y a otros antimicrobianos, aisladas en diferentes Servicios del Hospital Clínico Universitario (HCU) de Zaragoza durante el período de tiempo comprendido entre los años 1976 y 1984, se han seleccionado 60 con el fin de estudiar el mecanismo de resistencia al cloranfenicol. En un 60 % de los casos, dicha resistencia es de origen plasmídico y se debe a la producción del enzima inactivante cloranfenicol acetiltransferasa (CAT)

* A quien se dirigirá la correspondencia.

mientras que en el 40 % restante se trataría de un fenómeno relacionado con la permeabilidad de la membrana celular al antibiótico. En un 59 % de los aislamientos en los que no se detecta actividad CAT la resistencia al cloranfenicol está mediada por dos tipos de plásmidos transferibles a *Escherichia coli* K-12 por conjugación careciendo de DNA extracromosómico el 41 % restante.

El estudio de la actividad enzimática CAT en las cepas productoras del enzima, demuestra que ésta es máxima en la fase de crecimiento estacionaria. *In vitro* el pH óptimo es 7.8 y el máximo de estabilidad térmica corresponde a 40 °C. Los iones calcio y magnesio favorecen la actividad acetilante y ésta se inhibe en presencia de EDTA e iones cinc.

La valoración cuantitativa de los aislamientos realizados en el hospital, demuestra que el número de cepas con resistencia al cloranfenicol mediada por plásmidos R conjugativos fue máximo durante el período abril 1980-marzo 1981. La posterior disminución podría estar relacionada en parte con el descenso en la utilización del antibiótico. (Parte de este trabajo ha sido presentado en el X Congreso Nacional de Microbiología. Valencia, 1985).

Introducción

La resistencia al cloranfenicol en la mayor parte de las cepas patógenas es de origen plasmídico y se debe a la producción del enzima inactivante cloranfenicol acetiltransferasa (CAT).

Este enzima cataliza la 3-0 acetilación del antibiótico en presencia de acetil-coenzima A. Los tres tipos de CATs descritos en bacilos gram-negativos difieren en afinidad por el cloranfenicol, valores de K_m para el antibiótico y para el cofactor, sensibilidad al ácido 2,2'-dinitro-5,5' - ditiodibenzoico (DTNB), movilidad electroforética y reacción inmunológica cruzada (14). En general, las células productoras del enzima tipo I tienen los valores de CIM más elevados (125-325 μg/ml), las del tipo II los más bajos (75-150 μg/ml) y las del tipo III valores intermedios (150-200 μg/ml) (15). Todas ellas son proteínas tetraméricas compuestas por subunidades de 22.000-26.000 daltons.

Por otra parte, son frecuentes los aislamientos de bacilos gram-negativos resistentes al cloranfenicol en los que el mecanismo de resistencia es, probablemente, una barrera de permeabilidad al antibiótico a nivel de membrana citoplasmática (6). Las bases genéticas de esta resistencia pueden ser tanto plasmídicas como cromosómicas.

En el presente trabajo se han estudiado 60 cepas de bacilos gram-negativos resistentes al cloranfenicol con el fin de investigar la distribución del enzima CAT así como la frecuencia de otros mecanismos de resistencia a dicho antibiótico en el medio hospitalario.

Materiales y métodos

Cepas bacterianas

Las 60 cepas de bacilos gram-negativos fueron aisladas en el Servicio de Microbiología del HCU y procedían de pacientes hospitalizados en diferentes servicios. Como cepa

receptora para la transferencia de resistencia a antibióticos se utilizó *E. coli* K-12 J62 (F^-, Nal^r, pro, his, lac) (1).

Medios de cultivo

Medio LB (Difco), Brain Heart Infusión broth (Difco), McConkey agar (Difco), Mueller-Hinton (Difco). Todas las incubaciones se realizaron a 37 °C.

Antibióticos

Los antibióticos fueron suministrados por los siguientes laboratorios: cloranfenicol por Parke-Davis; ampicilina por Normon, S.A.; tetracilina por Pfizer; kanamicina por Bristol Myers; gentamicina y netilmicina por Schering; tobramicina por Eli Lilli; dibekacina por Lefa; estreptomicina por Antibióticos, S.A., ácido nalidíxico por Sigma; sulfametoxazol y trimetoprima por Difco.

Sensibilidad a antibióticos

La sensibilidad a antibióticos fue determinada por el método de difusión en agar Mueller-Hinton con discos de máxima concentración (2). Las concentraciones inhibitorias mínimas (CIMs) se determinaron por el Sistema Autobac-I (13).

Transferencia de la resistencia

La transferencia de la resistencia a los antibióticos se realizó por conjugación en medio líquido (5, 16). Las transconjugantes fueron seleccionadas en placas de agar McConkey conteniendo las siguientes concentraciones de antibióticos: cloranfenicol 30 μg/ml, ampicilina 50 μg/ml, tetraciclina 10 μg/ml, estreptomicina 10 μg/ml, kanamicina 25 μg/ml, gentamicina 10 μg/ml, tobramicina 10 μg/ml, y ácido nalidíxico 250 μg/ml.

Preparación y ensayo de CAT

Se partió de 100 ml de cultivo en medio BHI en fase estacionaria. Las células recogidas por centrifugación se resuspendieron en 2 ml de tampón (50 mM Tris. HC1, pH 7.0; 5 mM $Mg\ C1_2$; 2 mM B-mercaptoetanol) y se sometieron a ruptura por ultrasonidos (100 W, 3 min, 4 °C). El extracto enzimático crudo obtenido por centrifugación (100.000 x g, 60 min, 4 °C) fue utilizado para el ensayo radioenzimático de CAT (11). La composición de la mezcla de reacción fue la siguiente: 10 μl Tris. HC1 1 M (pH 7.8), 65 μl H_2O, 10 μl (^{14}C) acetilcoA (8 μCi/ μmol/ml), 10 μl extracto enzimático crudo y 10 μl cloranfenicol (16 mg/ml). El cloranfenicol acetilado se separó en la fase orgánica tras la adición de 3 ml de líquido de centelleo (PPO-POPOP-Tolueno). La concentración de proteínas en el extracto crudo fue determinada por el método de Lowry (9).

Factores que modifican la actividad CAT

El estudio de la influencia de determinados factores tales como pH, temperatura, iones y fase de crecimiento bacteriano en la actividad enzimática CAT se realizó mediante el ensayo radioenzimático.

i) *Actividad CAT en las fases de crecimiento bacteriano*

Fue determinada por el método radioenzimático en los extractos crudos correspondientes a cultivos bacterianos con períodos de crecimiento comprendidos entre 1 y 9 horas.

ii) *pH óptimo*

La influencia del pH en la actividad CAT se determinó a partir de un cultivo en fase estacionaria del que se separaron distintas fracciones con el fin de preparar extractos crudos a distintos valores de pH. Los valores estudiados oscilaron entre 3 y 9.

iii) *Estabilidad térmica*

Para determinar la estabilidad térmica del enzima CAT se tomaron distintas fracciones de un extracto crudo preparado a partir de un cultivo en fase estacionaria. Las fracciones se incubaron durante 10 min a temperaturas comprendidas entre 10 y 80 °C y se determinó la actividad CAT en cada una de ellas.

iv) *Iones*

El estudio de la influencia *in vitro* de distintos iones en la actividad CAT se llevó a cabo realizando la reacción enzimática en presencia de magnesio, calcio, sodio, potasio, cesio, amonio, cinc y EDTA.

v) *Curva de inactivación del antibiótico*

El grado de inactivación enzimática del cloranfenicol se estudió mediante la determinación de la actividad CAT en fracciones del extracto crudo mantenidas a distintos tiempos de reacción. El intervalo de tiempo analizado osciló entre 5 y 60 min.

Aislamiento e identificación de DNA plasmídico

Para el aislamiento del DNA plasmídico se siguió la técnica descrita por Crosa y Falkow (4). La lisis celular se realizó con dodecil-sulfato sódico (SDS) en medio alcalino (4 % SDS en 0.05 Tris. HCl-0.01 M EDTA, pH 12.4). El DNA plasmídico fue precipitado con isopropanol y posteriormente purificado con fenol.

El DNA plasmídico fue analizado mediante electroforesis en gel laminar vertical de agarosa al 0.7 % en tampon Tris-borato (89 mM Tris. HCl-2.5 mM EDTA-8.9 mM ácido bórico) (10).

Resultados

Resistencia a antibióticos

El estudio de la resistencia a antibióticos realizado por el método disco-placa demostró que las cepas seleccionadas son resistentes *in vitro* al cloranfenicol y a otros antimicrobianos. Los valores de las concentraciones inhibitorias mínimas para el cloranfenicol aparecen en la Tabla 1.

TABLA 1
CEPAS HOSPITALARIAS CON RESISTENCIA PLASMIDICA AL CLORANFENICOL

Cepa donadora	Origen	Servicio	Patrón de resistencia[a] transferido a *E. coli* K-12	CIM (mg/l)	Activ.[b] CAT (%)
Escherichia coli 1801	Orina	Med. Int.	Cm Km	64	1,08
Serratia marcescens 785	L.C.R.	Neuroc.	Cm Ap Tc Gm Km Sm Su	64	0,86
Klebsiella pneumoniae 19990	Orina	Urologia	Cm Ap Tc Gm Km Sm Su	64	0,35
Escherichia coli 4475	Orina	Med. Int.	Cm Tc Su	64	54,85
Klebsiella pneumoniae 5870	Orina	Pediatria	Cm Ap Su	64	63,29
Salmonella enteritidis 6893	Heces	Pediatria	Cm Ap Km	64	63,26
Acinetobacter calcoaceticus 5656	Asp. bron.	Med. Int.	Cm Ap Tc Gm Sm Su Sxt	64	61,68
Citrobacter freundii 3928	Orina	Cirugia	Cm Ap Tc Gm Tm Dkb Nt Sm Su	64	69,99
Serratia marcescens 4034	Drenaje	Nefrologia	Cm Ap Tc Gm Tm Dkb Nt Km Sm Su	64	78,45
Enterobacter cloacae 4402	Ex. pur.	H. Dia	Cm Ap Tc Gm Tm Dkb Nt Km Sm Su	64	68,94

[a] *Antibióticos:* Cm, cloranfenicol; Ap, ampicilina; Tc, tetraciclina; Gm, gentamicina; Km, kanamicina; Sm, estreptomicina; Tm, tobramicina; Dkb, dibekacina; Nt, netilmicina; Su, sulfamidas; Sxt, trimetroprim-sulfametoxazol.

[b] Porcentaje de actividad cloranfenicol acetiltransferasa en el extracto enzimático crudo, referido al (^{14}C)aceil-coenzyma A (100 %).

Los experimentos de conjugación bacteriana indicaron que de las 60 cepas estudiadas todas las pertenecientes a la familia *Enterobacteriaceae* son capaces de transferir a *E. coli* K-12 la resistencia al cloranfenicol y a otros antibióticos (Tabla 1). No se observó transferencia de material genético en ninguna de las 10 cepas de *Pseudomonas aeruginosa* incluidas en este estudio.

Producción de CAT

El estudio, mediante el ensayo radioenzimático, de la actividad CAT en los extractos enzimáticos crudos de las 60 cepas resistentes al cloranfenicol demostró la existencia de actividad CAT en 36 casos (60 %) mientras que las 24 cepas restantes (40 %) carecen de dicha actividad (Tabla 1).

Factores que influyen en la actividad CAT

El estudio de la actividad CAT en las distintas fases del crecimiento bacteriano indicó que ésta es máxima en la fase estacionaria (Fig. 1).

El pH óptimo para la actividad CAT en el extracto enzimático crudo resultó ser 7.8.

La máxima estabilidad térmica del enzima en el extracto crudo, correspondió a los 40 ºC. Gran parte de la actividad CAT se mantiene hasta los 60 ºC (Fig. 2).

El estudio de la influencia de determinados iones en la actividad CAT demostró que, a concentraciones adecuadas (1.5 mM), los iones calcio, magnesio, cesio y amonio potencian, en este orden, dicha actividad. La presencia de cinc y EDTA la inhiben. Los iones sodio y potasio apenas la modifican.

Los resultados obtenidos en el estudio del grado de inactivación enzimática del cloranfenicol indicaron que ésta es total a los 10 min de reacción.

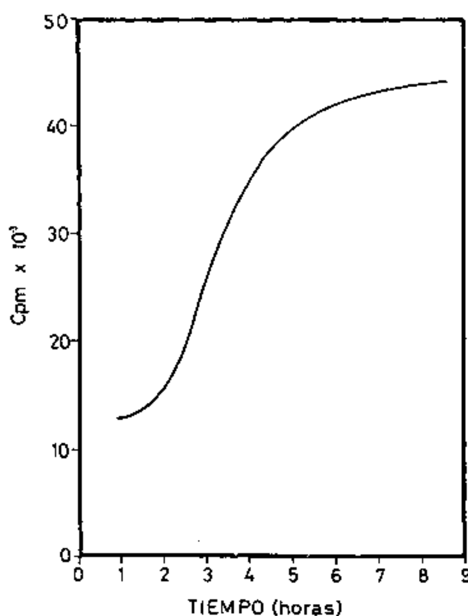

Fig. 1. Variación de la actividad enzimática CAT en las distintas fases del crecimiento bacteriano.

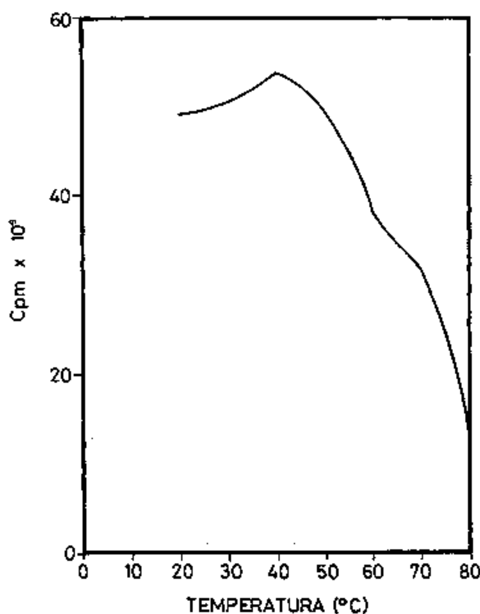

Fig. 2. Influencia de la temperatura en la actividad enzimática CAT del extracto crudo.

DNA plasmídico

El aislamiento de DNA extracromosómico y su posterior análisis mediante electroforesis en gel de agarosa, demostró que en las 36 cepas productoras de CAT la resistencia al cloranfenicol es mediada por plásmidos de diferente peso molecular. En el estudio de las 24 cepas resistentes al cloranfenicol sin producción de CAT no pudo observarse presencia de DNA extracromosómico en ninguna de las 10 cepas de *P. aeruginosa* (41 %), mientras que entre las 14 cepas de enterobacterias (59 %) se detectó la existencia de dos tipos de plásmidos. El primero de ellos media resistencia a ampicilina, tetraciclina, gentamicina, kanamicina, estreptomicina, sulfamidas y cloran-

fenicol y su peso molecular es de 45 megadaltons (Md). El segundo, observado sólo en la cepa de *E. coli* 1801, confiere resistencia a kanamicina y cloranfenicol y su peso molecular aproximado es de 60 Md (Tabla 1).

Discusion

En este trabajo se ha realizado una revisión de la resistencia al cloranfenicol en el H.C.U. Para ello se ha estudiado el mecanismo bioquímico y el origen genético de la resistencia a dicho antimicrobiano en 60 cepas seleccionadas de un total de 946 aislamientos con resistencia antibiótica múltiple correspondientes al período de tiempo comprendido entre abril de 1976 y marzo de 1984. Estas cepas procedían de diferentes servicios del hospital y de ellas, 50 pertenecen a distintos géneros y especies de la familia *Enterobacteriaceae* y 10 al género *Pseudomonas.*

La utilización del ensayo radioenzimático de Lietman *et al.* (8), modificado por Robison *et al.* (11), para detectar la presencia del enzima CAT resultó de gran eficacia por su sensibilidad y simplicidad. Este método está basado en la acetilación específica de cloranfenicol con un grupo acetilo marcado procedente del (^{14}C) acetil-coenzima A en presencia de CAT y permite extraer directamente el cloranfenicol acetilado radioactivo en el líquido de centelleo. Los resultados de la aplicación de dicho método al estudio del mecanismo de resistencia al cloranfenicol indican que en la colección de cepas seleccionadas no hay una prevalencia considerable del mecanismo de inactivación enzimática frente al de alteración de la barrera de permeabilidad, ya que sólo en un 60 % de los casos se detectó actividad CAT.

Estos resultados están relacionados con los obtenidos en el estudio del origen o determinantes genéticos de esta resistencia. Los experimentos de transferencia de material genético mediante conjugación y la identificación de DNA extracromosómico en las cepas no productoras de CAT demostraron ausencia de DNA plasmídico sólo en las 10 cepas de *P. aeruginosa* mientras que en las 14 cepas restantes, todas ellas enterobacterias, se pudieron detectar dos tipos de plásmidos conjugativos responsables de la resistencia al cloranfenicol. Es de destacar que, a excepción de la cepa *E. coli* 1801, todas las demás son portadoras de un mismo plásmido de 45 Md. Dicho plásmido, que confiere además resistencia a ampicilina, tetraciclina, gentamicina, kanamicina, estreptomicina y sulfamidas y pertenece al grupo de incompatibilidad P-1 (Inc P-1), se ha diseminado epidémicamente entre cepas de bacilos gram-negativos en el HCU, según ha sido demostrado previamente (Rivera, M.J. et al. 1984. III Reunión FISSS p. 119) (Martín, C. 1985. Tesis Doctoral. Universidad de Zaragoza, Zaragoza). Estos datos, la diseminación hospitalaria del plásmido y su pertenencia al grupo de Inc. P-1 normalmente asociado a un mecanismo de resistencia relacionado con la permeabilidad (6, 7), explican el relativamente elevado porcentaje de resistencia no enzimática obtenido en este estudio.

Respecto a las cepas en las que se detecta actividad CAT, la conjugación bacteriana y el aislamiento del DNA extracromosómico indicaron que en todas ellas este enzima está codificado por genes localizados en un plásmido, siendo éstos diversos tanto en su patrón de resistencia como en su peso molecular.

En las cepas con resistencia enzimática al cloranfenicol, se estudiaron los valores de la actividad CAT en las distintas fases del crecimiento bacteriano, así como la influencia *in vitro* de algunos factores tales como la presencia de EDTA e iones, el pH del medio y la temperatura de reacción.

Respecto a la actividad CAT en las fases de crecimiento, se detectó un aumento en la fase estacionaria, lo que se podría interpretar como un aumento en la concentración del enzima proporcional al número de copias del plásmido. Por otra parte, en todas las cepas estudiadas el enzima CAT es de naturaleza constitutiva ya que no se observó variación en la actividad al introducir cloranfenicol en el medio de cultivo. Este comportamiento es el correspondiente al de las especies gram-negativas y contrario al de las gram-positivas en las que hay que inducir su síntesis (14).

En cuanto a la presencia de EDTA y algunos iones en el medio de reacción se observó que los iones calcio y magnesio incrementan la actividad acetilante mientras que ésta se inhibe en presencia de iones cinc y EDTA. El pH óptimo para el enzima es 7.8, idéntico al descrito para los enzimas producidos por *Bacteroides fragilis* y *Staphylococcus aureus* (3, 12). La actividad enzimática es máxima a 40°C y se mantiene gran parte de ésta incluso hasta 60°C concordando este comportamiento con el descrito para otras CAT constitutivas.

Finalmente, otro de los resultados a destacar es la evolución de la resistencia al cloranfenicol de origen plasmídico en el H.C.U. La revisión de las cepas hospitalarias portadoras de plásmidos R demostró que en el intervalo de tiempo elegido (abril 76-marzo 84) el porcentaje de las resistentes a este antimicrobiano fue en aumento hasta llegar a un valor máximo de 53,7 % correspondiente al período abril 1980-marzo 1981. A partir de ese momento se observó un descenso progresivo llegándose a valores mínimos de 21,9 % (abril 82-marzo 83) y manteniéndose sólo ligeramente superior (30 %) en los meses siguientes. Estos datos podrían explicarse tanto por la restricción en el uso de cloranfenicol en el Hospital como por el menor número de cepas aisladas portadoras del citado plásmido epidémico durante los últimos años del período estudiado.

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SEM

Estudio ultraestructural de los componentes del citoesqueleto en *Paramecium putrinum* (Ciliado, Hymenostomido)

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Summary

Three dimensional cytoskeletal organization of Triton X-100-treated *Paramecium putrinum* was studied by electron microscope. A new network with filaments of 5 to 15 nm in diameter is demostrated; these filaments are in contact with other cytoskeletal structures. The detergent extraction strongly suggests that the cytoskeleton forms an heterogeneous network where might be in contacts all cytoskeletal structures.
Key words: Cytoskeletal organization, Paramecium putrinum, Triton X-100.

Resumen

Se estudia con microscopía electrónica la organización tridimensional del citoesqueleto de *Paramecium putrinum* tratado con Triton X-100. Se describe una red de filamentos de 5 a 15 nm de diámetro; dicha red está en contacto con las otras estructuras citoesqueléticas. La fuerte extracción con detergentes sugiere que el citoesqueleto está formado por una red heterogénea, donde parece ser que están en contacto todas las estructuras citoesqueléticas.

Introducción

Los microtúbulos (MTS) son componentes que ocupan las células eucarióticas formando una gran variedad de estructuras, tales como las redes citoplasmáticas que se han encontrado en células de cultivos celulares de mamíferos. En Protozoos los microtúbulos se han encontrado en diferentes tipos de estructuras (1, 2, 3, 4, 5, 6, 7, 8). Los microtúbulos en conexión con otros elementos citoesqueléticos, son los responsables de la forma y de la movilidad de la célula (10). La disposición de los microtúbulos es bien conocida en la mayoría de los Protozoos, pero no ocurre lo mismo con los sistemas microfibrilares.

Como es sabido, se han podido ver en las células los microtúbulos, microfilamentos y filamentos intermedios, cuando se han empleado los métodos de extracción con

detergentes, de los componentes menos estables del citoplasma. Schliwa y Blerkom (9), al utilizar Triton X-100 con PHEM como regulador del pH, consiguieron observar una interacción entre los diferentes tipos de filamentos en células de vertebrados.

Aquí damos algunos detalles que estaban sin resolver sobre el citoesqueleto de *Paramecium* aplicando la técnica de la extracción con detergentes.

Materiales y métodos

En este trabajo se ha empleado una estirpe de *Paramecium putrinum* que fue aislada en 1977 de una muestra de agua y todavía la mantenemos en cultivo. Esta estirpe la utilizó Patterson en 1981, ver página 525 (7).

La permeabilización de las células se hizo, en portas excavados, con Triton X-100 al 5 %, empleando PHEM como estabilizador del pH, durante 2 ó 3 minutos. El PHEM, que fue descrito por Schliwa y Blerkom (9), contiene: PIPES 60 nM, HEPES 25 mM, EGTA 10 mM y $MgCl_2$ 2 mM. Su pH es de 6,9. Después las células se fijaron con una mezcla reciente de glutaraldehido al 2 %, OsO_4 al 1 % y ácido tánico al 0,5 %. Las soluciones se prepararon de la siguiente manera: solución A, glutaraldehido al 4 % y ácido tánico al 1 % en PHEM; solución B, OsO_4 al 4 % en agua destilada más un volumen igual de estabilizador de cacodilato sódico. Por último se mezclan volúmenes iguales de las soluciones A y B. La fijación se hizo durante media hora y después las células se lavaron varias veces con PHEM.

Las células se incluyeron en Epon. Se cortaron secciones de 60-70 nm de espesor en un ultramicrotomo Reichert Ultracut. Los cortes se observaron en un microscopio Philips E.M. 300 a una potencia de aceleración de 60-80 KV.

Resultados

Células de *Paramecium putrinum* tratadas con Triton X-100 al 5 % mantienen su forma tridimensional aunque durante el tratamiento se extraen gran parte de las estructuras celulares (Fig. 1): los cilios pierden las membranas y sólo quedan los microtúbulos (Figs. 2, 9), de las tres cubiertas de la membrana celular (1) solamente queda una de ellas que por su aspecto parece ser el epiplasma (Figs. 2, 3). Muchos de los componentes del citoplasma desaparecen, tales como el retículo endoplasmático, las mitocondrias y gran parte del contenido del macronúcleo.

Elementos que permanecen en el interior de la célula después de la extracción cn Triton X-100:

Microtúbulos de los cilios (Figs. 2, 5, 9). Microtúbulos subpeliculares tanto de la zona cortical (Fig. 5) como de la zona oral (Fig. 6). Haces microfibrilares superficiales (Fig. 9, RMS), profundos (Fig. 8, RMP) y la red microfibrilar con nudos de condensación (Fig. 8, RFN). Por último, una red de microfilamentos que se encuentra por toda la célula (Figs. 1, 4, 5, 6, 7) y que se describe por primera vez en *Paramecium.*

Fig. 1. Corte longitudinal. (Composición de dos placas contiguas). Se observa la red de microfilamentos (RFI) que ocupa toda la célula. Todas las figuras corresponden a *Paramecium putrinum* tratado con Triton X-100.

Fig. 2. Corte superficial de la zona cortical. Los cilios han perdido la membrana. Microtúbulos de los cilios (Mc); epiplasma (Ep); fibra cinetodésmica (Kd); saco parasomal (Ps); tricocisto (T).

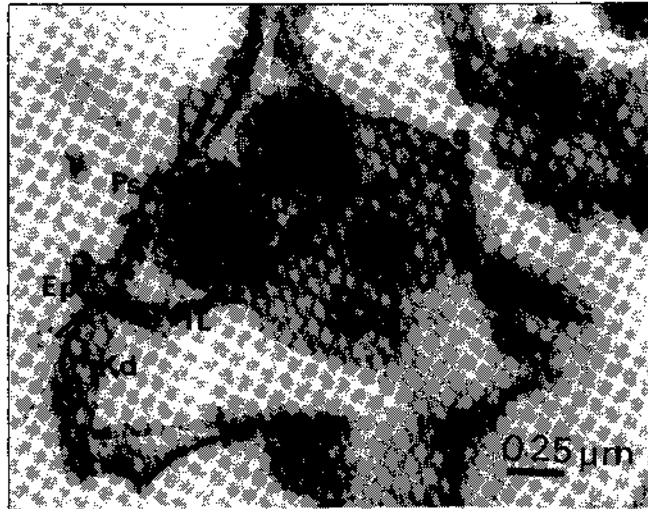

Fig. 3. Corte oblicuo de la zona cortical. La red infraciliar (IL) está en contacto (↑ corta) con el epiplasma (Ep). De la red infraciliar salen filamentos muy finos (↑ larga) que enlazan con los cinetosomas (K).

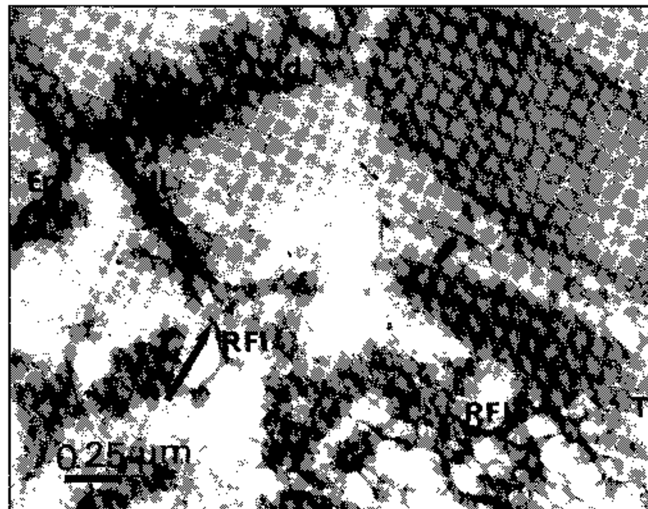

Fig. 4. Corte oblicuo de la zona cortical. La red infraciliar (IL) está en contacto (↑ corta) con el epiplasma. La red infraciliar también está en contacto (↑ larga) con la red de filamentos intermedios (RFI). Los tricocistos (T) están atrapados por la red RFI.

Red de microfilamentos intermedios

La red que se describe es posible sea el soporte de todo el citoesqueleto. Consta de microfilamentos más compactos en el interior de la célula (Fig. 1) y menos compactos en la zona más externa donde están alojados los tricocistos (Figs. 1, 4); los filamentos es posible que tengan cierta elasticidad. La red se ramifica en filamentos que tienen forma

de Y, un espesor de 5 a 15 nm (Figs. 4, 7) y por su tamaño se pueden comparar a los filamentos intermedios de células de vertebrados (Ver la Fig. 16 en Schliwa y Blerkom, 9), por lo que la denomino «red de filamentos intermedios RFI». Hay que destacar que dicha red está en contacto con las estructuras microtubulares y microfibrilares que permanecen en la célula después de la permeabilización.

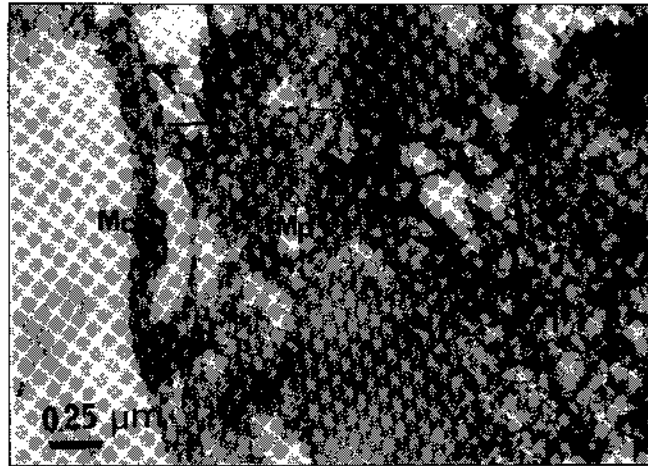

Fig. 5. Corte superficial de la zona cortical. Se ven bien los microtúbulos subpeliculares (Mp). La red de filamentos intermedios (RFI) está en contacto con los microtúbulos subpeliculares (↑). Microtúbulos de los cilios (Mc).

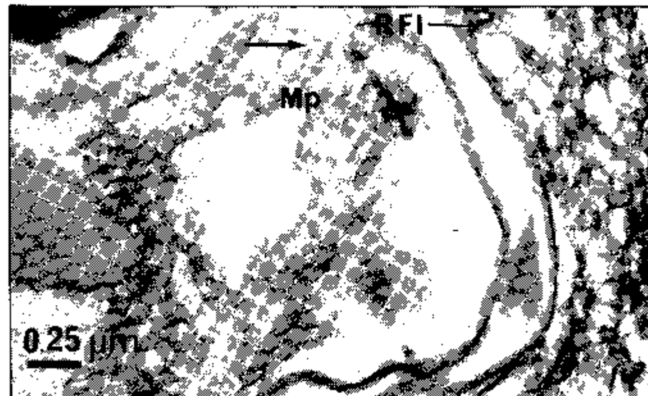

Fig. 6. Detalle de a, Fig. 1. La flecha señala el contacto entre los microtúbulos subpeliculares (Mp) y la red de filamentos intermedios (RFI).

Red infraciliar «Infraciliary lattice, IL» (5)

En este trabajo he podido medir el diámetro de las fibrillas que componen los haces de la red infraciliar, siendo su espesor de unos 3 nm (Fig. 3). Algunas fibrillas enlazan con los cinetosomas (Fig. 3, ↑ larga). La red infraciliar (IL), por su parte superior, está en

contacto con el epiplasma (Figs. 3, 4, ↑ corta), y por la parte inferior conecta con la red de filamentos intermedios RFI (Fig. 4 ↑ larga).

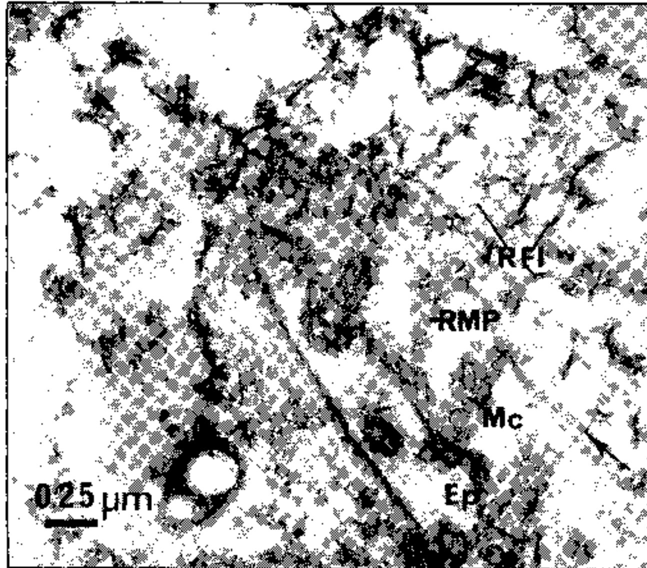

Fig. 7. Corte oblicuo profundo de la zona oral. Se observa el contacto (↑) entre la red microfibrilar profunda (RMP) con la red de filamentos intermedios (RFI). Epiplasma (Ep). Microtúbulos de los cinetosomas (Mc).

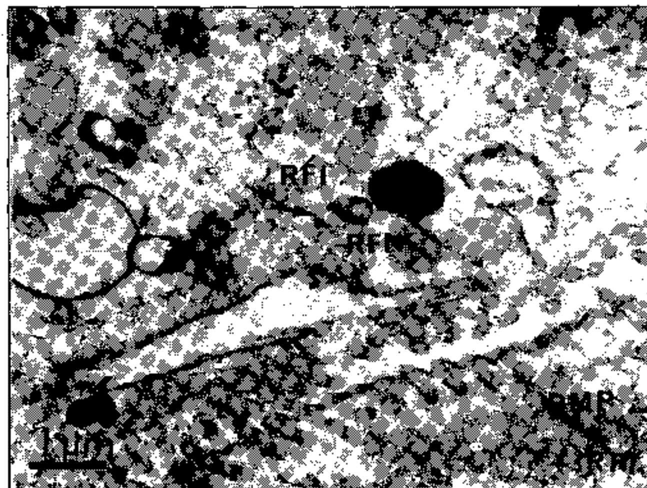

Fig. 8. Corte oblicuo profundo de la zona oral. La red de filamentos con nudos de condensación (RFN) está en contacto (↑) con la (RFI), también se observa el contacto entre la (RMP) y la (RFI).

Conexiones entre los diferentes elementos que componen el citoesqueleto

La red de filamentos intermedios RFI se encuentra por toda la célula y conecta con la mayoría de los elementos citoesqueléticos (Fig. 1). En la parte más externa de la célula

la red infraciliar (IL) está en contacto con el epiplasma y con las fibras cinetodésmicas (Fig. 3 Ep, Kd) por su parte anterior, y por la parte posterior con la red de filamentos intermedios RFI (Fig. 4). Los tricocistos se encuentran atrapados por la red de filamentos intermedios (Fig. 4). Los microtúbulos subpeliculares de la zona cortical (Fig. 5) y de la zona oral (Fig. 6) están en contacto con la red de filamentos intermedios. En las redes microfibrilares orales se observa un contacto entre la red de filamentos intermedios con las redes microfibrilar profunda (Fig. 9) y la red con gránulos de condensación (Fig. 8).

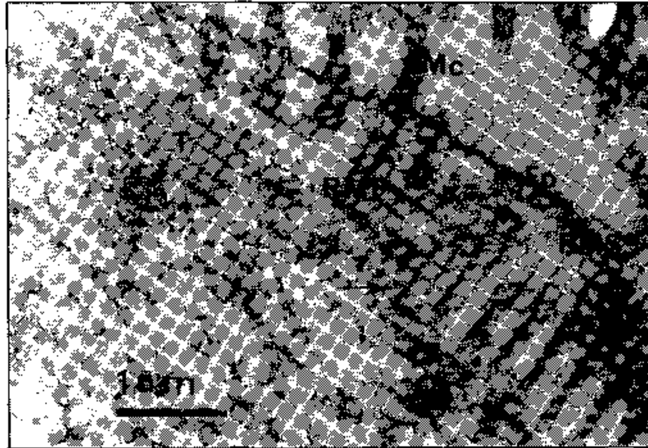

Fig. 9. Corte oblicuo en la parte superior de la zona oral. La red microfibrilar profunda (RMP) está en contacto (1) con la red de filamentos intermedios (RFI). Microtúbulos de los cilios (Mc). Epiplasma (Ep). Tricocisto (T). Red microfibrilar superficial (RMS).

Discusión

P. putrinum tratado con Triton X-100 mantiene su forma, aunque desaparecen un gran número de estructuras celulares.

Schliwa y Blerkom en 1981 (9) describieron un método de permeabilización de las células con Triton X-100 empleando como estabilizador de pH el PHEM. Con dicho método consiguieron una buena conservación de todos los microtúbulos, microfilamentos y filamentos intermedios sin una fijación previa. Para estudiar el citoesqueleto de *P. putrinum* he utilizado el método descrito por dichos autores pero con modificaciones en los tiempos y en las concentraciones de Triton X-100 según una comunicación personal de la Dra. M. Jerka Dziadosz en 1984.

Al comparar la ultraestructura de *P. putrinum* permeabilizado con la de *Paramecium* sin tratar con detergentes (1, 2, 7, 8) se observa que mantiene su forma. Esto es debido a que permanece el citoesqueleto aunque gran número de las estructuras celulares desaparecen.

En el citoesqueleto, resistente al Triton X-100, de *P. putrinum*, se encuentran microfibrillas comparables a las encontradas en células de vertebrados (9). Esto ocurre con la red que se describe en este trabajo como la red de filamentos intermedios, por tener un diámetro de 5 a 15 nm. También en la red infraciliar (IL) se describen unos filamentos

que salen de dicha red y que están en contacto con los microtúbulos de los cinetosomas. Dichos filamentos tienen un diámetro aproximado de unos 3 nm, Schliwa y Blerkom (9) describen unos filamentos de un tamaño similar, los cuales ponen en contacto los microtúbulos entre sí y los filamentos intermedios.

La red de filamentos intermedios que se describe en este trabajo está en contacto con los sistemas microfibrilares profundos de la zona oral descritos por Didier (6), con los sistemas microfibrilares de la zona cortical, con la red infraciliar (8) y también se encuentra en contacto con los microtúbulos de los cinetosomas y con los microtúbulos subpeliculares de la zona de crestas (2). Los tricocistos están atrapados en dicha red.

Los filamentos que describe Allen (2) enlazando los haces de microtúbulos con la membrana en la citofaringe (Fig. 11), pudieran ser filamentos pertenecientes a la red de filamentos intermedios.

Por los resultados obtenidos en este trabajo con *P. putrinum,* llegamos a la misma conclusión que Schliwa y Blerkom (9) llegan con células de vertebrados; que el citoesqueleto estaría formado por una red tridimensional de gran complejidad que podría relacionar entre sí a todas las estructuras celulares.

Agradecimientos

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SEM

NOTES

The protective effect of BCG pretreatment against *Staphylococcus aureus* induced pyelonephritis in rat

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Summary

A well defined and localized experimental pyelonephritis was produced in rats by intravenous administration of *Staphylococcus aureus*. This study shows that BCG pretreatment induced a significant resistance increase against experimental hematogenous pyelonephritis in rats.

Key words: Immunostimulation, experimental pyelonephritis, BCG.

Resumen

Una pielonefritis experimental bien definida y localizada fue inducida en ratas mediante la administración intravenosa de *Staphylococcus aureus.* Este estudio muestra que el pretratamiento con BCG indujo en ratas un significativo incremento en la resistencia frente a pielonefritis hematógenà experimental.

The active nonspecific immunostimulation has attracted great interest recently. Pullinger first showed in 1936 that animals infected with a tubercle bacillus expressed increased resistance to the unrelated pathogen *Brucella abortus* (3), and from then it has been found that various compounds have capability to stimulate immune system nonspecifically (2, 3, 7, 10, 13). These compounds include some microorganinsms such as Bacillus Calmette-Guerin (BCG) and *Corynebacterium parvum,* some microbial products such as muramyl dipeptide, some compounds elaborated by the immune system itself such as interleukins and interferon, and some synthetic agents such as levamisole and isoprinosine. The potential therapeutic value of nonspecific immunostimulation has been shown in various clinical syndromes such as malignancies, some chronic diseases and some infections which are induced by viruses and especially by intracellular gram-negative bacteria. Nevertheless the induction of increased resistance against facultative intracellular and gram-positive cocci and gram-negative rods which are grown ex-

* Corresponding author.

tracellularly is less impressive and to some extent controversial (3). In our previous works (5, 6, 14) we observed that a well defined and localized hematogenous pyelonephritis can be developed in rats by intravenous administration of ***Staphylococcus aureus*** which is the most abundant gram-positive bacterium. On the other hand, it has recently been reported in a number of studies that there is a marked suppression in the immune response during acute pyelonephritis (8, 12, 17), and an increased resistance can be produced by immunostimulation (11). In the present study whether the BCG pretreatment has any protective effect against *S. aureus* induced pyelonephritis in rats was investigated.

Male wistar rats weighing 220-250 g obtained from Experimental Research Center of Istanbul Medical Faculty were used in our experiments. All animals kept in a room at 22-23° C on a 12 hour light/dark cycle, were fed with a standard regime ad libitum and had free access to drinking tap water throughout the experiment. Inocula of *S. aureus* were prepared by subculturing and aliquot of the stock culture (Center for Resarch and Application of Culture Collections of Microorganisms of Istanbul Medical Faculty) in nutrient broth (Difco, Detroit, USA). Twenty-four hours after incubating at 37° C, bacteria were diluted with sterile physiological saline to the desired concentration of (5×10^8 colony-forming-units/ml). The rats were divided into two groups. Each rat of the first group injected with 0.5 ml fresh preparation of BCG (containing 7×10^8 viable Bacillus of Calmette-Guerin) intravenously. To the rats of the second group were given the same volume of sterile physiological saline through the same route. Ten days after BDG administration pyelonephritis was produced in both groups. To produce experimental pyelonephritis, each rat of both groups was injected with 1 ml of *S. aureus* suspension through one of the tail veins. Ten days later sterile heparinized blood from each rat of both groups was collected by cardiac puncture under anaesthesia (40 mg/kg pentobarbital, intraperitoneally). Sterile urine was taken using Pasteur pipette after urinary bladder had been exposed by means of a midline abdominal incision. The spleen and one kidney of each rat were removed and homogenized in a sterile glass-homogenizer. The remaining kidney was used for pathomorphologic examination. The sterile heparinized blood and urine, and the homogenates of spleeen and kidney were cultured on nutrient agar plates (Bacto, Nutrient Agar, Difco, Detroit, USA) at 37° C for 48 h. The number of the viable bacteria per ml of blood and urine, and per g of wet tissues of the spleen and the kidney of each rat were determined from the colonies count. After careful macroscopic and microscopic examination the pathomorphologic findings were rated as follows: no lesion «O», only microscopic lesions «1»; 1 or 2 abscesses seen macroscopically «2»; more than 2 abscesses «3»; widespread abscesses «4». The mean values of bacterial counts and pathomorphological scores were calculated for both groups. The statistical evaluations of the mean values were made by using Student's test.

Comparisons of mean bacterial counts, determined at the 10th day of infection in blood and urine and homogenates of spleen and kidneys of GCG-treated and nontreated rats, are shown in Table 1. As can be seen the viable numbers of bacteria in blood and urine, and homogenates of spleen and kidney of BCG-treated rats were sygnificantly lower than those of the BCG-nontreated group. The pathomorphological alterations which are obsrved in the kidneys of BCG-treated group were also significantly less than those of the nontreated group (Table 1). Typical hematogenous pyelonephritis picture was seen in the kidneys of BCG-nontreated group. This picture

TABLE 1

BACTERIAL COUNTS AND PATHOLOGICAL SCORES IN BCG —TREATED AND NONTREATED RATS[a]

	Bacterial counts In				Pathological[b] Scores
Groups[c]	Blood	Urine	Spleen	Kidney	
Nontreated (9)	1.7×10^3 $\pm 6.8 \times 10^2$	8.5×10^7 $\pm 3.3 \times 10^7$	3.5×10^3 $\pm 1.0 \times 10^3$	1.3×10^6 $\pm 3.9 \times 10^5$	2.77 ± 0.40
BCG Treated (10)	3.33 ± 1.66*	5.4×10^4 $\pm 4.4 \times 10^4$*	9.0×10^2 $\pm 2.6 \times 10^2$*	5.8×10^4 $\pm 3.1 \times 10^4$*	1.11 ± 0.42*

a The results are expressed as the mean ± SEM. *$P<0.05$ by Student's t test
b Rating of pathological findings (see text).
c Number of animals in each group are shown in brackets

was characterized macroscopically by gross abscesses formation and tissue scarring extending from cortex of kidneys to pelvis. The microscopic examination displayed gross tissue degenerations. There was irregular thickening in glomerular and tubular basement membrane and intima layer of medium to large sized arteriols. The thickened necrotic epithelium and multicellulare infiltrate consisting maintly of mononuclear cells and lymphocytes were observed in interstitial area, and tubular and arteriolar lumen. In contrast, no gross abcesses formation was see in BCG-treated rats. Only a small and localized cellular infiltration was observed in some rats of this group. Degenerative tissue changes which are seen in the kidneys of this group were also less evident.

We have previously observed that *S. aureus* is one of the most active bacteria in producing well defined and localized experimental pyelonephritis in rats (5, 6, 14). Therefore it might be suitable for assesing possible protective effect of BCG against pyelonephritis development. The findings obtained from this study suggest that BCG has significant protective effect against *S. aureus* induced experimental pyelonephritis in rats (Table 1). The protective effect of BCG which has been observed in this study may be explained on the basis of its well known powerful macrophage activating effects which have an essential role in host defence against infectious agents. On the other hand, the generation of fylly microbicidal effects of macrophages appears to be dependent on the cooperation of sensitized T-lymphocytes which secrete lymphokines (9, 15). Additionally it has been reported that cellular immune response is markedly depressed in acute pyelonephritis in both experimental animals and children (8, 11, 12, 17). Therefore for protection against pyelonephritis BCG seems to have a stimulatory action on cellular immunity in addition to its macrophage activating effects. The powerful stimulating effects of BCG, at the same doses and through the same route of administration which are also used in this study, on both macrophages and lymphocytes were observed in various in vitro and in vivo studies (2, 4). However, it has been reported that immunostimulation induced by various agents did not provide any significant protection against extracellular or facultative intracellular organisms such as *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, in contrast to their marked immunostimulatory effects against intracellular organisms or tumor cells (3). These contradictory results may be dependent

on various factors which are well defined by Bliznakov (1). For example in the been found that, for appreciable effects, BCG must be given intravenously (16). Nonspecific immunostimulatory effects of BCG were not observed when it was given intraperitoneally or subcutaneously (2, 4). In addition it has been determined that the immunostimulatory effects of BCG are strictly dose dependent. Thus some doses of BCG may show inhibitory effect instead of the generally displayed stimulation (1, 4).

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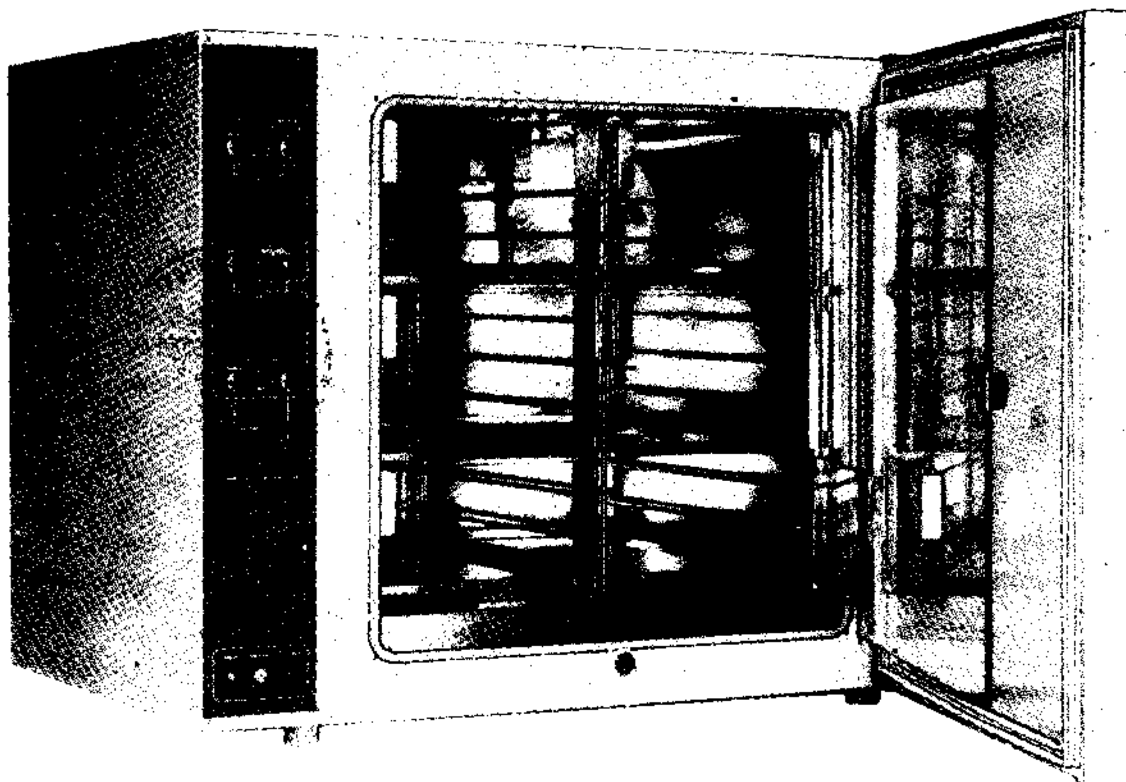

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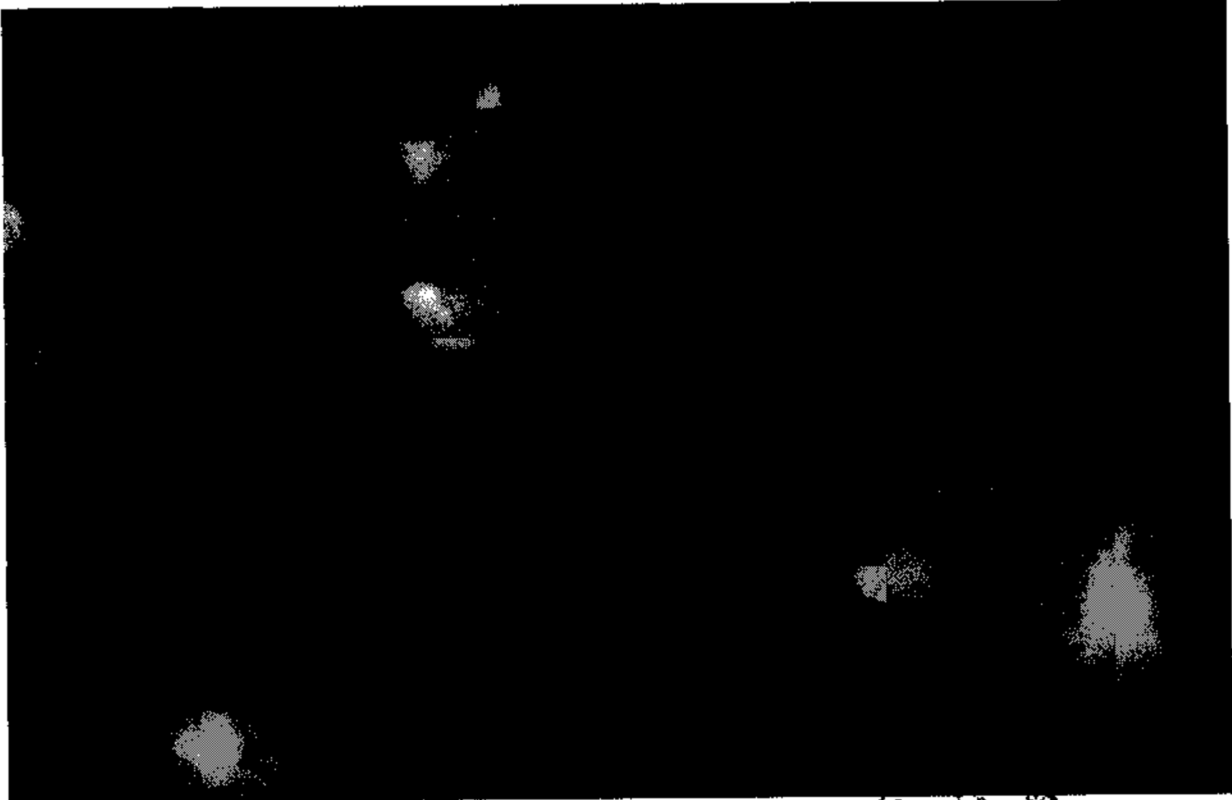

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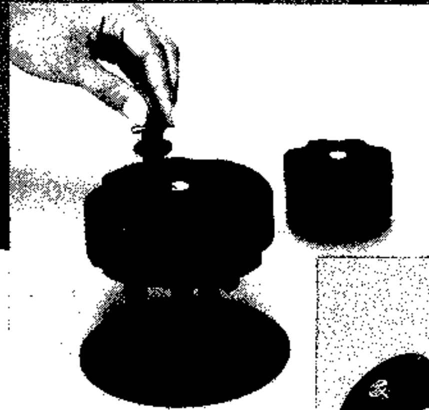

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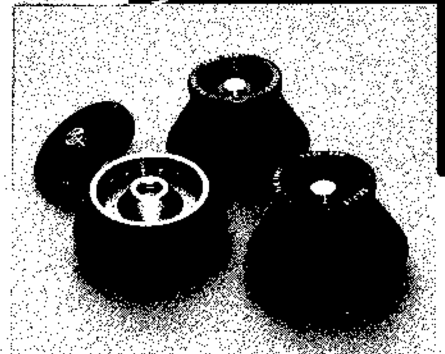

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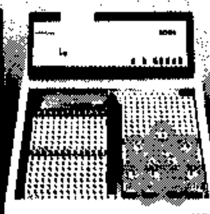

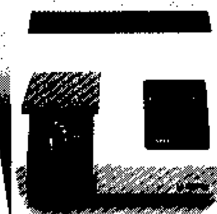

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