American Fisheries Society Special Publication 18:304–310, 1988

Monoclonal Antibodies: A Tool for Molluscan Pathology

ERIC MIALHE, VIVIANE BOULO, AND HENRI GRIZEL

Institut Français de Recherche pour l'Exploitation de la Mer
Laboratoire de Pathologie et de Génétique des Invertébrés Marins
17390 La Tremblade, France

HERVÉ ROGIER AND FRANÇIS PAOLUCCI

Laboratoire d'Immunodiagnostics, Sanofi, 34082 Montpellier, France

Abstract.—Hybridoma technology is reviewed, and the characteristics of monoclonal antibodies are compared with those of polyclonal antibodies. The contribution of monoclonal antibodies to molluscan pathology is developed with special emphasis on their use as diagnostic tools. The results of studies with monoclonal antibodies prepared against the protozoan oyster pathogen *Bonamia ostreae* are briefly described.

The development of hybridoma technology as elaborated by Kohler and Milstein (1975) has made an impact in many fields of biological research such as immunology, biochemistry, and pathology (Yelton and Scharff 1981; Krakauer 1985; Seiler et al. 1985). In pathology, mouse monoclonal antibodies have been used in diagnosis (Van Der Auwera 1987) and therapy (Blythman et al. 1981; Frankel 1985). In this article, we briefly review hybridoma technology. We compare the properties of monoclonal and polyclonal antibodies, and we consider the prospects for use of monoclonal antibodies in molluscan pathology, especially for the diagnosis of infectious diseases.

Hybridoma Technology and Production of Monoclonal Antibodies

The principle of hybridoma technology is the continuation of the nonproliferative line of antibody-producing lymphocytes by fusing the lymphocytes with a tumor cell line (myeloma cells; Figure 1). Hybrid cells, called hybridomas, are obtained which retain both the ability of individual lymphocytes to secrete antibody and the ability of myeloma cells to grow without limit. Thus, the homogenous antibody derived from a single clone of hybridomas is called a monoclonal antibody (MAB). The properties of the lymphocyte can also be retained by infecting the lymphocyte with a transforming virus or by transfecting it with tumorigenic DNA (Schonherr and Houwink 1984). The production of MAB has been reviewed by Kennett et al. (1980), Goding (1983), Pau et al. (1983), Schonherr and Houwink (1984), and Paolucci et al. (1986). The different steps in the production are shown in Figure 2.

Immunization, Preparation of Cells, and Fusion

A mouse is immunized by successively injecting it with antigenic preparations (Figure 2). Purified antigen is not necessary, but the hybridization yield is partly conditioned by the level of sensitization of the animal. After the last injection, lymphocytes isolated from the spleen are fused with myeloma cells either by chemical treatment with polyethylene glycol (Paolucci et al. 1986) or by electrical treatment (Vienken and Zimmermann 1985).

Selection of Hybridomas

Because the yield of stable hybridomas from parent cells is low, about 10^6–10^7, the nonfused myeloma cells deficient in hypoxanthine guanine phosphoribosyltransferase (HGPRT−) must be kept from overgrowth. The HGPRT-deficient cells cannot grow in a medium containing hypoxanthine aminopterin thymidine, so this medium is used to select the HGPRT+ hybridomas after fusion.

Selection of Hybridomas Producing Specific Antibody

The hybridomas must be screened as early as possible to distinguish and eliminate those producing nonspecific antibody from those producing specific antibody. The screening technique must be rapid, simple, and suitable for the large number of hybridomas grown in the wells of microculture plates. The techniques used most frequently are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunofluorescence (IF). Screening is a key step in lymphocyte hybridization, and its success depends entirely on the availability of purified antigen.

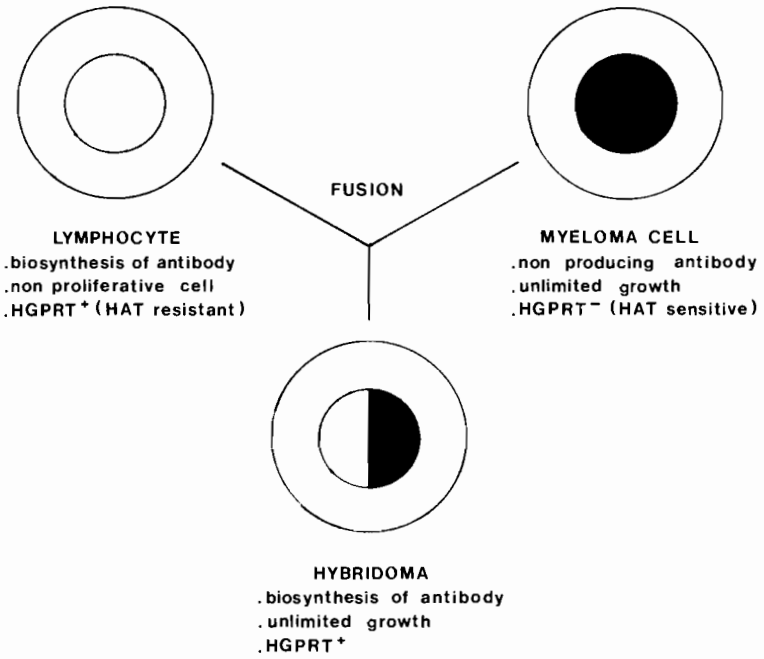

FIGURE 1.—Principle of preparation of hybridomas. HGPRT = hypoxanthine guanine phosphoribosyltransferase, HAT = hypoxanthine aminopterin thymidine.

Cloning of Hybridomas Producing Specific Antibody

As soon as positive hybridomas (i.e., those producing specific antibodies) are identified (Figure 2), they must be cloned to reduce the risk of overgrowth by negative cells (i.e., those producing nonspecific antibody). The cells can be cloned in soft agar, with an electronic cell sorter, or by limiting dilutions. The last method is used most frequently; cells are successively diluted to a point at which, statistically, there is less than one viable cell per microculture well. The capacity of the cloned cells to produce specific antibody is then assayed.

Production of Monoclonal Antibodies and Cryopreservation

Because the hybridomas are descended from a line of tumor cells, they can be grown indefinitely in culture and they can produce monoclonal antibodies in vitro or in vivo. Usually, the cloned hybridomas are grown in the ascites fluid of pristane-pretreated mice: On average, 3 mL of fluid or 3–30 mg of antibody per mouse are obtained. Monoclonal antibodies are purified by affinity chromatography on immobilized protein A, which selects immunoglobulins according to isotype. Cryopreservation of hybridomas is an essential safeguard against loss of valuable hybridoma lines. Cells that are cryoprotected with dimethyl sulfoxide (7.5%) in fetal calf serum can be stored in liquid nitrogen.

Comparison of Polyclonal and Monoclonal Antibodies

Polyclonal antisera obtained from immunized animals are characterized by a heterogeneity of antigen-specific immunoglobulins, a low titer of these specific antibodies, and variability between serum batches (Figure 3). Nevertheless, the use of highly purified antigens for immunization and in immune adsorption techniques has led to the development of specific and sensitive test systems, for example, indirect immunofluorescence (Boulo et al., in press). Polyclonal antibodies give rise to difficult problems in the development of quantitative and reproducible immunodiagnostic methods.

Because of their specificity, unlimited availability, and homogeneity (Figure 3), monoclonal antibodies can be standardized for use in highly sensitive and specific immunoassays, especially for detecting small amounts of infectious agents in clinical specimens. Also, they may be valuable in detecting antigenic variation between different stages or strains of parasites, which would be a more difficult procedure with polyclonal antibodies.

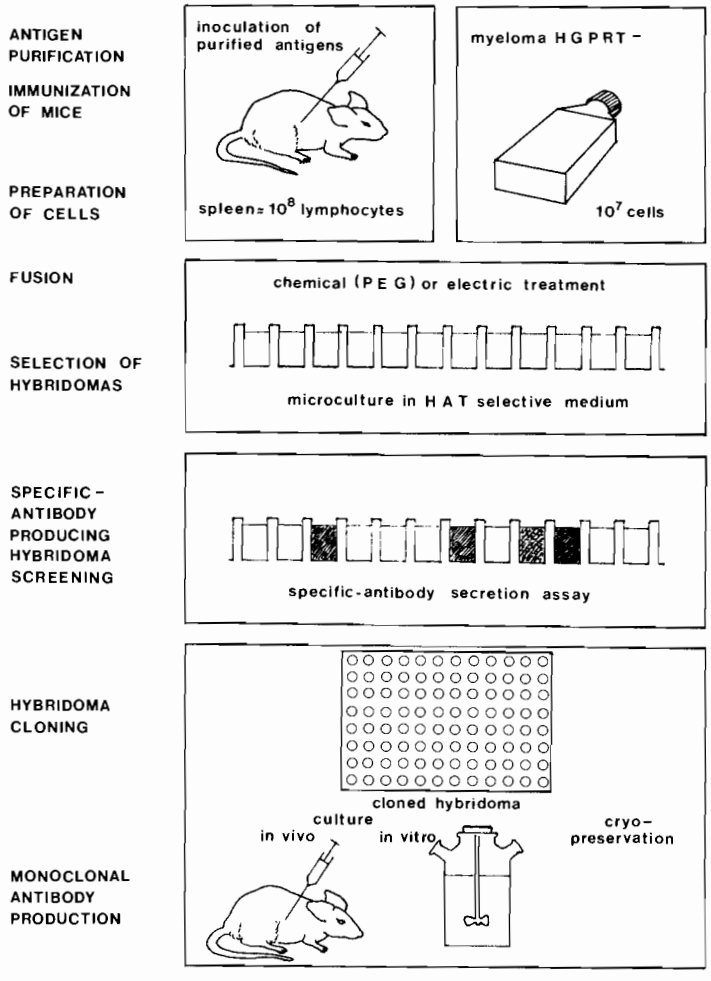

FIGURE 2.—Steps in hybridoma technology. HGPRT = hypoxanthine guanine phosphoribosyltransferase; PEG = polyethylene glycol; HAT = hypoxanthine aminopterin thymidine.

Monoclonal Antibodies for Diagnosis of Molluscan Pathogens

Heretofore, infectious diseases of molluscs have been diagnosed from histological preparations. Although parasites and procaryotes can be detected and identified by this technique, the method has several limitations and disadvantages, especially in epidemiological surveys. The preparation and observation of specimens are time-consuming. The availability of personnel and material is probably insufficient for useful disease prophylaxis. Moreover, it is difficult to precisely quantify infections by this procedure, and the method cannot be used to diagnose viral infections of molluscs (Johnson 1984; Elston and Wilkinson 1985).

Alternative methods used in human and veterinary pathology include immunoassays based on specific antigen–antibody reactions. Monoclonal antibodies are especially suitable for detecting antigens in epidemiological studies of parasitic (Ungar et al. 1985; Wirtz et al. 1985), procaryotic (Holley et al. 1984; Kotani and McGarrity 1985; Morris and Ivanyi 1985), or viral (Beards et al. 1984; Monath et al. 1986) diseases.

Because invertebrates have no demonstrable humoral immune response, immunodiagnosis depends on the utilization of specific antibodies to reveal the presence of infectious agents in them. Among molluscs, the barrier to the development of such technology has been the inability to prepare purified parasite suspensions to immunize

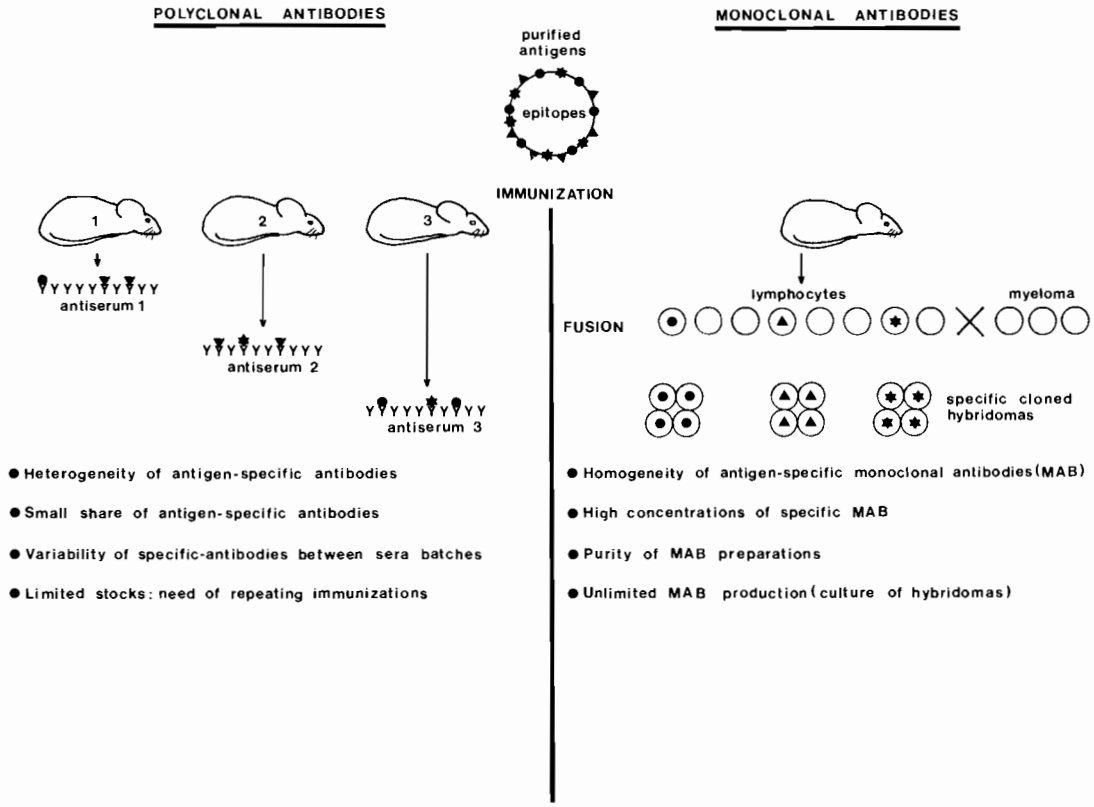

FIGURE 3.—Comparison of properties of polyclonal and monoclonal antibodies. Solid star, circle, or triangle = antigenic determinant; star, circle, or triangle + Y = immunoglobulin specific for antigenic determinant; circled star, circle, or triangle = lymphocyte secreting immunoglobulin specific for antigenic determinant.

mice and to assay hybridomas. However, the recent purification of two pathogens of oysters (Mialhe et al. 1985; Mialhe et al. 1988a) has overcome this barrier.

Pathogens can be detected by three types of immunoassays, direct, indirect, and direct sandwich (Figure 4). The different solid-phase immunoassay systems, IF, RIA, and ELISA, depend on the labeling element that is conjugated to the antibody. Immunofluorescence is mainly a qualitative and sensitive technique adapted for analyzing a few specimens (Nairn 1976), whereas RIA and ELISA are suitable for detecting and quantifying pathogens in many specimens. However, radioactive isotopes with short half-life radiation hazards limit the use of RIA to specially authorized laboratories. The sensitivity of ELISA (Voller et al. 1976; Voller et al. 1978; Yolken 1982) relies on the conversion of many substrate molecules by a single molecule of enzyme–antibody conjugate. These conjugates are stable and can be stored frozen. Microtiter-plate colorimeters are available to rapidly measure many samples. Substrates that give rise to colored and precipitated reaction products (Dao 1985; Turner 1986) are well adapted for simple assay without any instruments. Consequently, ELISA, especially the direct type (Figure 4), constitutes a good immunodiagnostic method for the quick, easy, and precise determination of the percentage and rate of infection of diseases in molluscs.

Monoclonal Antibodies against *Bonamia ostreae*

In several European countries, the breeding of the edible oyster *Ostrea edulis* is adversely affected by the intrahemocytic parasite, *Bonamia ostrea* (Ascetospora). In consideration of the limits of the histological diagnostic method and the recent purification of this pathogen (Mialhe et al. 1988a), it became important to produce monoclonal antibodies for immunodiagnosis. Some brief

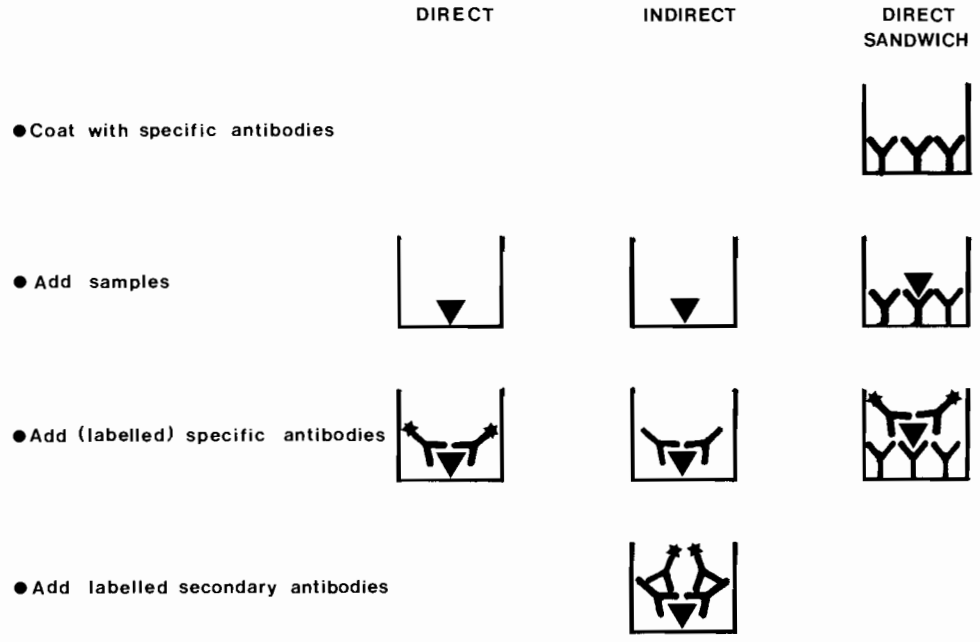

FIGURE 4.—Different types of solid phase immunoassays. Y = immunoglobulin; triangle = antigenic determinant; triangle + Y = immunoglobulin specific for antigenic determinant; Y + star = immunoglobulin labeled with an enzyme or radioisotope.

results are presented below to illustrate the applicability of monoclonal antibody technology to molluscan pathology. The details of the research will soon appear in print (Boulo et al., in press; Rogier et al., in press).

About 700 hybridomas were obtained from a fusion of lymphocytes of a hyperimmunized Balb/c mouse with myeloma cells. Eight hybridomas were then selected from these on the basis of their production of antibody specific for *B. ostreae*. The epitope specificity of these eight hybridomas was defined precisely by an inhibition RIA test and by IF antibody pattern analysis. Monoclonal antibodies 20B2 and 15C2, specific for cytoplasmic membrane epitopes, were retained for diagnosing *B. ostreae* by indirect IF in three clinical studies. The results were related to those diagnosed histologically. In light infections, when only a few *Bonamia* cells were observed on a smear, the IF test with MAB permitted quicker detection of *B. ostreae* because it could be observed at lower magnification.

The two radiolabeled monoclonal antibodies differentiate between hemolymph of diseased oysters from that of healthy oysters (Figure 5). On this basis, we are now developing an enzymatic immunoassay for detecting *Bonamia* disease.

Discussion

The economic impact of infectious diseases on bivalve culture and the absence of both resistant races and antiparasitic treatment indicate how necessary preventive measures are to insure the continuity of bivalve culture. Such measures depend mainly on the development of quantitative

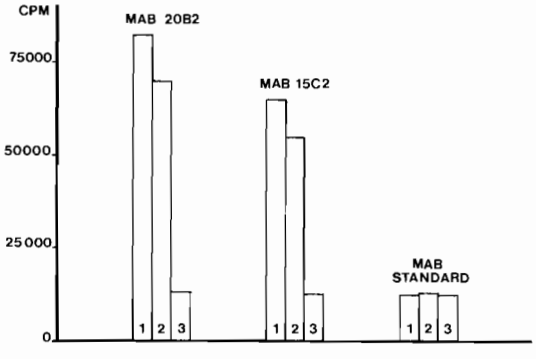

FIGURE 5.—Specificity analysis of monoclonal antibodies specific to *Bonamia ostreae* by direct radioimmunoassay. CPM = counts/min; MAB = monoclonal antibody. Bar 1 = purified *B. ostraeae* cells immobilized on nitrocellulose; bar 2 = parasitized oyster hemolymph immobilized on nitrocellulose; bar 3 = healthy oyster hemolymph immobilized on nitrocellulose.

immunodiagnostic methods such as ELISA, which is quick, reliable, and applicable by all investigators concerned with a specific disease. Monoclonal antibody methods provide a desirable alternative to polyclonal immunosera techniques. Our first results with *B. ostreae* suggest opportunities for other investigations into monoclonal antibodies against the major identified pathogens of economically important bivalves.

The value of monoclonal antibodies for fundamental research in molluscan pathology must also be noted. Monoclonal antibodies constitute new and powerful tools with which to study the antigenic variation of parasites during development or from different geographical areas (Mialhe et al. 1988b). Also, such antibodies are suitable reagents for discerning the role of receptors implicated in the recognition process between host cell and parasite. Finally, collaboration between molluscan pathologists and specialists in hybridoma technology is essential to quickly elaborate prophylactic strategies on an international scale.

References

Beards, G. M., and eight coauthors. 1984. Enzyme-linked immunosorbent assays based on polyclonal and monoclonal antibodies for rotavirus detection. Journal of Clinical Microbiology 19:248–254.

Blythman, H. E., and six coauthors. 1981. Immunotoxins: hybrid molecules of monoclonal antibodies and toxin subunits specifically kill tumour cells. Nature (London) 290:145–146.

Boulo, V., H. Rogier, F. Paolucci, E. Mialhe, and H. Grizel. In press. Immunodiagnostic of *Bonamia ostreae* (Ascetospora) parasite of *Ostrea edulis* and subcellular identification of epitopes by monoclonal antibodies. Journal of Fish Diseases.

Dao, M. L. 1985. An improved method of antigen detection on nitrocellulose. In situ staining of alkaline phosphatase conjugated antibody. Journal of Immunological Methods 82:225–231.

Elston, R. A., and M. T. Wilkinson. 1985. Pathology, management and diagnosis of oyster velar virus disease (OVVD). Aquaculture 48:189–210.

Frankel, A. E. 1985. Antibody–toxin hybrids: a clinical review of their use. Journal of Biological Response Modifiers 4:437–446.

Goding, J. W. 1983. Monoclonal antibodies: principles and practice. Academic Press, New York.

Holley, D. L., S. D. Allen, and B. B. Barnett. 1984. Enzyme-linked immunosorbent assay, using monoclonal antibody, to detect enterotoxic *Escherichia coli* K99 antigen in feces of dairy calves. American Journal of Veterinary Research 45:2613–2616.

Johnson, P. T. 1984. Viral diseases of marine invertebrates. Helgoländer Meeresuntersuchungen 37: 65–98.

Kennett, R. H., T. J. McKearn, and K. B. Bechtol. 1980. Monoclonal antibodies. Hybridomas: a new dimension in biological analysis. Plenum, New York.

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibodies of predefined specificity. Nature (London) 256:495–497.

Kotani, H., and G. J. McGarrity. 1985. Rapid and simple identification of mycoplasmas by immunobinding. Journal of Immunological Methods 85: 257–267.

Krakauer, H. 1985. Clinical applications of monoclonal antibodies. European Journal of Clinical Microbiology 4:1–9.

Mialhe, E., E. Bachère, D. Chagot, and H. Grizel. 1988a. Isolation and purification of the protozoan *Bonamia ostreae* (Pichot and coll. 1979), a parasite affecting the flat oyster *Ostrea edulis* L. Aquaculture 71:293–299.

Mialhe, E., E. Bachère, C. Le Bec, and H. Grizel. 1985. Isolement et purification de *Marteilia* (Protozoa: Ascetospora) parasites des bivalves marins. Comptes Rendus de l'Académie des Sciences, Série III, Sciences de la Vie 301:137–142.

Mialhe, E., and seven coauthors. 1988b. Serological analysis of *Bonamia* in *Ostrea edulis* and *Tiostrea lutaria* using polyclonal and monoclonal antibodies. Aquatic Living Resources 1:1–3. (Gauthier Villars, Montrouge Cedex, France.)

Monath, T. P., and six coauthors. 1986. Sensitive and specific monoclonal immunoassay for detecting yellow fever virus in laboratory and clinical specimens. Journal of Clinical Microbiology 23: 129–134.

Morris, J. A., and J. Ivanyi. 1985. Immunoassays of field isolates of *Mycobacterium bovis* and other mycobacteria by use of monoclonal antibodies. Journal of Medical Microbiology 19:367–373.

Nairn, R. C. 1976. Fluorescent protein tracing. Churchill-Livingstone, London.

Paolucci, F., B. Basuyaux, C. Clavies, M. Piechaczyk, and B. Pau. 1986. Lymphocyte hybridization: basic principles and selection of monoclonal antibodies directed against hormones. Serono Symposia Publications from Raven Press 30:3–13.

Pau, B., P. Poncelet, and P. Gros. 1983. L'hybridation lymphocytaire: principe et potentiel d'application en biologie clinique. Revue Française des Laboratoires 115:5–16.

Rogier, H., and seven coauthors. In press. Monoclonal antibodies against *Bonamia ostreae* (Ascetospora) parasite of the flat oyster *Ostrea edulis*. Hybridoma.

Schonherr, O. T., and E. H. Houwink. 1984. Antibody engineering, a strategy for the development of monoclonal antibodies. Antonie van Leeuwenhoek Journal of Microbiology 50:597–623.

Seiler, F. R., and seven coauthors. 1985. Monoclonal antibodies: their chemistry, functions and possible uses. Angewandte Chemie International Edition in English 24:139–160.

Turner, B. M. 1986. Use of alkaline phosphatase-conjugated antibodies for detection of protein antigens on nitrocellulose filters. Methods in Enzymology 121: 848–855.

Ungar, B. L. P., R. H. Yolken, and T. C. Quinn. 1985.

Use of monoclonal antibody in an enzyme immunoassay for the detection of *Entamoeba histolytica* in fecal specimens. American Journal of Tropical Medicine and Hygiene 34:465–472.

Van Der Auwera, P. 1987. Rapid diagnosis of infectious diseases. Internal Clinic Products Review 1:12–27.

Vienken, J., and U. Zimmermann. 1985. An improved electrofusion technique for production of mouse hybridoma cells. FEBS (Federation of European Biochemical Societies) Letters 182:278–280.

Voller, A., A. Barlett, and D. E. Bidwell. 1976. Enzyme immunoassays for parasitic diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene 70:98–106.

Voller, A., A. Barlett, and D. E. Bidwell. 1978. Enzyme immunoassays with special reference to ELISA technique. Journal of Clinical Pathology (London) 31:507–520.

Wirtz, R. A., T. R. Burkot, R. G. Andre, R. Rosenberg, W. E. Collins, and D. R. Roberts. 1985. Identification of *Plasmodium vivax* sporozoites in mosquitoes using an enzyme-linked immunosorbent assay. American Journal of Tropical Medicine and Hygiene 34:1048–1054.

Yelton, D. E., and M. D. Scharff. 1981. Monoclonal antibodies: a powerful new tool in biology and medicine. Annual Review of Biochemistry 50: 657–680.

Yolken, R. H. 1982. Enzyme immunoassays for the detection of infectious antigens in body fluids. Current limitations and future prospects. Reviews of Infectious Diseases 4:35–68.