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PREPARATION OF AN IMMUNOTOXIN FOR ACANTHAMOEBA CASTELLANII

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Summary. Acanthamoeba castellanii (Neff) is a small free-living amoeba which is closely related to pathogenic organisms that produce almost invariably fatal human infections (1). The f(ab)' fragment of a monoclonal antibody (designated A9) specifically reactive to the A. castellanii cell surface, was covalently linked to the A chain of diphtheria toxin. This immunotoxin inhibited cell division completely, whereas nonspecific mouse IgG or nonspecific f(ab)' derivatized with diphtheria toxin A chain (DTA), A chain alone, or unlinked A chain and A9-f(ab)' in combination, had no effect on cell division.

The immunotoxins are a group of cytotoxic agents which possess a far greater potential than traditional antibiotics with respect to the selective killing of eukaryotic cells. These antibiotics, formed by the covalent linkage of a general eukaryotic toxin to a cell-type specific antibody, exploit antigenic differences in cell surfaces for their selective cytotoxicity. Considerable effort is presently being expended to apply immunotoxins to cancer therapy (2). But, there has been little investigation of the possibility of applying these potential therapeutic agents to parasitic diseases which quite often represent greater worldwide problems than neoplastic disease. Because of insufficient information concerning endocytosis of surface bound molecules, the mechanisms of toxin escape from endocytotic vesicles, and the toxicity of mammalial toxins for protozoa, investigation in this area is presently empirical. Recently, we reported data which indicated that ricin A chain and α-amanitin, unlike diphtheria toxin A chain, were unsuitable candidates for the toxic moiety of A. castellanii immunotoxins (3). We report here that immunotoxins can be prepared that are effective for protozoa.

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Methods

The monoclonal antibody used in this study, designated A9, was secreted by a hybridoma prepared by polyethylene glycol mediated fusion of A. castellanii (Neff) plasma membrane-immunized BALB/c mouse splenocytes (4) with SP2/0-Ag14 myeloma cells (5). The antibody was purified from ascites fluid by 40% saturated ammonium sulfate precipitation followed by protein A-sepharose affinity chromatography (6). The f(ab)'-A9 fragments were prepared by (a) reduction of $f(ab)_2'$-A9 in 0.05M sodium phosphate, pH 5, with 0.01M mercaptoethylamine for 80 min at 37°, followed by (b) alkylation of free sulfhydryl groups in 0.4M tris, pH 8.3, with 20mM iodoacetic acid for 30 min at 0°. The $f(ab)_2'$-A9 was prepared from purified A9 antibody by digestion with a 40:1 (mg:mg) antibody:pepsin ratio in 0.1M sodium acetate, pH 4.5, for 16 hours at 45°. Diphtheria toxin A chain was prepared by selective heat precipitation of the B chain under reducing conditions (7). Diphtheria toxin A chain was linked to f(ab)'-A9 using the heterobifunctional crosslinking reagent N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (8).

A. castellanii (Neff) were obtained and cultured as previously described (9-10), and the membranes for immunization prepared by the procedure of Ulsamer et al (11). Cell number was determined, in duplicate upon replicate samples, by counting aliquots with an improved Neubauer haemocytometer (average deviation is 17%).

Results and Discussion

Diphtheria toxin A chain derivatives of f(ab)'-A9 inhibit Acanthamoeba castellanii (Neff) cell division (fig. 1). Non-specific mouse IgG, non-specific mouse f(ab)' derivatized with DTA, as well as f(ab)'-A9, had no effect upon cell division at any concentration tested which includes concentrations higher than those used in fig. 1. In addition, this organism is immune to diphtheria toxin although A. castellanii cell free protein-synthesizing systems are inhibited by diphtheria toxin A chain (3). Diphtheria toxin A chain as well as a combination of f(ab)'-A9 and diphtheria toxin A chain (unlinked) also had no effect upon cell division at any comparable concentrations. The results clearly indicate that the binding of f(ab)'-A9 diphtheria toxin A chain immunotoxin to the cell surface was the direct cause of the complete inhibition of cell division observed in fig. 1. A9 antibody selectively binds to this strain of A. castellanii. Binding assays indicate no binding to A. culbertsoni or three different strains of A. castellanii (samples of the latter cell lines were provided by Dr. Govinda Visvesvara, Center for Disease Control, Atlanta, Georgia). This immunotoxin is, then, a selective cytotoxic agent.

The immunotoxin used in experiments giving results illustrated in fig. 1, was a derivative that had 1.2 (DTA) moieties per f(ab)'-A9 moiety. Another

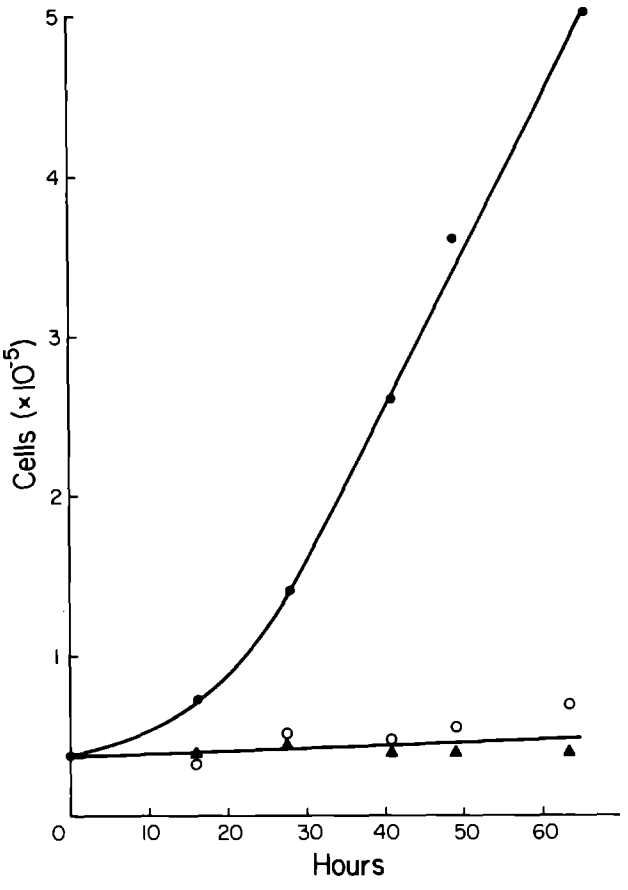

Fig. 1 Inhibition of Cell Division by Immunotoxin.
Log phase A. castellanii cells were grown from an initial cell concentration of 37,500 per ml in 2.5ml growth media (9) to which 0.35 ml of phosphate buffered saline containing the additives indicated, was added. The samples were prepared in duplicate. No additives, ●-●; 61μg/ml immunotoxin, o-o; 26μg/ml immunotoxin, ▲-▲. The immunotoxin was a diphtheria toxin A chain derivative of the f(ab)' fragment of A9 monoclonal antibody (see text). A chain to f(ab)' ratio was 1.2.

series of experiments performed with a DTA-f(ab)'-A9 immunotoxin that had a DTA:f(ab)'-A9 ratio of 1.6 was much less effective, inhibiting cell division by only 70% at slightly higher concentrations. Binding assays of the second derivative indicated a much lower binding to the cell surface per unit protein than the 1.2 derivative. The PDP derivative of the f(ab)'-A9 used in the second preparation of this immunotoxin demonstrated little difference in antigen binding activity from the original f(ab)'-A9. A third derivative with a DTA: f(ab)'-A9 ratio of 3.7 was completely inactive, both with regard to inhibiting cell division and binding to the cell surface. Presumably,

oversubstitution of the antibody fragment with DTA molecules produced either a steric inhibition of antibody binding, or, alternatively, a partial denaturing of the antibody molecule resulting from antibody-A chain interactions. Intuitively, it appears that the ratio of toxin to antibody which results in maximum inhibition of cell growth should be as characteristic of a particular antibody as a pH optimum is for an enzyme, for analogous reasons.

"Western" blot analysis (12) of A9 indicated that the antigen was an A. castellanii membrane protein in the 100,000 dalton range. No information is presently available concerning the function of that protein. Preliminary* indications that immunotoxins could be used to kill protozoans were obtained in this laboratory earlier (13). The recognition moiety of that immunotoxin was the f(ab)' fragment of a different monoclonal antibody, C_5, that binds to the major (15,000 dalton) protein in A. castellanii plasma membrane. Since these immunotoxins bind to two different randomly selected, plasma membrane proteins,and individually inhibited cell division, it seems likely that most cell surface binding sites could serve the purpose.

The results presented here demonstrate that immunotoxins can be prepared that will selectively inhibit the growth of parasitic organisms. Screening of available catalytic toxins could result in the discovery of a more effective toxic moiety. Also, screening of cell surface binding sites may indicate that some categories result in faster uptake of the immunotoxin, or present a biological route of uptake that is more favorable for the particular toxin being used. Further, in vivo effectiveness of these antibiotics must be investigated before the possibility of therapeutic potential can be estimated.

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* Results presented in the Ph.D. thesis of Dr. Mark Howell were obtained with a f(ab)'-C5 antibody fragment that was naturally produced in occasional ascites fluid. C5 is an IgM and, consequently, no defined technique is presently available to produce f(ab)'-C5. Therefore, it is extremely difficult to reproduce those experiments at will conveniently.

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