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Predation on Planktonic Protists Assessed by Immunochemical Assays

Mark D. Ohman

INTRODUCTION

Measuring the sources and rates of predation on planktonic protists is of interest from a number of perspectives. In studies of protist population dynamics, mortality rates as well as division rates need to be assessed.¹ From a predator's perspective, the abundance (and specific composition of assemblages) of planktonic protists can markedly influence a predator's growth rate. From the point of view of ecosystem studies, the intensity and selectivity of predation on planktonic protists can alter the pathways of material and energy transfer in pelagic food webs.

The present method was developed to measure predation on ciliate protists by planktonic suspension-feeding copepods. This prey-predator interaction is addressed because of the suspected importance of ciliates as a prey resource for planktonic metazoans^{2,3,4,5} and the likelihood that ciliates are significant intermediaries in the transfer of microbial loop production to larger suspension feeders. The need for new methods to study predation on planktonic ciliates arises because many ciliates are quite fragile and lack recognizable hard parts. Conventional methods for collecting, concentrating, incubating, and fixing planktonic organisms frequently result in lysis of these cells.⁶ This is particularly true for ciliates in the order Oligotrichida and those members of the Choreotrichida that lack a lorica. Despite the particular focus of the method outlined here, the principles of this immunochemical method should also be applicable to other protist-predator studies.

The principle of this method is that antibodies raised against ciliate antigens can be used as a probe to detect the presence of ciliate antigens from intact cells or the gut contents or fecal pellets of a predator. The reaction between antigen and antibody (Ag-Ab) can be visualized in a number of ways. The procedure presented here employs dot blots, using an enzyme-linked conjugate to visualize the intensity of the reaction. In a dot blot procedure the antigen solution (e.g., gut contents of a predator) is applied (blotted) onto nitrocellulose membranes. The immobilized antigen is reacted with a primary antibody (which recognizes the target Ag), then with a secondary antibody (which recognizes the primary Ab) conjugated to a reporter molecule (in this case the enzyme alkaline phosphatase). The Ag-Ab reaction is then visualized by allowing the dot blot to react with a chromogenic substrate solution.

The advantages of this immunochemical method include: (1) *in situ* predation on ciliates can be measured; (2) there is no need to collect, concentrate, incubate, or preserve ciliates,

any of which can result in cell lysis and lead to experimental artifacts; (3) immunochemical probes can be developed with different levels of specificity, depending on the question being addressed; and (4) individual predators can be probed, rather than relying on ensemble measures of ingestion.

Disadvantages of the method include: (1) the targeted prey organisms usually must be cultured for production of antibodies; (2) crossreactions must be screened carefully in advance; and (3) the predator digestive tract may have to be dissected and removed to minimize crossreactions between the antibodies and predator tissues.

MATERIALS REQUIRED

Equipment

- Orbital shaker
- Microliter pipette
- Reflectance densitometer
- Dot blot manifold (optional)

Solutions

- Nitrocellulose (0.45 μm ; from Schleicher and Schuell)
- Blotto (concentration of merthiolate increased over that in Reference 17):
 - 5% (w/v) nonfat dry milk prepared in buffer (e.g., phosphate-buffered saline, PBS; pH 7.4).
 - 0.01% (v/v) Antifoam A
 - 0.00025% (w/v) Merthiolate
- Primary antibody diluted in Blotto
- Secondary antibody (e.g., alkaline phosphatase conjugated goat anti-rabbit IgG) diluted 1:1,000 in Blotto
- 3% (v/v) normal goat serum, in Blotto
- Stock stain solutions:¹⁸
 - 5-bromo-4-chloro-3-indolyl phosphate (BCIP), *p*-toluidine salt (5 mg/ml in dimethylformamide); nitro blue tetrazolium (NBT) (1 mg/ml in either Veronal-acetate or 0.15 *M* Tris buffer, pH 9.6); and 2 *M* MgCl_2

PROCEDURES

These procedures assume that the objective is to produce a probe consisting of polyclonal antisera. In predation studies, polyclonal antisera can be advantageous because detection of the presence of prey remains does not depend upon the immunoreactivity of a single epitope. For production of monoclonal antibodies consult the current literature. For the basic elements of immunochemistry, including antibody production and antiserum purification, see Warr^{7,8} and Johnstone and Thorpe.⁹ Only those aspects unique to the application used here are specified in any detail.

Antigen Preparation

Generally it will be necessary to culture ciliates or other protists to obtain sufficient monospecific antigen for antibody production. As noted below, care must be taken when

preparing ciliate antigens for immunization. The specificity of the antisera will be influenced by procedures at this step. In particular:

- Avoid contamination by bacteria and other microorganisms by use of monoxenic cultures and harvesting techniques that retain only the ciliates of interest.
- Avoid inclusion of particulate debris egested by ciliates by harvesting only from fresh cultures.
- Maintain antigen extracts on ice to minimize proteolytic activity; if protease inhibitors are used, ensure that they are used at concentrations that will not adversely affect the host organism producing antibodies (e.g., 1 mM phenylmethyl sulfonyl fluoride, 2 mM EDTA used in Reference 4).

Formaldehyde-fixed cells are commonly used for antibody production. However, some aloricate ciliates do not survive fixation and preservation.⁶ In such cases extracts of live or frozen cells should be used as the immunogen.

Particular care must be taken in concentrating and harvesting aloricate ciliates for antibody production because of their fragility. The following procedure has worked effectively in the author's laboratory.

1. Allow cultured ciliates to reduce their prey to background levels.
2. For bacterivorous taxa, harvest ciliates separately from bacteria by differential centrifugation (e.g., $270 \times g$ for 15 min for *Strombidium*, $1000 \times g$ for 20 min for *Uronema*, at 4°C).
3. Discard the supernatant and quickly resuspend cells in buffer (e.g., PBS). (For herbivorous or carnivorous ciliates use other methods to concentrate ciliates and to separate them from their prey. Alternatives include gentle filtration, reverse flow filtration, centrifugation, pipetting, or perhaps chemotaxis.)

Often it will be useful to characterize the molecular mass of antigens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and to characterize the specific antigens recognized by the antibodies on Western blots.⁹

Antibody Production

Several different immunization protocols and mammalian hosts can be used for antibody production.⁹ The approach of Vaitukaitis¹⁰ requires small quantities of antigen, injected intradermally at multiple sites in New Zealand white rabbits. It has been used successfully to produce antibodies to yolk protein from larvae of the northern anchovy.¹¹ A modified version of the protocol permitted Ohman et al.⁴ to produce sensitive antisera using just 90 µg of protein extracted from oligotrich ciliates as the immunogen. In the protocol used by Ohman et al.,⁴ 6 to 8 intradermal injections were made in Freund's complete adjuvant on day 0. This was followed by multiple intramuscular booster injections using Freund's incomplete adjuvant on day 21. Additional booster injections followed at 14-d intervals, until the antiserum titer was acceptably high.

It is advisable to have adequate antigenic material available for titer checks and for calibrating the immunoassays. Two or more rabbits should be immunized with any immunization protocol to allow for genetic and physiological differences in the animals' immune response to particular antigens. Pre-immune serum should be withdrawn to serve in control assays. The titer of test bleeds and the specificity of the antibodies can be assessed by dot blot assays (below).

Purification of Antibodies

The resulting crude antisera can be used for immunoassays, but purification can lead to greater specificity. Also, it is sometimes desirable to remove the targeted immunoglobulins (usually IgG) from serum albumin, proteases, other proteins, carbohydrates, and other serum constituents. A variety of techniques can be used to accomplish this purification, including ion exchange chromatography, high-performance liquid chromatography (HPLC), and ligand affinity columns (see References 9, 12, and 13). Perhaps the simplest is the use of Protein A or Protein G affinity columns. Specific purifications may be required for special applications or where undesired crossreactions occur.

Immunoassay

The reaction between antibodies and the prey antigens from a predator's gut contents is visualized by dot blots. General discussions of dot blotting techniques can be found in References 14, 15, and 16. Specific applications can be found in References 4 and 11. The procedure entails, in order: homogenization of gut contents in a buffer and application onto nitrocellulose; reaction with primary antibody; reaction with secondary antibody conjugated to alkaline phosphatase; incubation with chromogenic substrate; and quantification of the reaction intensity.

An additional dot blot series should be incubated with pre-immune serum as a control for false positives. This is particularly important when assaying antibody titer and testing for crossreactions. If the reaction is to be quantified by densitometry, a standard of known reactivity with the primary antibody should be blotted along with each batch of samples to ensure consistent reaction intensity. The original immunogen serves well for this purpose.

1. Immerse nitrocellulose in distilled water for 5 min and air dry.
2. Blot gut contents onto nitrocellulose and air dry.
3. Block with Blotto for 30 min.
4. Bind primary Ab for 60 min.
5. Wash with Blotto for 10 min ($2\times$).
6. Block with 3% normal goat serum for 20 min.
7. Bind secondary Ab for 60 min.
8. Wash with Blotto for 10 min ($2\times$).
9. Add alkaline phosphatase substrate for 20 min. (From stock solutions combine immediately before use in the following proportions: 9.0 ml buffer, 1.0 ml NBT, 0.10 ml BCIP, and 0.02 ml MgCl_2 .)
10. Stop reaction with deionized or distilled water rinse ($3\times$).
11. Record results. A qualitative scale can be established for the intensity of precipitated dye product.
12. Air dry, then read with reflection densitometer.

Quantification

The presence or absence of a significant reaction is sufficient information for some predator-prey studies. In this case, dot blot intensity can be evaluated visually and compared with the reaction blank obtained from assay of the gut contents of starved predators. The detection limit can be determined from serial dilution and dot blot assay of known quantities of antigen.

To quantify the amount of antigenic material in a predator's gut and estimate ingestion rates further requires: (1) calibration of densitometric measures of the intensity of dot blots;

Figure 1. Dot blots of extracts of the ciliate *Strombidium* sp. reacted with anti-*Strombidium* serum. Serial dilutions (left to right) were blotted in duplicate (top and bottom rows).

(2) knowledge of changes in immunoreactivity of antigens with time during digestion; and (3) knowledge of the gut residence time of antigenic material. Changes in immunoreactivity (2) and gut passage (3) may or may not occur simultaneously. If early digestion alters epitopes sufficiently to change the immunoreactivity of the ingested prey prior to complete assimilation, then the timing of these two processes needs to be resolved separately. Feller and Ferguson¹⁹ point out additional concerns when quantifying immunochemical estimates of predator gut contents.

The intensity of the dot blot is proportional to the amount of antigenic material present, provided antibodies and chromogenic substrate are available in excess. With an appropriate calibration curve, the reaction intensity can be expressed in terms of the quantity of the original antigen (e.g., mass of protein if the primary antigenic substance is proteinaceous). A calibration curve is prepared by serial dilution of a known antigen which is blotted onto nitrocellulose and taken through the assay procedure in the immunoassay section (Figure 1). When the reaction is read by reflectance densitometry the response is typically curvilinear (rectilinear on a semi-log plot, Figure 2). Because of this log-linear relation, the assay must be calibrated carefully over the range of antigenic material found in samples. Dot blots should be uniform in shape and surface area for densitometry; blotting templates or manifolds are helpful for this purpose.

Areas for Further Development

The Ag-Ab reaction can be read in a number of ways. The primary antibody can be reacted with a secondary antibody coupled to a variety of reporter molecules (enzymes,²⁰ fluorochromes, immunogold, or radiotracers). For some purposes different reporters may be more advantageous than that presented here. For example, immunofluorescence may be useful for visualizing the sites of prey assimilation in a predator's digestive tract, or for enumerating intact ciliate cells by flow cytometry. Further enhancement of the sensitivity of the dot blot assay may come from the use of chemiluminescence (rather than histochemical stains) to visualize the reaction.

The reaction can also be carried out in solution by ELISA (enzyme-linked immunosorbent assay) techniques rather than in a solid phase bound to nitrocellulose. ELISAs have the advantage of greater sensitivity than dot blots and the availability of automated procedures for plate washing and spectrophotometric or fluorometric reading of results. However, care must be taken to select microtiter plates that bind the antigens (or antibodies) of interest with high affinity. Two advantages of the solid-phase dot blot approach over ELISA are the excellent protein-binding characteristics of nitrocellulose and the permanent record of the dot blot that is obtained.

Figure 2. Relationship between the optical density of immunochemical dot blots and the quantity of ciliate protein. Extracts of the ciliate *Strombidium* sp. were reacted with anti-*Strombidium* serum that had been immunoadsorbed against extracts of larval anchovy to eliminate cross-reacting antibodies (see text). The two points indicated by open squares were excluded from the regression. (Reprinted from Ohman, M. D., Theilacker, G. H., and Kaupp, S. E., *Biol. Bull.*, 181, 500, 1991. With permission of *The Biological Bulletin*.)

NOTES AND COMMENTS

1. Immunoassay. Assay can be done at 21 or 37°C, but the temperature must be consistent for all assays. Incubation times should be optimized for the temperature and antibodies employed. Agitate on orbital shaker throughout incubations. Use a dot blot template if applying numerous samples to a single piece of nitrocellulose. Blot samples in duplicate or triplicate, provided sufficient gut material is available. Control dot blots should be carried out using pre-immune serum as a control for false positives.
2. Cross reactions. It is essential to screen antisera carefully for crossreactivity with non-homologous antigens prior to use in predation studies. Predator tissues should also be screened for crossreactions with the antibodies. To minimize such reactions, the gut contents of the predator can be dissected away from predator tissues onto a microscope slide, homogenized in a few microliters of a suitable buffer, and blotted onto nitrocellulose. Undesired crossreactions can sometimes be eliminated. For example, Ohman et al.⁴ used an affinity column to eliminate a strong, unexpected crossreaction between the antiserum raised against ciliates and extracts of teleost larvae. This procedure entailed, first, binding soluble extracts of fish larvae to Sepharose and packing the beads into a microaffinity column. The antiserum was then immunoadsorbed against the immobilized larval extracts until the eluted antiserum had virtually no remaining crossreactivity with the teleost tissues. The antiserum used for the calibration relation in Figure 2 had been immunoadsorbed in this manner.
3. Ethical concerns. Alternatives to the production of antibodies in mammalian hosts are being discussed,²¹ but these are not practical options at present. Since mammalian hosts remain necessary, the activity should not be carried out frivolously. The user should ensure that an immunochemical approach is the best method available for the question at hand prior to the immunization of experimental animals. Also, proper facilities and protocols are needed for animal care. If these facilities are not available, it is possible to have custom antibodies produced by an experienced commercial laboratory.

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