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Immunological Studies on Hartmannellid Amoebae

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Abstract. Two types of complement fixation antigens—cellular and extracellular—were prepared from three strains of *Hartmannella castellanii:* the original strain (CASTELLANI 1930), Neff strain (NEFF 1957) and A 1 strain (CULBERTSON et al. 1959). Sera of rabbits immunised with these amoebae were absorbed with homologous and heterologous organisms and extracellular antigens and cross examined in CF reaction. The results of these reactions are leading to the following conclusions:

1. The A 1 strain of *H. castellanii* differs distinctly from the Neff and Castellani strains.
2. Neff and Castellani strains show the nearest relationship.
3. Extra- and intra-cellular antigens have a thermostable component of similar CF reactivity.
4. Superficial antigens of hartmannellae do not participate in the CF reaction.
5. The preparation of extracellular antigens is simple and economic. It secures the same results in CF reaction in specificity and sensibility as antigens prepared of amoeba cells.

Satisfactory antibody response of rabbits in pathogenetical experiments with *Hartmannella castellanii* (Douglas 1930) A 1 strain (CULBERTSON et al. 1959)*) led us to the idea of comparing immunologically this strain with the original strain isolated by CASTELLANI (1930)**) and a strain isolated from soil by NEFF (1957)*). All these strains are practically identical in their morphology. Results of a very interesting immunological study gained by means of the immobilization test have been published by ADAM (1964) recently.

Considering certain disadvantages of the immobilization test—very slow movement of the hartmannellid amoebae, difficulties with the determination of the endpoint of this reaction, nonspecific immobilization with sera of healthy animals (CULBERTSON 1965), the participation of agglutinating and lytic antibodies and the necessity to work with living antigen only—we have chosen the complement-fixation (CF) reaction for our studies. The sensibility of this method enables a more detailed antigen and antibody tracing.

*) Obtained from Dr. J. H. Sandground, Haskins Laboratories, New York, U.S.A.

**) Obtained from Prof. Aldo Castellani, Institute of Tropical Medicine, Lisbon, Portugal.

MATERIAL AND METHODS

Cultivation of amoebae. A medium consisting of 2 % Bacto Casitone (Difco) and 0.5 % NaCl in distilled water was used for the cultivation of amoebae. 1200 ml Roux-bottles hydrophobized with silicone and filled with 100 ml of this medium were applied for mass production of this organism.

Preparation of the cellular antigen. CF antigen prepared from homogenized suspensions of formolized amoebae after the method of CULBERTSON et al. (1961) did not yield satisfactory results. The insufficient mechanical homogenization of amoeba-cells is the possible reason of differences in the quality of antigens. We have found that antigens from live amoebae which were desintegrated by means of lyophilization possess superior CF activity. The amoebae from the cultivation medium were concentrated by means of centrifugation at 2000 rpm. for 10 min. and the sediment washed three times with phosphate-buffered isotonic salt solution (PBS)—pH 7.2. This suspension was counted in Bürker's counting chamber, diluted on the concentration of 2,500,000 amoebae per ml or more, filled into ampules and lyophilized. The amoeba cells are perfectly homogenized in antigen rehydrated to the original volume with distilled water. Optimal dilutions of antigen for CF reaction were determined in antigen titration. Lyophilized antigen is storable practically without limitation and any changes of activity. It is almost completely transparent after diluting. Young cultures are most convenient for its preparation, especially in such strains, which spontaneously produce great numbers of cysts in later phases of growth (strain Neff). Cysts are not destroyed by lyophilization and antigens containing excessive numbers of cysts appear to be complementary in CFR.

Preparation of the extracellular antigen.—The extracellular antigens were gained from the Bacto Casitone medium in which the amoebae were growing for 10 days. The amoebae were removed from the medium by means of centrifugation (10 min. at 2000 rpm.). Supernatant fluid was dialyzed against distilled water for three days in a refrigerator (+1 °C). The fluid with nondialyzable components was concentrated by means of Carbowax 20 M to one twentieth of the original volume. It has been found later, that the same CF activity of this type of antigen is preserved if the dialyzed medium is evaporated to the required volume in a thermostat at 60 °C for 2 or 3 days. Concentrated antigens were then extracted with 3 volumes of ethylether and deetherized under an air-pump. Some of the antigen samples appeared anticomplementary occassionally. Two parts of such antigens were absorbed with one part (v./w.) of pure minced guinea-pig muscular tissue overnight at +1 °C. Optimal dilution of antigens for CF reaction was determined in antigen titration.

Preparation of immune sera. Young rabbits weighing from 2500 to 3000 grams were immunized with three injections of whole live amoebae into the ear vein. 5,000,000 of organisms washed in PBS were administered as one dose, the intervals beeing 8 and 4 days. The animals were bled three weeks after the first injection. Antibody titers of their sera ranged between the values of 1 to 128 and 1 to 512. The development of antibodies does not substantially differ in animals inoculated with the nonpathogenic strains and with the pathogenic A 1 strain. The animals did not suffer from any symptoms of infestation, although infections of mice, guinea-pigs and rats with such doses of the same A 1 strain are usually fatal.

Technique of complement-fixation reaction. The CF reaction was produced in a total volume of 0.5 ml with 2 units of complement, 2 units of antigen and 2 units of haemolysine. Tubes were incubated 60 minutes at 37 °C.

Absorption of sera with live amoebae. One ml of fresh serum was mixed with a sediment of 10 million of living amoebae, washed three times with PBS. This suspension was incubated at 4 °C for 24 hours, amoebae separated by means of centrifugation and the supernatant serum inactivated at 56 °C for 30 minutes.

Absorption of sera with extracellular antigens. Immune sera and extracellular antigens were mixed in the same volumes, incubated at 4 °C for 24 hours and then inactivated for 30 minutes at 56 °C.

RESULTS

Cellular antigens. The sera selected for our experiments presented approximately the same antibody titer with homologous antigens (Fig. 1). In cross-reactions, however, the A 1 strain differs distinctly from the other two. Anti-A 1 serum gives

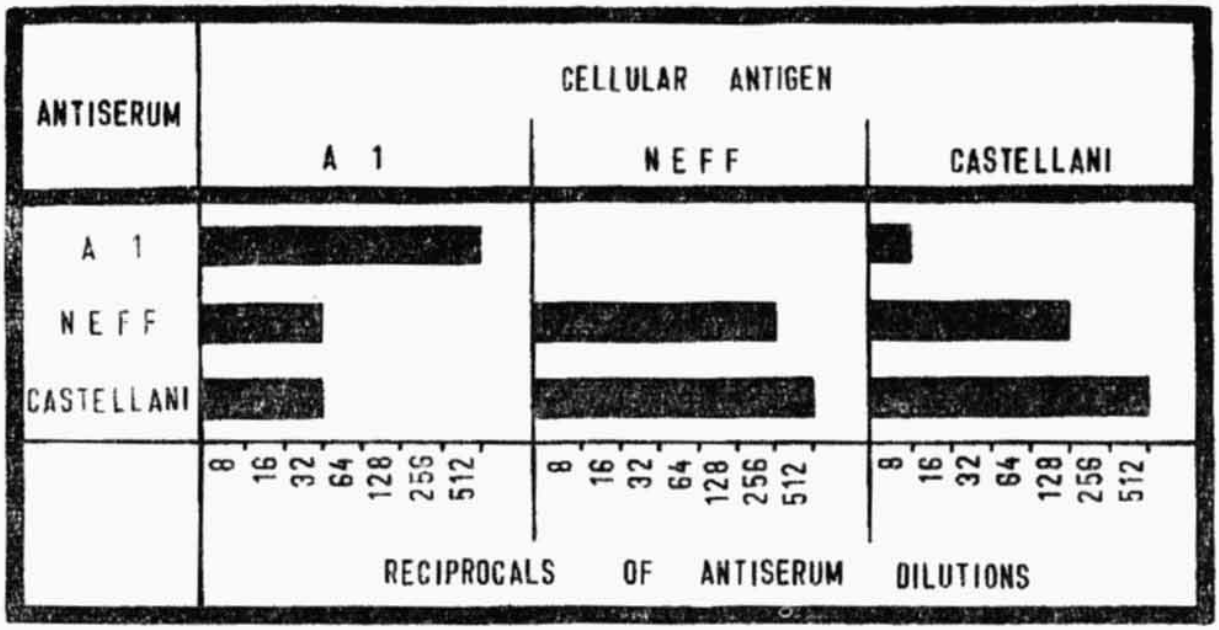

Fig. 1. — CF titers of immune sera with cellular antigens.

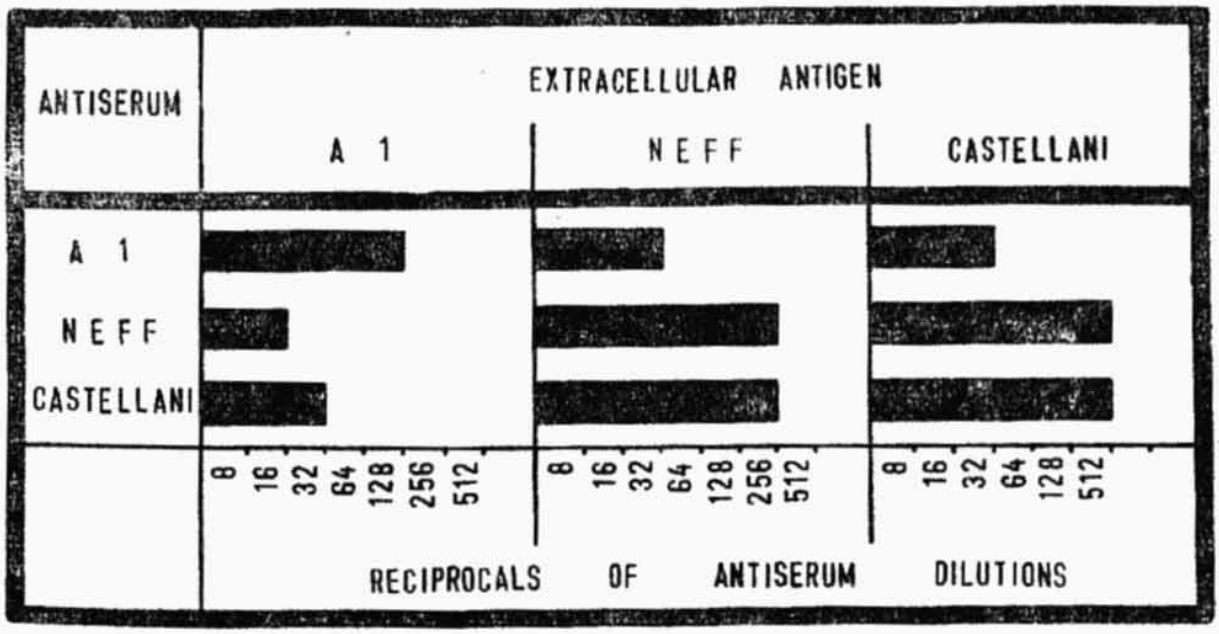

Fig. 2. — CF titers of immune sera with extracellular antigens.

a negative reaction with the Neff antigen and a very weekly positive one with the Castellani antigen. The positive titers of Neff and Castellani antisera with homologous antigens are much higher. The reaction of A 1 antigen with both heterologous sera is, nevertheless, distinctly positive. This antigen seems to have the broadest activity. The differences between Neff and Castellani antigens are insignificant.

A detailed structure of the cellular antigens has not been defined so far, but a substantial part of the CF activity is bound to a thermostable component. The precipitable protein component has a subordinate position.

Extracellular antigens. Results of CF cross-reactions with immune sera are very similar to those obtained with cellular antigens (Fig. 2). Only the A 1 antiserum gives a significantly higher positive reaction with heterologous antigens and lower titer with homologous antigen. The Bacto Casitone medium is constituted mostly of amino acids. After dialysis, thermal preparation and ether extraction of this medium, the CF active component is, with the greatest probability, formed by materials of a polysaccharide character. It is stable also after long heating at 100 °C. Four days after the inoculation of fresh medium we were able to prove the first traces of this antigen. Its concentration is relatively low and does not increase substantially between the 7th and 30th day after inoculation. Traces of antigen activity were demonstrated in dried ether extracts of antigens dissolved in PBS. Dialyzable components of antigens are not CF active.

Absorption experiments. We tried to absorb the antisera on the surface of whole live amoebae. This way of absorption did not cause any significant changes in the CF titer of sera with homologous antigens. Only small differences in titers with heterologous antigens were visible (Fig. 3). In a further experiment, extracellular antigens were used for absorption of antisera. This absorption was followed in homologous antisera by a decrease of the CF titer, the differences being 1 or 3 dilutions (Fig. 4). Cross absorption of Neff and Castellani antisera shows only little differences between these two strains.

There are two apparently paradoxical phenomena in the results: the remarkable decrease or disappearence of homologous CF titer of all antisera absorbed with heterologous antigens and the increase of CF titer of these sera in the reaction with heterologous antigens. Several technical factors in the CF reaction may participate in these changes. The definitive explanation of these phenomena is not simple and will remain only within the sphere of a hypothesis until the structure of antigens is precisely known.

DISCUSSION

Results of our experiments with cellular and extracellular antigens give more evidence of a distinct difference between the A 1 strain of *Hartmannella castellanii* and the other two strains tested. Immunologically active components specific for this strain are connected chiefly with some intracellular materials. Surface antigens are probably identical in all three strains (Červa, 1966) and do not participate in the CF reaction. This was demonstrated in the absorption experiments.

The thermostable component of extracellular and cellular antigens is almost identical in its CF reactions and seems to be a polysaccharide synthetized by the amoebae. Further studies of the structure of both antigens are programed. Strains isolated by Neff and Castellani may be taken for identical. Differences in cross-

Fig. 3. — CF titers of immune sera absorbed with whole live amoebae.

Fig. 4. — CF titers of immune sera absorbed with extracellular antigen.

reactions of absorbed sera are analogous to the differences usually found by means of immunological procedures among various strains belonging also to another protozoan species. Our results agree with the conclusions of ADAM (1964), who used the immobilization test. This reaction is probably not bound to the surface of amoebae only. A practical conclusion of our experiments may be of importance for immunological studies of hartmannellid amoebae and differentiation of the pathogenic strains. Extracellular antigen-active materials isolated from the cultivation medium are more easily attainable and more convenient for use than cellular antigens.

Detailed studies of the A 1 strain (CULBERTSON 1961, CULBERTSON et al. 1958, 1959, 1965a, 1965b, ADAM 1964) have shown that its specific features are not only in immunological reactions, but chiefly in its biological properties, as the ability to penetrate into and multiply in the tissue of experimental animals, the optimal temperature for growth and also growth requirements in defined media. Only the fact that sufficient morphological differences have not been found so far prevent the assignment of this strain for a fully defined species.

REFERENCES

ADAM K. M. G., A comparative study of hartmannellid amoebae. J. Protozool. 11: 423—430, 1964.

CASTELLANI A., An amoeba found in cultures of a yeast. Preliminary note. J. Trop. Med. Hyg. 33: 160, 1930.

CULBERTSON C. G., Pathogenic Acanthamoeba (*Hartmannella*). Am. J. Clin. Pathol., 35: 195—202, 1961.

—, ENSMINGER P. W., OVERTON W. M., The isolation of additional strains of pathogenic *Hartmannella* sp. (Acanthamoeba). Proposed culture method for application to biological material. Am. J. Clin. Pathol. 43: 383—387, 1965.

—, HOLMES D. H., OVERTON W. M., *Hartmannella castellani* (Acanthamoeba sp.). Preliminary report on experimental chemotherapy. Am. J. Clin. Pathol. 43: 361—364, 1965.

—, SMITH J. W., MINNER J. R., Acanthamoeba: Observations on animal pathogenicity. Science 127: 1506, 1958.

—, —, COHEN H. K., MINNER J. R., Experimental infection of mice and monkey by Acanthamoeba. Am. J. Pathol. 35: 185—197, 1959.

ČERVA L., Use of fluorescent antibody technique to identify pathogenic hartmannellae in tissue of laboratory animals. Folia parasitol. (Praha) 13: 328—331, 1966.

DOUGLAS M., Notes on the classification of the amoeba found by Castellani in cultures of a yeast-like fungus. J. Trop. Med. Hyg. 33: 258—259, 1930.

NEFF R. J., Purification, axenic cultivation and description of a soil amoeba, Acanthamoeba sp. J. Protozool. 4: 176—182, 1957.

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