On the Mechanisms of Cytotoxicity by Cationic Tissue Proteins for Cryptococcus Neoformans

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(Z. Naturforsch. 21 b, 1048—1051 [1966]; eingegangen am 20. Mai 1966)

A number of cationic constituents of mammalian tissue origin have been shown to possess potent in vitro anticyptococcal activity. Lysosomal cationic protein from rabbit leukocytes and plakin from human platelets altered membrane permeability resulting in release of ultra-violet-absorbing material from the cryptococcus. In addition, oxygen uptake was significantly inhibited. Muramidase, the histones and, to a lesser extent, ribonuclease inhibited the uptake of radioactively-labelled amino acid.

GADEBUSCH and JOHNSON have recently shown that a number of cationic proteins from mammalian tissues possess potent in vitro and in vivo anticyptococcal activity. Microscopic examination of Cryptococcus neoformans cells treated with these agents revealed cytological alterations ranging from overt lysis of the cells (polymorphonuclear leucocyte lysosomal cationic protein) to changes in plasticity (muramidase) and/or vacuolation (plakin) or granulation of the cytoplasm (histone). This study will attempt to shed some light on the possible mechanisms responsible for these alterations.

Materials and Methods

The isolates of C. neoformans (BRI and HU), the culture medium and staining procedures have all been described previously:

**Cationic Protein.** Lysosomal cationic protein (LCP) was prepared from rabbit polymorphonuclear leucocytes; plakin from human platelets and muramidase from rat kidneys. LCP and plakin were essentially free from ribonuclease (RNase) and muramidase. The muramidase preparation used consisted of the beta peak of enzyme activity after gradient elution re-chromatography and was essentially free from cytochrome c.

The lysine-rich histone (subgroup f1) and the arginine-rich histone (subgroup f3) prepared by the method of JOHNS as well as the whole, unfractonated histone (HLY) from calf thymus was obtained from Worthington Biochemical Corp., Freehold, New Jersey. All histones exhibited ultraviolet absorption maxima at 277 nm, and were heterogeneous on polyacrylamide gel electrophoresis. Ribonuclease (5X crystallized, salt and protease free, 42 Kanitz units/mg) and spermine (tetra-HCl) both from beef pancreas were obtained from Sigma Chemical Company, St. Louis, Mo.

Measurement of In Vitro Antijungal Activity. All agents were reconstituted in 0.15 M NaCl, filter-sterilized (Millipore HA 0.22 filter, Millipore Filter Corp., Bedford, Mass.) and serially diluted in basal salt-glucose-glutamate (BSGG) broth before addition of approximately 2.5 x 10⁶ C. neoformans HU cells to 3 ml of medium. After 48 hours incubation at 37 °C, each sample was plated out to distinguish between fungistic and fungicidal activity.

Manometric Respiration Studies. C. neoformans HU was grown in liquid BSGG medium using Erlenmeyer flasks and a rotating shaker at 37 °C for 3—4 days. The cells were harvested, washed repeatedly with M/15 phosphate buffer, pH 6.8 (μ 0.1) and finally with 0.15 M NaCl until reducing sugar as glucose was not detected in the supernatant fluid by the method of PARK and JOHNS.

The oxygen uptake of one ml quantities of such cell suspensions (7—15 mg/ml dry wt. of cells) were studied in Warburg vessels by the conventional Warburg technique.

Glucose (0.055 M) containing 1 μg/ml thiamine HCl served as the substrate and was compared with various cationic proteins. Q₁₀₂ values were calculated to represent μ 1 of oxygen uptake/mg dry wt. of organisms/hour.

Isotopic Amino Acid Uptake Studies. C. neoformans HU was grown in carbon-free basal salt medium con-

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5 E. W. Johns, Biochem. J. 92, 55 [1964].
taining thiamine at 37°C for 48 hours and washed once in fresh medium. Sterile, 13 × 100 mm screw cap tubes containing the same medium, plus 1.1 mM sodium glutamate, 0.1 ml l-glutamic-C¹⁴ acid (Nuclear-Chicago, Des Plaines, Ill.) and basic proteins were inoculated with 5 × 10⁸ of the carbon-depleted Cryptococci and allowed to incubate at 37°C for 72 hours. Two controls (0.15 M NaCl) were included: one to measure unrestricted uptake and the other, heated at 80°C for 10 minutes prior to incubation, served as an absorption control.

After the incubation period, all tubes received 0.5 ml neutral buffered formalin and were allowed to stand for 12 hours at 25°C. The cells were washed well with M/15 phosphate buffer (pH 7.0) to remove untagged radioactive glutamate, lysed with 1 ml Hyamine 10-X (Rohm and Haas Co., Philadelphia, Penna.) diluted in 15 ml scintillator solution and counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co., La Grange, Ill.). All analyses were run in duplicate.

**Results**

**In Vitro Antifungal Activity.** All cationic proteins (except RNase) exhibited some fungistatic activity under the conditions of the experiment (Table 1) and many were fungicidal in microgram quantities. When slide preparations of these treated Cryptococci were examined, it was found that morphological alterations paralleled those of earlier experiments. Spermine, which had not been tested before, induced lysis of Cryptococci without significant cytological alteration.

**Effect of Cationic Proteins Upon Oxygen Uptake.** When 1 mg/ml solutions of muramidase, RNase, whole or arginine-rich histones were incorporated in Warburg vessels with viable isolate HU cells (equivalent to 10.2 mg dry weight) for 7 hours, the uptake of oxygen was unaltered in the presence of phosphate buffer (pH 6.9), glucose, or 0.15 M NaCl (Table 1). On the other hand, modest to severe reductions in oxygen uptake were demonstrated by spermine, lysine-rich histone, plakin and LCP respectively.

**Effect of Cationic Proteins on Uptake of C¹⁴ Glutamic Acid.** When 1 mg/ml concentrations of muramidase, RNase and whole or fractionated histones were evaluated for their ability to block the uptake of glutamate by carbon-depleted Cryptococci, it was found (Table 1) that muramidase, the lysine-rich histone fraction, whole histones, arginine-rich fraction, and RNase in that order of effectiveness, reduced the uptake of this amino acid.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Minimum Fungistatic Concentration [γ/ml]</th>
<th>Minimum Fungistatic Concentration [γ/ml]</th>
<th>Respiration Difference from mean</th>
<th>Amino Acid Uptake C.P.M. × 10³ (Corrected for Absorption)</th>
<th>Permeability</th>
<th>Colony Count per ml</th>
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<tbody>
<tr>
<td>Lysosomal</td>
<td>Rabbit Poly-</td>
<td>16.0</td>
<td>32.0</td>
<td>-31</td>
<td>7.10 + 0.160 + 0.097 3.2 × 10⁴</td>
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<tr>
<td></td>
<td>morpho-</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>degmophoenuclear</td>
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<td></td>
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<td>Leucocyte</td>
<td>Rat Kidney</td>
<td>8.0</td>
<td>32.0</td>
<td>+34</td>
<td>6.90 NC NC 5.6 × 10⁶</td>
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<tr>
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<td>&gt; 1000</td>
<td>&gt; 1000</td>
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<td>Spermine</td>
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<td>16</td>
<td>100</td>
<td>+13</td>
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<tr>
<td>Plakin</td>
<td>Human Platelets</td>
<td>32.0</td>
<td>64.0</td>
<td>-25</td>
<td>6.90 + 0.127 + 0.098 3.8 × 10⁶</td>
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<td>Histone *</td>
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<td>64.0</td>
<td>+28</td>
<td>7.20 NC NC 2.8 × 10⁶</td>
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<td>Unfractionated</td>
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<td>16.0</td>
<td>125.0</td>
<td>+32</td>
<td>6.55 NC NC 5.1 × 10⁶</td>
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<tr>
<td>ARG fraction</td>
<td></td>
<td>4.0</td>
<td>32.0</td>
<td>-4</td>
<td>7.25 NC NC 1.4 × 10⁶</td>
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<tr>
<td>LYS fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ 0.270</td>
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<td>Perclohar Acid</td>
<td>Glucose, 0.055 M NaCl</td>
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<tr>
<td>Control</td>
<td>(0.15 M NaCl)</td>
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</table>

Table 1. Effect of mammalian cationic tissue constituents upon respiration, amino acid uptake and permeability of Cryptococcus neoformans HU. * Histone ARG = arginine-rich; LYS = lysine-rich; ** These values represent absorbance of difference spectra; NC = No significant change in absorbancy; muramidase solutions had to be diluted 1 : 5 before spectra could be run for comparison.
Release of Ultraviolet-Absorbing Materials from the Cryptococcal Cell. Stock solutions of the cationic proteins were made in distilled water and in 0.15 M NaCl, the $p_H$ was checked electrometrically, the ultraviolet spectrum recorded for each sample in a DK-2 recording spectrophotometer (Beckman Instruments, Fullerton, California), and the solutions filter sterilized (Millipore HA, 0.22 μ filter). Portions of the filter-sterilized material were re-run on the spectrophotometer to ascertain retention on the filter. No reduction in absorbancy was noted for any sample.

Five ml of the sterile solutions were subsequently inoculated with C. neoformans HU to give a final concentration of 1 mg of the cationic protein per 1 mg of wet packed cells ($5 \cdot 10^6$). The suspensions were mixed and incubated at 25 C for 60 minutes, a portion was plated on BSGG medium, and the remainder centrifuged at 5000 g for 20 minutes to sediment cells and cellular debris. The supernatant was filter sterilized as before and the filtrates were subjected to ultraviolet spectroscopic analysis. The control cells, (those suspended in the solvents only) after decantation and filtration of the supernatant, were extracted with 3 ml of 1.5 N perchloric acid for 60 minutes at 25 C and handled as before to obtain sterile filtrates. Slides were made from all sediments and stained with alcian blue-basic fuchsin and alkaline fast green-azure A to ascertain changes in cell morphology.

In unbuffered water solutions, the cationic proteins tested all had a marked killing effect on C. neoformans HU. With the exception of muramidase, all cationic protein activity in this medium was related, in part, to the release of ultraviolet (A260) absorbing material. When the solvent was changed to unbuffered 0.15 M NaCl, the increases in ultraviolet absorption at 260 m/ were inhibited except for LCP and plakin (Table 1), but the viable counts did not completely reflect the inhibition. The $p_H$ never dropped more than 0.15 units in either solvent. No gross morphological changes were observed.

Specific Studies with Muramidase. Earlier efforts in this study have shown that this protein interferes with the viability of a cryptococcal population very slowly. This observation is based partly upon the fact that increasing dosage of the protein does not increase the rate of fungicidal activity proportionately, since presumably the cryptococcal cells were capable of taking up only a certain amount of the enzyme. Another possibility was suggested from staining procedures; namely, that the enzyme at low concentration exerts its activity on only certain cells, e.g. the cell about to reproduce.

Inasmuch as the generation time of C. neoformans BRI is 4.7 hours, it would be reasonable to assume that only a small portion of a culture would be attacked at one given interval, the budding stage, in a para-synchronous population.

To increase the chance for maximum exposure and activity, cultures of isolate BRI were brought into the log phase in Fernbach flasks at 32 C with vigorous chemical agitation. Under these conditions population densities of $4.5 \cdot 10^9$ cryptococci/ml were routinely obtained in 72 hours.

At this time, flasks containing 1 mg/ml muramidase in BSGG were inoculated with one tenth their volume of the log phase isolate BRI and placed on the shaker and sampled at intervals. Such samples were examined with eosin Y or India ink wet mounts and on alcian blue-fuchsin stained slides. In this manner, it was ascertained that cryptococci indeed seem to be most vulnerable to muramidase at the budding stage, resulting in a multiplicity of aberrant forms. These included ovoid or elongated cells, pseudohyphal elements, multiple budding cells, as well as partially broken or lysed ghosts.

In order to ascertain if overt lysis of muramidase-treated cryptococci occurred, the enzyme (in concentrations ranging from $162.5 \mu g/ml$ to $5000 \mu g/ml$ in doubling increments was allowed to react with C. neoformans BRI at 25 C in phosphate buffer ($p_H$ 6.2). Absorbancy at 540 m/ was not reduced after 2 hours. Each sample was adjusted to $p_H$ 10.75 by the addition of IN NAOH (Naka mur reaction). No significant difference from suitable controls was noted after incubation at 25 C for 18 hours. While experiments just presented gave evidence that the test organism could be killed by muramidase, gross dissolution of the cell wall in the majority of cells was apparently not the direct effect. The addition of 100 or 250 $\mu g/ml$ of ethylenediamine tetracetate (EDTA) (di-sodium salt) to 1 mg/ml solutions of muramidase as suggested by Repaske similarly did not result in gross dissolution of the cryptococcal cell wall in vitro. Antifungal activity of muramidase, however, was enhanced by EDTA.

Discussion

Comparison of various basic tissue constituents has shown all of these substances to interact with *C. neoformans* under suitable conditions *in vitro*. Thus, potent anticytotoxic activity was demonstrated for LCP, muramidase, spermine, plakin and a number of histones. Subsequently, it was shown that *cryptococci* were especially sensitive to muramidase during the budding process resulting in cells which suggested (a) osmotic instability; (b) interference with capsular polysaccharide synthesis, and (c) inability of reproductive units to separate. Spontaneous lysis of *cryptococci* even in the presence of EDTA could not be shown under isotonic conditions.

LCP and plakin were demonstrated to be capable of releasing nucleic acids and oligo-nucleotides absorbing at 260 m\(\mu\) from viable *cryptococci* under physiological conditions. Similar, but less dramatic permeability changes were shown to occur when *cryptococci* were exposed to unfractionated or lysine-rich histone, spermine or RNase in water or low ionic strength salt solutions. This observations for RNase recalled a similar recent demonstration of this phenomenon in *Candida utilis* and *Saccharomyces cerevisiae*.

In addition, LCP and plakin rapidly inhibited oxygen uptake of *C. neoformans* HU.

When histones, muramidase and RNase were compared for their ability to inhibit uptake of radioactively-labelled glutamic acid, they were all found to be active in this respect. The concomitant intracellular formation of granular (void) bodies which stained for RNA (acridine orange) and the finding that oxygen consumption at the same time was only minimally affected in histone-treated *cryptococci* led to the conclusion that the histone complexes with nucleic acid and the fungus undergoes irreversible cellular injury.

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