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Preparation of Monoclonal Antibodies to Xanthine Oxidase and Other Proteins of Bovine Milk-Fat-Globule Membrane

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Nine hybridomas secreting monoclonal antibody to proteins of bovine milk-fat-globule membrane were isolated. All nine cell lines continued to secrete monoclonal antibody after serial transfer in culture and after passage as solid tumours in Balb/cJ mice. Four of the cell lines secreted monoclonal antibody specific for xanthine oxidase, one of the major proteins of milk-fat-globule membrane.

In the mammary gland, large quantities of membrane-associated protein are discharged from the apical surface of secretory cells into milk in the form of fat-globule membrane (Anderson & Cawston, 1975; Patton & Keenan, 1975). Recent evidence suggests that some of these membrane proteins possess antigenic determinants unique to mammary secretory cells (Ceriani *et al.*, 1977, 1978). As a consequence, specific antibodies to these proteins are potentially valuable markers of secretory cells and both the plasma membrane and endomembranes of these cells. Some of the proteins of fat-globule membrane are also expressed on the surface of mammary-tumour-cell lines, which raises the possibility of isolating tumour-specific antibodies by using fat-globule-membrane proteins as immunogens (Ceriani *et al.*, 1977, 1978).

Antisera prepared by conventional methods, however, often lack specificity, and non-specific antibodies have to be removed by adsorption on to appropriate immobilized antigens. Since the work of Köhler & Milstein (1975, 1976) it is now possible to isolate antibodies specific for single antigenic determinants on proteins by fusing mouse myeloma cells with spleen cells from immunized donors. The resulting cloned hybrids can then be grown as solid tumours in mice and monoclonal antibody isolated from the serum.

The present paper describes the isolation of monoclonal antibody to fat-globule-membrane proteins and discusses the practical importance of such antibody for both membrane and tumour biology.

Materials and Methods

Materials

Reagents for electrophoresis and electrofocusing were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.), and Ultrodex was from LKB Instruments, (Rockville, MD, U.S.A.). Xanthine was from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and all culture medium was purchased from Gibco (Grand Island, NY, U.S.A.).

Balb/cJ mice (8–12 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). The non-secreting azaguanine-resistant hybridoma SP-2/0 derived by Shulman *et al.* (1978) was used as the established cell parent in the construction of the hybrids reported in the present paper.

Methods

Immunization of mice. Fat-globule-membrane was prepared from bovine milk as described by Mather (1978). Female Balb/cJ mice (12 weeks old) were primed by intraperitoneal injection of 2mg of membrane protein, that had been emulsified in Freund's complete adjuvant. Subsequent intraperitoneal injections of 200μg of membrane protein were given 30 and 60 days later. Spleens were removed from three immunized mice 5 days after the last injection.

Hybridization and culturing techniques, and collection of antisera. Spleen cells (6×10^8) from immunized mice were fused with SP-2/0 cells (6×10^7) essentially as described by Goldsby *et al.* (1977). After fusion the cells were diluted in the HAT (hypoxanthine/aminopterin/thymidine) medium of Littlefield (1964) and distributed in 0.1ml aliquots to the wells of 96-well microtitre plates with the aid of a mechanical cloning and sampling apparatus of the type described by Goldsby & Mandell (1973). The first hybrid clones appeared 12 days after fusion. Selected hybrid clones elaborating antibody to fat-globule mem-

Abbreviations used: IgA(etc.), immunoglobulin A(etc.); SDS, sodium dodecyl sulphate.

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brane were re-cloned by limiting dilution as outlined by Goldsby & Zipser (1969).

Antibody-secreting tumours were produced by subcutaneous inoculation of 3–5-month-old Balb/cJ mice with approx. 5×10^6 hybrid cells. Animals were tail-bled when tumours became palpable and exsanguinated between 3–5 days later. The tumours were removed, minced, a portion passaged in 3–5-month-old Balb/cJ recipients, and the remainder frozen in liquid N_2.

Radioimmunoassay. Antibody to fat-globule membrane and purified xanthine oxidase was detected by a solid-phase radioimmunoassay based on the procedure of Rosenthal *et al.* (1973). An ^{125}I-labelled rabbit antibody to mouse immunoglobulins (IgA, IgG_1, IgG_{2a}, IgG_{2b}, and IgM) that had been purified by adsorption to a mouse immunoglobulin affinity column was used as a detecting agent.

Preparation of xanthine oxidase. Xanthine oxidase was purified by extracting washed fat-globules with butan-1-ol as described by Waud *et al.* (1975) and separating the solubilized extract by preparative electrofocusing in flat beds of Ultrodex (I. H. Mather, D. S. Armstrong & M. G. Irving, unpublished work). The active fractions, in 50mM-K_2HPO_4 buffer (adjusted to pH 7.8 with NaOH), were then chromatographed on concanavalin A immobilized by Sepharose 4B. Xanthine oxidase, which does not bind to concanavalin A was eluted in the void volume, and the active fractions were pooled and concentrated. The final preparation had a specific activity of 0.9 μmol of xanthine hydrolysed/min per mg of protein and an A_{280}/A_{450} ratio of 5.02.

The purified enzyme separated as a single major component during electrophoresis in SDS/polyacrylamide gels (Laemmli, 1970), and was estimated to be at least 95% pure by densitometric procedures.

Assays. Xanthine oxidase was assayed spectrophotometrically at 293 nm by measuring the formation of uric acid from xanthine. Samples were incubated in 50mM-Tris/HCl, pH 7.5, containing 0.1 mM-xanthine at 37°C.

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

The fusion of spleen cells from immunized Balb/cJ mice with SP-2/0 cells led to the isolation of 215 cloned hybrids. Of these hybrids, 73 clones secreted antibody that bound to unfractionated

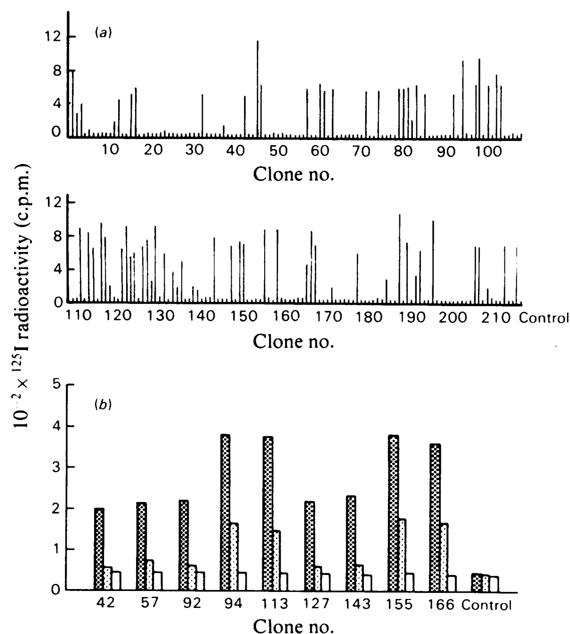

Fig. 1. *Radioimmunoassay of supernatants*
(*a*) Culture supernatants from cloned hybridomas were tested for binding to unfractionated bovine fat-globule membrane. Cell lines that secreted antibody specific for fat-globule-membrane antigens (^{125}I radioactivity at least 3-fold greater than control values) were grown in culture and subsequently re-cloned. (*b*) Supernatants from re-cloned hybridomas were tested for binding to unfractionated fat-globule membrane (▩), xanthine oxidase (▨), or uncoated plates (□).

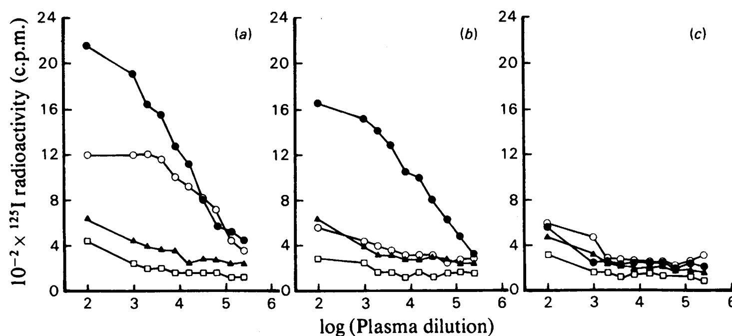

Fig. 2. *Immunotitration of plasma from mice carrying antibody-secreting tumours*
Numbering of cell lines corresponds to original clones as described in Fig. 1(*b*): (*a*) cell line 166; (*b*) cell line 42; (*c*) control plasma from tumour-free mice. Plasma was diluted down to 1:256000 with phosphate-buffered saline [1.47mM-KH_2PO_4/8mM-Na_2HPO_4,$7H_2O$, pH 7.2, containing 2.68mM-KCl, 137mM-NaCl and bovine serum albumin (0.1%)] and tested for binding to unfractionated bovine fat-globule membrane (●), bovine xanthine oxidase (○), rabbit erythrocyte 'ghosts' (▲) and uncoated plates (□).

fat-globule membrane (Fig. 1*a*). Of these positive clones, 47 were re-tested for the production of antibody to unfractionated fat-globule membrane and additionally tested against purified xanthine oxidase. Of the twelve clones retained, nine were still positive for monoclonal-antibody production after re-cloning and growth in culture (Fig. 1*b*). Clones 94, 113, 155 and 166 secreted antibody that bound to purified xanthine oxidase.

All nine hybrids were successfully transplanted as solid tumours in Balb/cJ mice. Fig. 2 compares the results of titrating the plasma of mice carrying clones 166 and 42 with control plasma obtained from tumour-free mice. Monoclonal antibody could be detected in the plasma from tumour-bearing mice down to a dilution of 1:256000. Plasma containing monoclonal antibody had only a slightly greater tendency to bind to an unrelated antigen (rabbit erythrocyte 'ghosts') than did plasma from control mice. Results were essentially similar with all nine cell lines, and the specificity for xanthine oxidase was retained by four hybrids as in Fig. 1(*b*).

Discussion

Antibodies to proteins of mouse, bovine and human milk-fat-globule membrane have been prepared previously in several species by using conventional procedures (e.g. Ceriani *et al.*, 1977, 1978; Kanno & Yamauchi, 1978; Nielsen & Bjerrum, 1977; Freudenstein *et al.*, 1979). Such antibody has been used for a number of purposes, including the analysis and comparison of fat-globule-membrane proteins by immunological procedures (Nielsen & Bjerrum, 1977; Freudenstein *et al.*, 1979), the identification of mammary secretory cells and certain mammary-tumour-cell lines by immunofluorescence microscopy (Ceriani *et al.*, 1977, 1978), and determination of the distribution of membrane antigens in mammary tissue (Freudenstein *et al.*, 1979). It has also been suggested that specific antibodies to fat-globule-membrane proteins may be used to monitor the spread of malignant tumours and act as carriers of cytotoxic agents to the surfaces of tumour cells in breast-cancer patients (Ceriani *et al.*, 1977).

The demonstration that hybridomas can be isolated that secrete monoclonal antibody to proteins of fat-globule membrane is therefore of general significance, because the success of the above procedures is largely dependent on the preparation of antibody of rigorously defined specificity. The methods we have used permit the use of unfractionated fat-globule membrane as an immunogen for preparing highly specific antibody to individual membrane proteins. Since fat-globule membrane can be easily isolated from the milk of many species, it will therefore be possible to prepare specific antibody to a number of physiologically important proteins without initially having to purify the individual antigens.

The demonstration that four of the nine hybridomas isolated secrete antibody specific for xanthine oxidase is specially noteworthy. This protein is present in large quantities in milk and has been purified by several procedures, some of which have involved the use of proteinases (e.g. Gilbert & Bergel, 1964). The solubilization of xanthine oxidase by proteolysis has led to erroneous estimations of its molecular weight, and there has therefore been some controversy about the size of the native form of this enzyme (Waud *et al.*, 1975; Mangino & Brunner, 1977).

There is also little known about the function of xanthine oxidase in mammary tissue. Recent work suggests that the enzyme forms part of the inner-

coat material associated with fat-globule membrane (Mather & Keenan, 1975; Nielsen & Bjerrum, 1977; Freudenstein *et al.*, 1979), and may catalyse the formation of superoxide at the cell surface (Löw & Crane, 1978).

The preparation of monoclonal antibodies to xanthine oxidase will provide new probes for estimating the size and determining the function of this enzyme in mammary tissue. Thus chromatography of solubilized xanthine oxidase from mammary tissue or milk by using monoclonal antibody immobilized on Sepharose should allow the preparation of the enzyme in its undegraded form. By using radioimmunoassays and immunocytochemical procedures it should also be possible to determine the distribution of xanthine oxidase in milk and in mammary tissue. Knowledge of the location of xanthine oxidase in mammary secretory cells may well prove to be the key to understanding the function of this enzyme at the cellular level.

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