

Purification of Mouse Alpha₁-Fetoprotein and Preparation of Specific Peroxidase Conjugates for Its Cellular Localization

W. D. KUHLMANN *

Immunocytochemistry SFB 136 and Institut für Nuklearmedizin, DKFZ Heidelberg, Germany

Histochemistry 44, 155-167, 1975

Summary

Mouse alpha-1-fetoprotein (AFP) was isolated from amniotic fluid by immunoabsorbent columns and preparative electrophoresis. Specific antibodies were isolated from monospecific hyperimmunera by use of immunoabsorbents, and subsequently coupled with horseradish peroxidase. At the light microscopic level, purified antibody-peroxidase conjugates were used for the cellular localization of AFP in fetal liver by direct and indirect staining methods. Fixatives containing ethanol or aldehydes were tried for antigen staining. Prior to immunocytological reactions, endogenous peroxidases were inhibited by hydrogen peroxide.

Introduction

Synthesis of alpha₁-fetoprotein (AFP) occurs in embryonic liver, the yolk sac and the gastrointestinal tract, and this protein is a normal constituent in fetal serum and amniotic fluid of many species (Pedersen, 1944; Bergstrand and Czar, 1956; Gitlin and Boesman, 1967). Under physiological conditions, this protein disappears after birth. However, following hepatocellular damage after ingestion of hepatotoxins, fetal-type protein synthesis can be observed in regenerating adult liver (Bakirov, 1968; Nechaud and Uriel, 1971; Nechaud and Uriel, 1972; Pihko and Ruoslahti, 1974). Also, development of hepatocellular cancer as initiated by different agents is often associated with reversion to the embryonic-type antigens. Thus, AFP, a fetal protein can be observed during the process of malignant transformation (Abelev *et al.*, 1963; Stanislawski-Birencwajg *et al.*, 1967; Abelev, 1971; Watabe *et al.*, 1972; Kroes *et al.*, 1972).

Studies on cellular localization of AFP synthesis are necessary to understand its role in both physiological and cancerous conditions. Several attempts have been made to describe the morphological basis of AFP by use of fluorescent labeled antibodies (Gitlin *et al.*, 1967; Engelhardt *et al.*, 1972; Branch and Wild, 1972). However, the main drawback of immunofluorescence is the uncertainty in determining cytological details.

The purpose of the present paper is to make use of recent developments in peroxidase-labeling techniques for immunocytological studies (Kuhlmann, 1973; Kuhlmann *et al.*, 1974). Procedures for purification of AFP, production and isolation of specific antibodies and peroxidase conjugates

* Supported by grants of the Deutsche Forschungsgemeinschaft (SFB 136, publ. No. 3) Bad Godesberg, Germany

are described. Subsequent immunocytological staining of AFP is performed on mouse embryonic liver at the light microscopic level. A method is presented to inhibit endogenous peroxidases prior to immunocytochemical staining.

Materials and Methods

Animals. C3H inbred mice were used.

Anti-AFP Immunsera. One year old New Zealand rabbits were used for immunization with AFP. The first specific anti-AFP immunsera were prepared according to this schedule: (a) amniotic fluid from 14 days old embryos was subjected to preparative electrophoresis in 5% acrylamide-0.8% agarose plates (Uriel, 1966) and the AFP containing fraction in α_1 -region was determined by Ouchterlony's gel diffusion technique (Ouchterlony, 1953) utilizing cross-reactivity with an immunserum against rat AFP (Watabe, 1974) obtained from Nordic Immunological Laboratories (Netherlands); (b) rabbits were immunized in both hind foot pads with AFP (1 mg protein) which was emulsified with complete Freund's adjuvant. Then, animals were boosted three times at monthly intervals using 0.5 mg of antigen emulsified with incomplete Freund's adjuvant; (c) immunsera were absorbed successively with glutaraldehyde cross-linked normal adult C3H plasma (Avrameas and Ternynck, 1969) until no immunological reaction was seen with normal adult serum proteins in the Ouchterlony test.

These monospecific immunsera were used to prepare the first immuno-adsorbent column in the beginning of the experiments. Later on, specific anti-AFP immunsera were produced in rabbits using specifically isolated AFP (see purification of AFP) by the same immunization schedule. Booster injections at monthly intervals were made with 0.5 mg of AFP per animal, and 1 week after the fifth injection rabbits were bled.

Anti-normal C3H Plasma Immunsera. Rabbits were immunized with 20 mg protein of pooled normal adult C3H plasmas per injection and boosted according to the schedule described above.

Anti-rabbit IgG Immunsera. One year old sheep were immunized with 5 mg of rabbit IgG (Nordic Lab., Netherlands) in complete Freund's adjuvant and boosted once a month for half a year using 0.2 mg of antigen in incomplete adjuvant. One week after the last injection, blood was taken from the jugular vein.

Purification of AFP. Using immuno-adsorption techniques and preparative electrophoresis, α_1 -fetoprotein was purified from amniotic fluid by three steps.

In the first step, amniotic fluid was incubated with the immuno-adsorbent column prepared by coupling of γ -globulins of specific anti-AFP immunsera (obtained by DEAE ion exchange chromatography) to Sepharose 4B (Pharmacia, Sweden) according to published procedures (Cuatrecasas, 1970). After washing the immuno-adsorbent extensively with phosphate buffered saline (PBS) and 2 M NaCl in 0.1 M NaOH-glycine buffer pH 9 until $A_{280\text{nm}}$ was no longer measurable, the adsorbed AFP was eluted with 3 M NaSCN in 0.1 M phosphate buffer pH 7.4.

In the second step, the eluted AFP was incubated with another immuno-adsorbent column from coupled γ -globulins of anti-normal adult C3H plasma immunsera to Sepharose 4B, and

AFP was obtained in the effluent. Traces of normal plasma constituents were adsorbed to the column.

In the third step, this preparation was submitted to preparative electrophoresis in 0.8% agarose gel, and the α_1 -fraction containing AFP was eluted and further utilized.

Protein determinations were made with the Folin-phenol reagent (Lowry *et al.*, 1951) using bovine serum albumin as standard.

Isolation of Specific Antibodies. For immunocytochemical purposes, pure rabbit anti-AFP and pure sheep anti-rabbit IgG antibody molecules were isolated from whole immunera using immunoabsorbents of copolymers of bovine serum albumin and amniotic fluid or normal rabbit serum respectively according to published procedures (Avrameas and Ternynck, 1969).

Peroxidase-labeling of Antibodies. Isolated antibodies (IgG and IgM classes) were chromatographed on a Sephadex G 200 superfine column (95 x 2 cm), calibrated for molecular weight determination (Determann, 1969). Fractions containing IgG molecules of the purified antibodies were concentrated to 5 mg protein/ml and conjugated with horseradish peroxidase (HRP, RZ 3; Boehringer-Mannheim, Germany) using glutaraldehyde in a two-step procedure (Avrameas and Ternynck, 1971): the molar ratio of peroxidase to antibody was 10 to 1. First, 25 mg of peroxidase were dissolved in 0.5 ml of 0.1 M phosphate buffer pH 6.8, mixed with 0.5 ml of 2% aqueous glutaraldehyde at room temperature, and after 18 hours unreacted glutaraldehyde was removed by filtration on Sephadex G 25 fine. The "activated" HRP was concentrated to 1 ml. In the second step, this preparation was allowed to react with 5 mg of isolated antibody using 0.5 ml of 0.5 M carbonate buffer pH 9.5 for 24 hours at 4° C. Unreacted aldehyde groups were subsequently blocked by dialysis of conjugates against 0.1 M ethanolamine-HCl pH 7.4.

Finally, peroxidase-labeled antibodies were separated from uncoupled antibody and peroxidase on a Sephadex G 200 superfine column as described above.

Immunodiffusion Techniques. Antigens and antibodies purified or not were submitted to immunoelectrophoretic analyses according to Grabar and Williams (1953), using 1% agarose in Veronal-HCl buffer pH 8.6.

Tissue Fixation and Processing. Various fixatives were examined. Formaldehyde, freshly prepared from paraformaldehyde (Merck, Germany) and vacuum distilled glutaraldehyde (Fahimi and Drochmans, 1965) with a purification index of P.I.= 0.1 to 0.2 were used. In detail, fixation schedules were as follows: (a) absolute ethanol for 10-15 hours; (b) 96% ethanol containing 1 % acetic acid for 10 to 15 hours; (c) Bouin's and Carnoy's fixatives (Romeis, 1968) for 1, 4 and 10 hours; (d) 4% formaldehyde and 0.5% picric acid in 0.1 M cacodylate buffer pH 7.2 for 30 min, followed by 4% formaldehyde and 0.5% picric acid supplemented with 0.25% glutaraldehyde in cacodylate buffer for 30 min (Kuhlmann, 1975).

After aldehyde fixation, the tissues were washed at 4° C for at least 3 days with frequent changes of the buffer solutions. In some cases, blocks were treated for 8 days with 30% sucrose (Deng and Beutner, 1974).

Tissue blocks were dehydrated in ascending ethanol, cleared in benzene and embedded in paraplast (Sherwood Ind., USA). 5 to 7 μ thick sections were cut, mounted on acetone cleaned slides, deparaffinized in xylene and passed from absolute ethanol into PBS before immunocytochemical incubation.

Immunocytochemical Procedures. Prior to incubation in antibodies, endogenous peroxidases in tissue sections were blocked by incubation in (a) 1 % sodium nitroferricyanide in absolute methanol containing 0.2% acetic acid (Straus, 1972); (b) absolute methanol for 20 min, followed by 0.03% hydrogen peroxide in PBS for 20 min (Streefkerk, 1972); (c) 0.5, 1 or 2% H₂O₂ in PBS during 30, 45 or 60 min at room temperature. Then, the slides were washed in PBS and reacted following either direct or indirect (Sandwich) methods. In the direct method, sections were incubated with peroxidase-labeled anti-AFP antibodies for 20 min. In the indirect method, incubation was first in unlabeled anti-AFP antibodies, then in peroxidase-labeled anti-rabbit IgG antibodies. Not reacted antibodies were in both cases eliminated by 3 successive washings, 5 min each, in PBS.

Peroxidase activity was revealed by incubation in 3,3'-diaminobenzidine (Merck, Germany), 0.5 mg/ml in 0.2 M Tris-HCl buffer pH 7.2 containing 0.01% hydrogen peroxide (Graham and Karnovsky, 1966). After washings in cacodylate buffer, sections were dehydrated in ethanol and mounted in resin. In some cases, sections were osmicated for 5 to 10 min with 0.5% OsO₄ in cacodylate buffer, then dehydrated and mounted under coverglass.

Controls. Inactivation efficiency of endogenous peroxidases using the described procedures was controlled by subsequent incubation of treated sections in Graham and Karnovsky's medium (1966).

Specificity of the immunocytological reactions was examined on tissue sections incubated with HRP alone, with peroxidase conjugates of sheep anti-rabbit IgG antibodies and with normal sheep IgG labeled with HRP.

Results

AFP containing fraction, eluted from preparative acrylamide-agarose electrophoresis was used for preparation of the first anti-AFP immunoserum. After absorption of this immunoserum with glutaraldehyde cross-linked normal mouse plasma proteins, this serum was rendered monospecific and utilized for preparing the first immunoadsorbent column.

Purification of AFP. In the first purification step, amniotic fluid with a mean protein content of 3 mg/ml was passed through the anti-AFP column and the adsorbed AFP was eluted by NaSCN. Then, proteins other than AFP were adsorbed in a second cycle using an immunoadsorbent column from anti-normal mouse plasma proteins. AFP was obtained in the effluent.

Finally, a third purification step was run using preparative electrophoresis in 0.8% agarose in order to eliminate contaminating γ -globulins, which might occur from leakage of the immunoadsorbent column. From 15 ml (45 mg protein) of amniotic fluid we isolated a total amount of 5 to 6 mg AFP.

After immunization of rabbits with purified AFP, immunosera gave a single precipitation reaction with amniotic fluid in either Ouchterlony's double diffusion tests or in immunoelectrophoretic

analyses. No immunological reactivity was obtained with normal adult mouse plasma proteins (Fig. 1 a).

Isolation of Antibodies and Peroxidase-labeling. Specific antibody molecules of the IgG class from rabbit and sheep immunsera were obtained using the immunoabsorbent technique, followed by subsequent gel chromatography on Sephadex G 200 (Fig. 1 b). From 10 ml whole immunserum (protein content: 50-60 mg/ml) we could isolate 5-7 mg pure antibodies of the IgG class.

Covalent linkage between antibody and peroxidase molecules were obtained by the two-step procedure. Conjugates were purified from uncoupled material by gel chromatography on Sephadex G 200 superfine. The active antibody-peroxidase complexes were collected in the void volume. Immuno-electrophoretic analyses of these conjugates revealed both antibody and enzyme activities in the same test (Fig. 1 c-d).

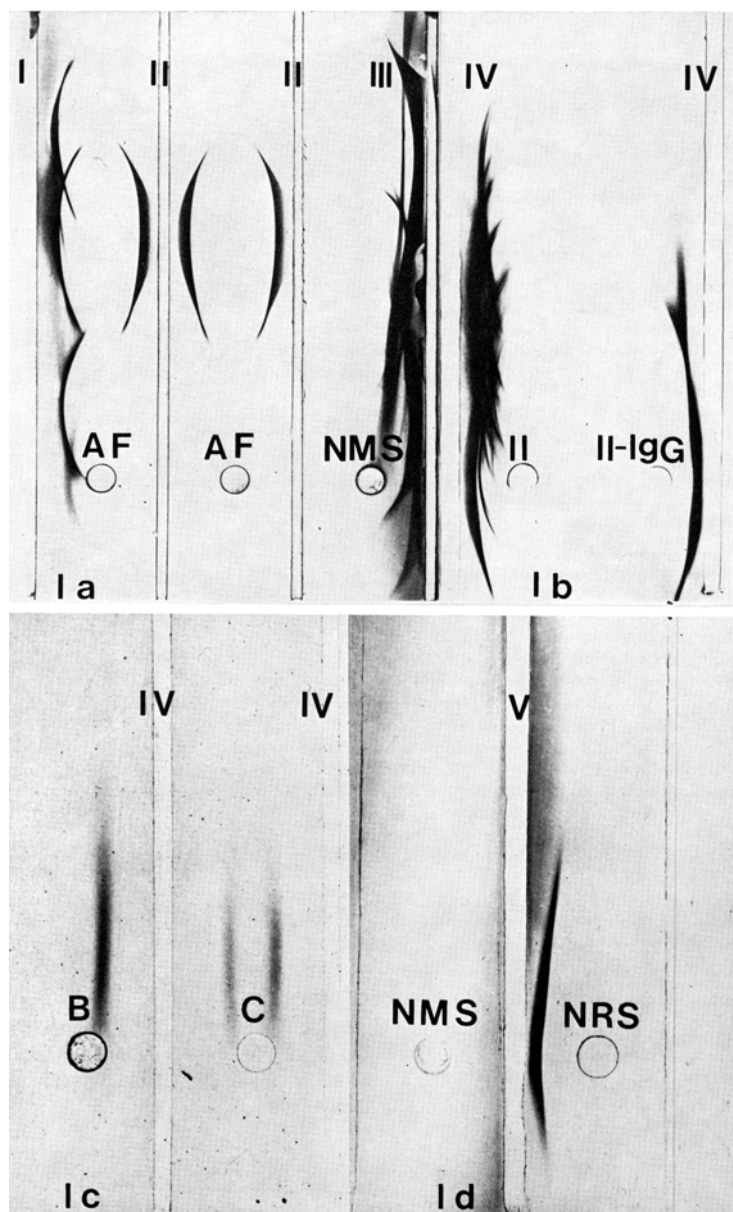


Fig. 1 a-d. Immuno-electrophoretic analyses. (a) Identification of AFP in amniotic fluid (AF); amido-black staining. NMS normal adult mouse serum; I rabbit anti-amniotic fluid; II rabbit anti-AFP; III rabbit anti-normal mouse serum. (b) immunoelectrophoretic pattern of whole rabbit anti-AFP immune serum (II) and its isolated antibodies (III/IgG) as revealed by goat anti-normal rabbit serum (IV); amido-black staining. (c) identification of peroxidase-labeled rabbit anti-AFP antibodies before (B) and after purification (C) of antibody-peroxidase complexes. Conjugates were revealed with goat anti-normal rabbit serum (IV); peroxidase staining. (d) detection of rabbit IgG in normal rabbit serum (NRS) as revealed by use of specific sheep anti-rabbit IgG antibodies coupled with peroxidase (V). No reaction with normal mouse serum (NMS); peroxidase staining.

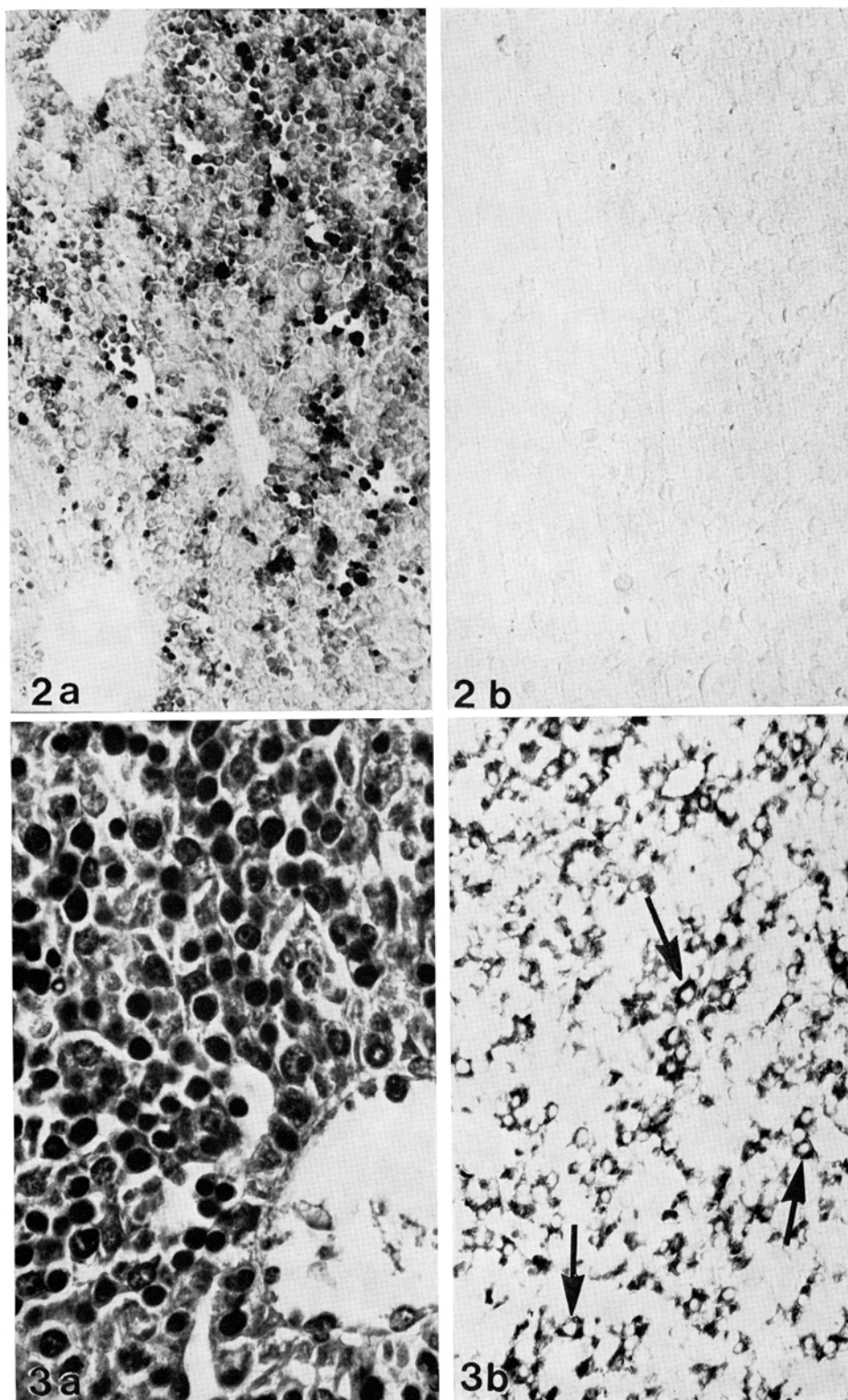


Fig. 2a and b. Control reaction in sections of fetal liver. (a) localization of endogenous peroxidases as revealed by Graham and Karnovsky's medium. Original x 250. (b) inhibition of endogenous peroxidases by hydrogen peroxide. Subsequently, section was incubated in sheep anti-rabbit IgG-peroxidase conjugate, then revealed with enzyme substrate; no staining reaction. Original x 250

Fig. 3a and b. Fetal liver preservation after fixation in ethanol-acetic acid mixture and localization of α_1 -fetoprotein by indirect antigen staining (a) H. E. staining; original x 540 (b) detection of AFP in hepatocytes (\leftarrow); original x 250

Immunocytochemical Staining. Endogenous peroxidase activity was completely inhibited by incubation of the sections in 0.5% hydrogen peroxide for 45 min (Fig. 2). Procedures using 1% sodium nitroferricyanide in absolute methanol containing 0.2% acetic acid or absolute methanol for 20 min followed by 0.03% H₂O₂ in PBS for 20 min did not result in complete inhibition of peroxidases in erythrocyte precursor cells.

After inhibition of endogenous peroxidases, specificity of the immunocytological reactions was observed, and tissue sections which were treated with either HRP alone or peroxidase conjugates of sheep anti-rabbit IgG antibodies or normal sheep IgG did not stain (Fig. 2).

Both localization of AFP and tissue preservation were satisfactory when embryonic liver blocks were fixed with ethanol-acetic acid (Figs. 3-4) or with formaldehyde-picric acid-glutaraldehyde mixture. AFP staining after treatment with the latter fixative was less pronounced than in ethanol-acetic acid fixed specimens (Fig. 5). Sucrose treatment of aldehyde fixed liver blocks did not influence tissue antigenicity. No differences were noted in staining of cellular AFP when either direct or indirect labeling methods were employed.

Alpha₁-fetoprotein was detected in hepatocytes randomly distributed throughout the fetal liver tissue (Fig. 3). Frequently, clusters of positive hepatocytes were seen with the tendency to accumulate around sinuses (Fig. 4).

Discussion

A prerequisite of immunocytology is the monospecificity of the immunsera used for visualization of the cellular components. The most frequently used methods to produce monospecific immunsera are immunization of animals with crude preparations followed by subsequent absorption. However, a more elegant procedure for high yields of monospecific immunsera is immunization of animals with purified antigens. Furthermore, instead of γ -globulins, pure antibodies might be labeled with the marker substance of choice, followed by purification of the active conjugate.

The present paper describes procedures for the cytological detection of alpha₁-fetoprotein by use of immunoperoxidase labeling. For this purpose, highly specific immunocytochemical reagents were prepared, and the main preparative stages are described: (a) purification of AFP by combined immunochemical and physicochemical means; (b) isolation of specific antibody molecules from immunsera and conjugation with horseradish peroxidase; (c) immunocytochemical staining of AFP in embryonic mouse liver at the light microscopical level.

Purification of AFP. Because of the narrow range of specificity of antigen-antibody reactions, immunological methods offer valuable procedures for the isolation of antigenic compounds. On the basis of this concept, isolation of AFP from various species was obtained (Lehmann *et al.*, 1971; Nishi and Hirai, 1972; Aussel *et al.*, 1973; Watabe, 1974).

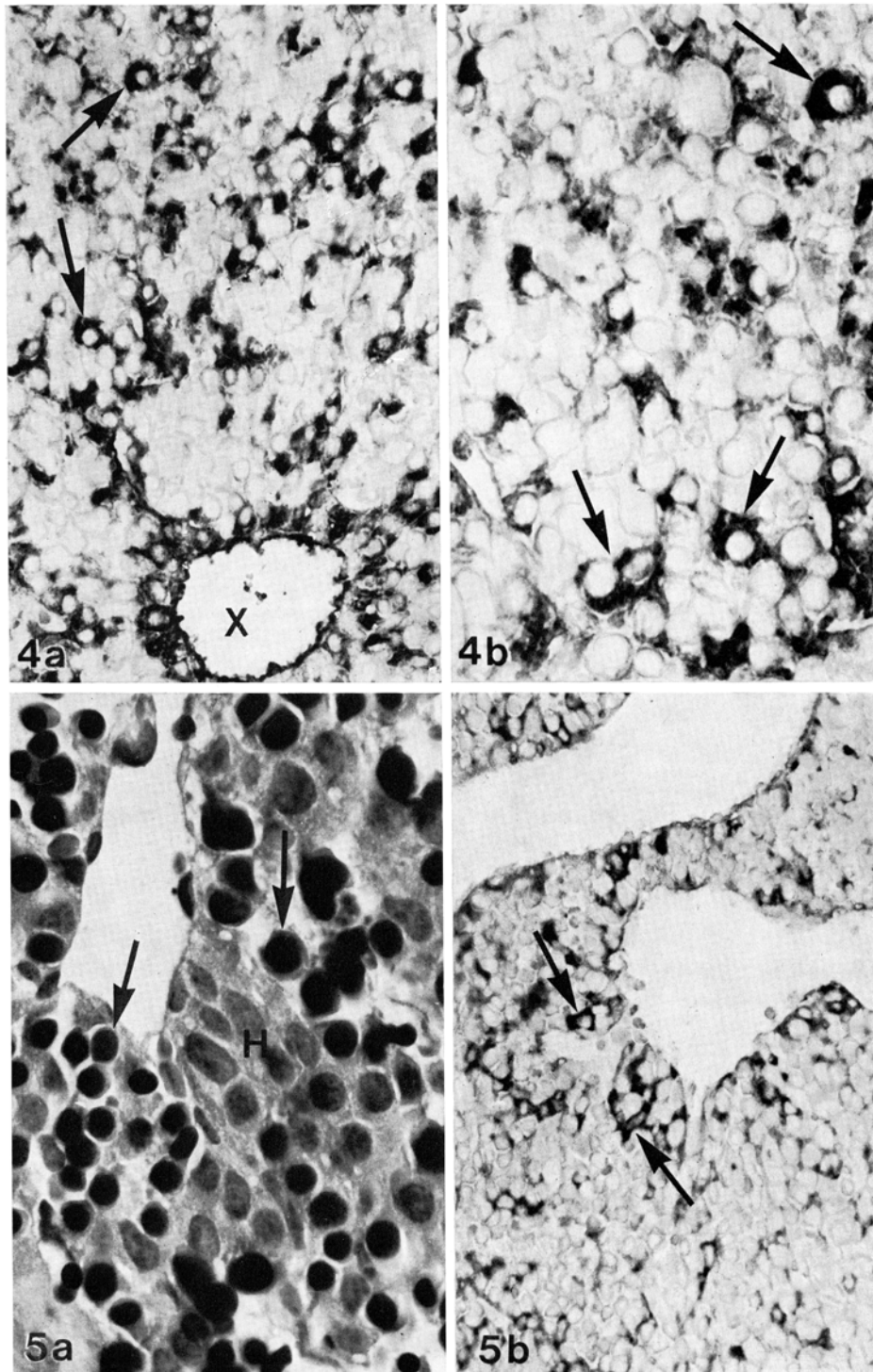


Fig. 4a and b. Detection of AFP in hepatocytes (←) by indirect antigen staining; blood sinus (x). Fixation was in ethanol-acetic acid. (a) Original x 250; (b) Original x 400

Fig. 5a and b. Liver preservation after fixation in formaldehyde-picric acid-glutaraldehyde mixture and localization of AFP by direct antigen staining. (a) H. E. staining; hepatocytes (H), erythrocyte precursor cells (←); original x 540. (b) positive AFP reaction in hepatocytes (←); original x 250

First of all, monospecific immunsera must be prepared. In the beginning of our experiments, monospecific immunsera against AFP were obtained by injection of rabbits with an

eluate from an α_1 -fraction of amniotic fluid after running in preparative acrylamide-agarose electrophoresis. Immunsera were rendered monospecific by absorption with glutaraldehyde insolubilized normal adult plasma proteins, and monospecificity was proved by gel diffusion methods. Then, the γ -globulins of these immunsera were coupled to Sepharose 4B to give the first immunoabsorbent in the isolation process.

When in the first isolation step amniotic fluid was incubated with this immunoabsorbent, AFP became adsorbed to its insolubilized antibody. After elution, AFP was passed in a second cycle using an immunoabsorbent prepared from γ -globulins of anti-mouse normal plasma proteins in order to adsorb possible traces of proteins other than AFP.

It was reported that covalently-linked ligand may leak from agarose matrix (Sepharose 4B), which is used for affinity chromatography (Smith and Kelleher, 1974). Thus, possible contamination of AFP from leaked γ -globulins was removed in a final run of AFP in a preparative electrophoresis of 0.8% agarose.

Isolation of Antibodies and Peroxidase-labeling. Hyperimmunization of rabbits using purified AFP resulted in monospecific immunsera, which gave a single precipitation arc in the α_1 -region when reacted in immunoelectrophoretic analyses with amniotic fluid. No precipitation reaction was obtained using normal mouse plasma proteins in either gel diffusion techniques employed. Also, upon immunization of sheep with rabbit IgG, monospecific immunsera were obtained.

It is obvious that for cytological labeling pure antibodies are preferred because other proteins which are present in the whole immunsera or in γ -globulin fractions may interfere with the tissue preparation (Avrameas, 1970). Thus, specific antibodies were isolated and subsequently conjugated with HRP. We have chosen the two-step procedure using glutaraldehyde activated peroxidase (Avrameas and Ternynck, 1971) because purification of the active conjugate was readily obtained by molecular filtration on Sephadex G 200. The conjugate was mainly composed of a homogenous fraction of a molecular weight of 210 000 and immunocytochemical staining using those conjugates was reliable as could be demonstrated also for ultrastructural studies (Kuhlmann *et al.*, 1974).

Immunocytochemical Staining of AFP. Procedures for the cytological detection of antigens require adequate preservation of cell structure and minimal alteration from the living state. Thus, the adaption of a fixation method is in most cases necessary. For immunocytological studies, however, one of the limiting factors impeding utilization of immunocytochemical reagents, is fixation of specimens. In any case, tissue preparation will depend on the type of antigen and its localization, and the choice of technique depends to a great extent on the information that one expects to obtain (Kuhlmann *et al.*, 1974).

Liver structure was well preserved when fixed in Bouin's or Carnoy's fixatives, but AFP staining was only faint. When we used absolute ethanol as fixative, positive AFP staining was achieved, however, best results with our peroxidase-conjugates were obtained when fixation was carried out in ethanol-acetic acid mixture, a fixative known to be useful for immunofluorescent studies (Engelhardt *et al.*, 1971). After fixation in formaldehyde-picric acid-glutaraldehyde, preservation of liver structure was excellent, and enough antigenicity was retained for the localization of AFP. However, the staining reaction was somewhat less than in ethanol-acetic acid fixed specimens, and prolonged storage in 30% sucrose did not restore the antigenicity as reported to be the case for several other antigens (Deng and Beutner, 1974).

Buffered picric acid-formaldehyde was introduced by Zamboni and DeMartino (1967) as a rapid fixative for electron microscopy, and in later studies it was also employed for immunocytological studies (Kawarai and Nakane, 1970). This fixative is a modification from Bouin's mixture. In our hands, better tissue preservation was obtained when small amounts of purified glutaraldehyde were added, as here reported, and staining of AFP was still possible. However, it must be kept in mind that glutaraldehyde in particular may cause changes in properties of proteins due to cross-linkage, and consequently, antigenic determinants may be masked. On the other hand, careful tissue fixation is necessary for adequate localization of proteins, otherwise these will leak out, thus increasing the possibility of staining artifacts (Kuhlmann, 1973; Kuhlmann *et al.*, 1974).

Utilization of peroxidase-conjugates requires complete inhibition of endogenous peroxidase activities in order to avoid interference with the specific antigen staining. Techniques described by Straus (1972) and Streefkerk (1972) did not result in complete inhibition of peroxidase activities in our material. However, higher concentrations of hydrogen peroxide alone in PBS were successful. Then, specificity of the immunological reactions was examined by incubation of sections in the above control conjugates. False positive staining due to non specific adsorption or cross-reactivity of the media with tissue compounds was not observed.

Norgaard-Pedersen *et al.* (1974) used peroxidase-labeled immunoglobulins for detection of AFP in two cases of human hepatoblastoma. In their material, only weak antigen staining could be seen and considerable background reaction occurred. Furthermore, inhibition of endogenous peroxidases was not performed; thus, specific localization of AFP is not convincing. From our results we deduce that pure antibodies, pure peroxidase of high specific activity and purified antibody-peroxidase conjugates are necessary for satisfactory immunocytology. We prefer conjugates prepared by a two-step procedure because the molar ratio of antibody to peroxidase is one, whereas conjugates following a one-step coupling contain highly heterogeneous populations of antibody conjugates (Avrameas, 1969).

Specific AFP staining was found to occur in single or grouped hepatocytes throughout the fetal liver tissue with the tendency to accumulate around liver sinuses. The importance of the distribution of positive hepatocytes in fetal liver as well as in hepatoma liver, and the high estrogen affinity of AFP (Uriel *et al.*, 1972; Uriel *et al.*, 1973) is difficult to assess and needs further experimentation. In this connection, peroxidase-labeling techniques at the cellular level may be useful. The advantage of enzyme-labeled antibody over immunofluorescent technique is that determination of cytological detail is often difficult with the latter one. Furthermore, rapid bleaching of fluorescent preparations and the occurrence of autofluorescence make them difficult for thorough investigations, whereas no such difficulties are encountered with enzyme techniques. In the case of peroxidase-labeling, stable reaction products are obtained which are resistant and insoluble to physicochemical treatment during usual embedment. Also, counterstaining procedures are easily performed, thus giving the possibility for further characterization of cells. Finally, peroxidase-labeled antibodies prepared as described here can be used for both light and electron microscopic studies (Kuhlmann *et al.*, 1974).

References

- Abelev, G. I.: Alpha-fetoprotein in ontogenesis and its association with malignant tumors. *Advanc. Cancer Res.* **14**, 295-358 (1971)

- Abelev, G. I., Perova, S. D., Khramkova, N. L., Postnikova, Z. A., Irlin, I. S.: Production of embryonal α -globulin by transplantable mouse hepatomas. *Transplant.* **1**, 174-180 (1963)
- Aussel, C, Uriel, J., Mercier-Bodard, C.: Rat alpha-fetoprotein: isolation, characterization and estrogen-binding properties. *Biochimie* **55**, 1431-1437 (1973)
- Avrameas, S.: Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochem.* **6**, 43-52 (1969)
- Avrameas, S.: Immunoenzyme techniques: enzymes as markers for the localization of antigens and antibodies. *Int. Rev. Cytol.* **27**, 349-385 (1970)
- Avrameas, S., Ternynck, T.: The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochem.* **6**, 53-66 (1969)
- Avrameas, S., Ternynck, T.: Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochem.* **8**, 1175-1179 (1971)
- Bakirov, R. D.: Appearance of embryonal serum α -globulin in adult mice after inhalation of carbon tetrachloride. *Byull. eksp. Biol. Med.* **2**, 45-47 (1968)
- Bergstrand, C. G., Czar, B.: Demonstration of a new protein fraction in serum from the human fetus. *Scand. J. clin. Lab. Invest.* **8**, 174 (1956)
- Branch, W. R., Wild, A.E.: Localisation and synthesis of α -fetoprotein in the rabbit. *Z. Zellforsch.* **135**, 501-516 (1972)
- Cuatrecasas, P.: Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *J. biol. Chem.* **245**, 3059-3065 (1970)
- Deng, J. S., Beutner, E. H.: Effect of formaldehyde, glutaraldehyde and sucrose on the tissue antigenicity. *Int. Arch. Allergy* **47**, 562-569 (1974)
- Determann, H.: Molecular weight determination. In: *Gel chromatography* (Determann, H., ed.), p. 107-130. Berlin-Heidelberg-New York: Springer 1969
- Engelhardt, N. V., Goussev, A. I., Shipova, L. J., Abelev, G. I.: Immunofluorescent study of alpha-foetoprotein (afp) in liver and liver tumors. I. Technique of afp localization in tissue sections. *Int. J. Cancer* **7**, 198-206 (1971)
- Fahimi, H. D., Drochmans, P.: Essais de standardization de la fixation au glutaraldehyde. I. Purification et determination de la concentration du glutaraldehyde. *J. Microscopie* **4**, 725-736 (1965)
- Gitlin, D., Boesman, M.: Fetus-specific serum proteins in several mammals and their relation to human α -fetoprotein. *Comp. Physiol.* **21**, 327-336 (1967)
- Gitlin, D., Kitzes, J., Boesman, M.: Cellular distribution of serum α -fetoprotein in organs of the foetal rat. *Nature (Lond.)* **215**, 534 (1967)

- Grabar, P., Williams, C. A.: Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines. Applications au sérum sanguin. *Biochim. biophys. Acta (Amst.)* **10**, 193-194 (1953)
- Graham, R. C, Karnovsky, M. J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291-302 (1966)
- Kawarai, Y., Nakane, P. K.: Localization of tissue antigens on the ultrathin sections with peroxidase-labeled antibody method. *J. Histochem. Cytochem.* **18**, 161-166 (1970)
- Kroes, R., Williams, G. M., Weisburger, J. H.: Early appearance of serum α_1 -fetoprotein during hepatocarcinogenesis as a function of age of rats and extent of treatment with 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res.* **32**, 1526-1532 (1972)
- Kuhlmann, W. D.: Ultrastructural localization of antigens by peroxidase-labeled antibodies. In: *Electron microscopy and cytochemistry* (Wisse, E., Daems, W. Th., Molenaar, I., van Duijn, P., eds.), p. 155-158. Amsterdam: North-Holland Publ. Comp. 1973
- Kuhlmann, W. D.: Cytological localization of antigens: Tissue fixation and processing in immunoenzyme techniques. In: *Immunoenzymatic techniques* (Feldmann, G. et al., eds.), p. 91-98. Amsterdam: North-Holland Publ. Comp 1976
- Kuhlmann, W. D., Avrameas, S., Ternynck, T.: A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates. *J. Immunol. Meth.* **5**, 33-48 (1974)
- Lehmann, G., Lehmann, D., Martini, G. A.: α_1 -foetoprotein. Isolierung und Kristallisation aus menschlichem Plasma. *Clin. chim. Acta* **33**, 197-206 (1971)
- Lowry, O. H., Rosebrough, N.J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265-269 (1951)
- Néchaud, B. de, Uriel, J.: Antigènes cellulaires transitoires de foie de rat. I. Sécrétion et synthèse des protéines sériques foetospécifiques au cours du développement et de la régénération hépatique. *Int. J. Cancer* **8**, 71-80 (1971)
- Néchaud, B. de, Uriel, J.: Antigènes cellulaires transitoires du foie de rat. II. Effet des inhibiteurs de synthèse sur les foetoprotéines sériques à la fin du premier mois de vie et après intoxication hépatique aigue. *Int. J. Cancer* **10**, 58-71 (1972)
- Nishi, S., Hirai, H.: Purification of human, dog and rabbit α -fetoprotein by immunoadsorbents of Sepharose coupled with anti-human α -fetoprotein. *Biochim. biophys. Acta (Amst.)* **278**, 293-298 (1972)
- Norgaard-Pedersen, B., Dabelsteen, E., Edeling, C. J.: Localization of human α -foetoprotein synthesis in hepatoblastoma cells by immunofluorescence and immunoperoxidase methods. *Acta path. microbiol. scand., Sect. A* **82**, 169-174 (1974)
- Ouchterlony, O., Antigen-antibody reaction in gels. IV. Types of reaction in coordinated systems of diffusion. *Acta path. microbiol. scand.* **32**, 231-240 (1953)

- Pedersen, K. O.: Fetuin, a new globulin isolated from serum. *Nature (Lond.)* **154**, 575 (1944)
- Pihko, H., Ruoslahti, E.: Alpha fetoprotein production in normal and regenerating mouse liver. In: *Proc. Int. Conf. Alpha-fetoprotein* (Masseyeff, R., ed.), p. 333-336. Paris: INSERM 1974
- Romeis, B.: Die Fixierung histologischer Präparate. In: *Mikroskopische Technik* (Romeis, B., ed.), p. 47-83. München-Wien: R. Oldenbourg Verlag 1968
- Smith, C. J., Kelleher, P. C.: Purification of the two molecular variants of rat alpha-fetoprotein by affinity chromatography. In: *Proc. Int. Conf. Alpha-fetoprotein* (Masseyeff, R., ed.), p. 85-94. Paris: INSERM 1974
- Stanislawski-Birencwajg, M., Uriel, J., Grabar, P.: Association of embryonic antigens with experimentally induced hepatic lesions in the rat. *Cancer Res.* **27**, 1990-1997 (1967)
- Straus, W.: Improved staining for peroxidase with benzidine and improved double staining immunoperoxidase procedures. *J. Histochem. Cytochem.* **20**, 272-278 (1972)
- Streefkerk, J. G.: Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol. *J. Histochem. Cytochem.* **20**, 829-831 (1972)
- Uriel, J.: Méthode d'électrophorèse dans les gels d'acrylamide-agarose. *Bull. Soc. Chim. Biol.* **48**, 969-982 (1966)
- Uriel, J., Aussel, C, Bouillon, D., Néchaud, B. de, Loisillier, F.: Localization of rat liver α -fetoprotein by cell affinity labelling with tritiated oestrogens. *Nature (Lond.)* **244**, 190-192 (1973)
- Uriel, J., Néchaud, B. de, Dupiers, M.: Estrogen-binding properties of rat, mouse and man fetospecific serum proteins. Demonstration by immunoautoradiographic methods. *Biochem. biophys. Res. Commun.* **46**, 1175-1180 (1972)
- Watabe, H.: Purification and chemical characterization of α -fetoprotein from rat and mouse. *Int. J. Cancer* **13**, 377-388 (1974)
- Watabe, H., Hirai, H., Satoh, H.: α -fetoprotein in rats transplanted with ascites hepatoma. *Gann* **63**, 189-199 (1972)
- Zamboni, L., De Martino, C: Buffered picric acid-formaldehyde: a new rapid fixative for electron microscopy. *J. Cell Biol.* **35**, 148A (1967)