esophagus, which is fused with the posterior wall of the trachea, must be separated. Next, having fixed the trachea with a dissecting meedle, the thyroid gland, whose capsule is firmly fused with the larynx and trachea, is due away from them.

PRODUCTION OF MONOCLONAL ANTIBODIES TO HORSERADISH PEROXIDASE AND THEIR USE IN IMMUNOHISTOCHEMISTRY AND IMMUNOBLOTTING

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The unlabeled antibodies method, or peroxidase-antiperoxidase method (the PAP test) [10] is widely used in immunohistochemistry, because of its high sensitivity and the low level of nonspecific (background) staining. The introduction of monoclonal mouse and rat antibodies into immunohistochemical practice has necessitated the obtaining of large quantities of mouse or rat antibodies to horseradish peroxidase (HRP) in order to prepare reagents for the PAP test. Unlimited quantities of standard antibodies to HRP can be obtained by hybridoma technology [5, 9].

The aim of this investigation was to obtain monoclonal antibodies (MCAB) to HRP, suitable for use in different kinds of immunoenzyme assays.

To obtain hybridomas, BALB/c mice were immunized by intraperitoneal injection of HRP (type VI, from Sigma, USA); at the first immunization the antigen was injected in Freund's complete adjuvant, but during subsequent immunizations, in incomplete adjuvant (Gibco, USA). The mice were stimulated 4 days before fusion of the immune spleen cells with myeloma cells, by intravenous injection of HRP. Fusion was carried out with the aid of polyethylene-glycol 1500 (Merck, West Germany) by the method described in [1]. After hybridization the cells were transferred to 96-well plates (Linbro, England) in medium RPMI-1640. The cells were grown at 37°C in an atmosphere with 5% CO_2. The presence of antibodies to HRMP in the culture fluids (CF) was determined by solid-phase radioimmunoassay (RIA) [9]. Positive CF were tested for suitability for use in the PAP test with the aid of monoclonal antibody L9 [3, 9].

For immunohistochemical staining cryostat sections of biopsy specimens of human breast, frozen in liquid nitrogen, were fixed with 4% formalin in buffered physiological saline (BPS) for 5 min at room temperature, washed with PBS, and then incubated with CF from a hydridoma producing MCAB PK S-12 to prekeratins [4], rabbit antiserum against mouse immunoglobulins (RAM; 1:50), and monoclonal PAP reagent, obtained by addition of HRP (Reanal, Hungary), up to a final concentration of 50 μg/ml, to CF of hybridoma clone AP-FC-2B4, producing MCAB to HRP.

Electrophoresis of the microsomal fraction of rat liver cells was carried out in a polyacrylamide gel gradient (6-9%) in the presence of sodium dodecylsulfate by the method in [7]. The separated proteins were transferred to a nitroclelulose membrane [12] and stained with MCAB to cytochrome P-450 [2], RAM (1:100), and monoclonal PAP complex.

Peroxidase activity was revealed in all cases by the diaminobenzidine test [6].

After hybridoma fusion, 106 primary hybridoma cultures were tested by RIA for production of antibodies binding with HRP. Of this number, 16 were positive. However, testing by immunocytochemical staining showed that only four cultures produced antibodies suitable for obtaining an active PAP reagent. The very first testing made it clear that the most intense staining could be obtained with MCAB of the clone designated AP-FC-2B4. This result also was confirmed on testing FC from subclones for all four cultures, and accordingly MCAB of clone

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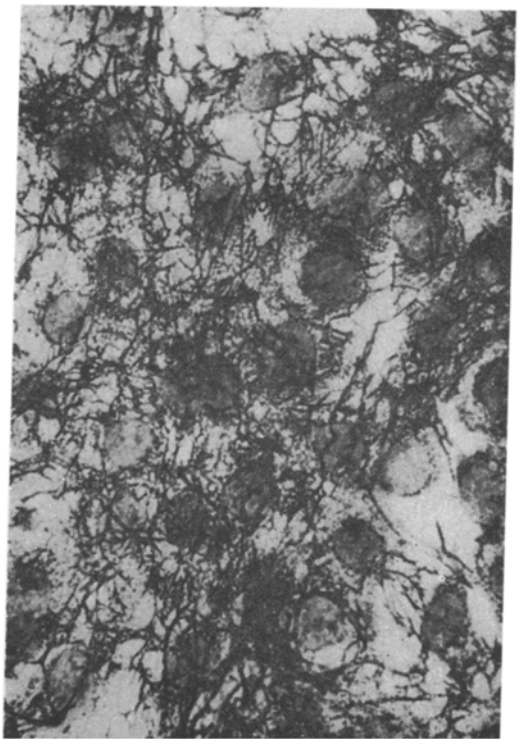

Fig. 1

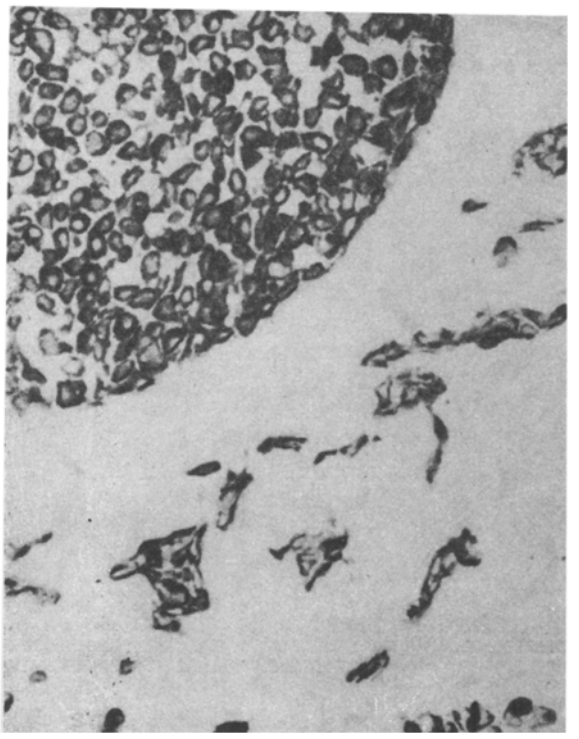

Fig. 2

Fig. 1. Immunocytochemical staining of fibronectin in culture of embryonic fibroblasts by the PAP method. Nuclei stained with gentian violet. 450 ×.

Fig. 2. Infiltrating lobular carcinoma of the human breast. Cryostat section. Fixation with 10% formalin. Staining with MCAB KS S-12 by the PAP method. 200 ×.

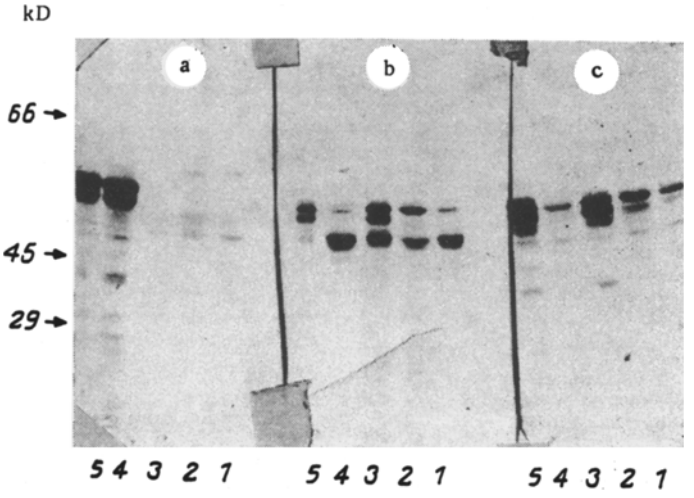

Fig. 3. Immunoblotting of microsomal fractions of rat liver after injection of various inducers. 1) Intact rat; 2) pregnenalone-16-α-carbonitrile; 3) phenobarbital; 4) 3-methylcholanthrene; 5) Araclor 1254. Stained by the PAP method using MCAB to cytochrome P-450 11E4 (a), 3FE (b), and 6A7 (c).

PA-FC-2Ba (IgG1) were used to obtain the PAP reagent. Testing hybridoma CF by immunohistochemical staining, incidentally, is an essential stage in the selection of MCAB suitable for use in the PAP method, and it is usual in this case to stain sections [5, 9], which is not very convenient if a large number of CF have to be tested after cloning. Immunocytochemical

staining of cultures grown in 96-well plates enables a large number of CF to be tested quickly, and the characteristic staining of the fibronectin fibrils (Fig. 1) makes the results of the test very easy to read.

The monoclonal PAP reagent can be used for histophathological diagnosis with the aid of MCAB. For instance, staining sections of a breast with an infiltrating lobular carcinoma with MCAB PK S-12 (Fig. 2) both the proliferating lobular growth and small groups and chains of tumor cells and also single tumor cells, infiltrating the connective-tissue stroma, were clearly seen.

Data illustrating how the PAP reagent can be used to detect antigens in the immunoblotting test are given in Fig. 3. A set of MCAB against isoforms of cytochrome P-450, arising in liver microsomes after injection of various inducers *in vivo* [2], was used as the model. By the PAP method it is possible to detect qualitative and quantitative differences in the expression of P-450 isoforms. For instance, the P-450a isoform is found in the liver of intact rats by MCAB 2F2, but not by MCAB 11E4 and 6A7 (Fig. 3). Meanwhile MCAB 11E4 react with two isoforms: P-450c (with a higher molecular weight) and P-450d (with a lower molecular weight), induced only by 3-methylcholanthrene and Araclor 1254, but not by other inducers. Parallel with the PAP method, staining by an indirect free-stage method using RAM and conjugates of goat antibodies to rabbit immunoglobulins with HRP (Sigma, USA) was used, and in this case background staining of proteins adsorbed on the nitrocellulose was rather stronger in degree than when the PAP method was used.

The hybridoma clone AP FC-2B4 which we obtained thus produces MCAB suitable for use in obtaining a PAP reagent, which can be used in immunohistochemistry and immunoblotting. The use of MCAB to HRP in solid-phase immunoenzyme assay for quantitative determination of antibodies [11] and for testing hybridoma clones during production of MCAB [8] has also been described in the literature.

The ease of obtaining a monoclonal PAP complex, the fact that chemical binding of the antibodies to the enzyme is unnecessary, and the possibility of using peroxidase preparations with a low level of purity, indicate that there are wide prospects ahead of the use of the MCAB produced in this manner in different fields of biology and medicine, in which methods of immunoenzyme assay are applicable.

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