

# ULTRASTRUCTURAL DETECTION OF ALPHA<sub>1</sub>-FETOPROTEIN IN HEPATOMAS BY USE OF PEROXIDASE-LABELLED ANTIBODIES

W. D. KUHLMANN

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

Int. J. Cancer 22, 335-343, 1978

---

## Summary

Hepatomas were induced in rats by feeding N-nitrosomorpholine. Nodules from alpha<sub>1</sub>-fetoprotein (AFP)-producing hepatoma were taken and subcutaneously injected into syngeneic hosts. From the first generation, the hepatoma producing most AFP was selected for further transfers. Four weeks after transplantation, the AFP concentration in the host rats was in the range of 0.8 to 1.5 mg AFP/ml serum. Transplanted hepatomas of the 14<sup>th</sup> to 19<sup>th</sup> generations were used for ultrastructural localization of AFP. Various methods of tissue processing were examined under the light and electron microscopes for intracellular detection of AFP by use of specific anti-AFP antibodies conjugated with peroxidase. AFP was localized in the perinuclear space, the rough-surfaced endoplasmic reticulum and the Golgi apparatus. Non-membrane-bound ribosomes did not stain for AFP. Fixation with 6% formaldehyde for 5 hours followed by 6% formaldehyde plus 0.25-0.5% glutaraldehyde for 60-90 minutes proved best both for satisfactory ultrastructural conservation of the organs and for immunocytochemical localization of AFP. Concentrations of glutaraldehyde which exceeded 0.5% led to a significant decrease in immunocytochemical AFP reactions. On the other hand, weak aldehyde fixation poorly preserved ultrastructural detail and leakage of insufficiently fixed material caused staining artifacts. Under our conditions, thick frozen sections from well-fixed hepatomas gave consistently reproducible results in that intracellular penetration of antibody-peroxidase conjugates was superior to hand-cut small tissue fragments. Tissue fixation and sampling were most important above all for other experimental steps.

## INTRODUCTION

Synthesis of embryo-characteristic molecules by human and animal tumors is an oncologic phenomenon, and reexpression of fetal antigens has been reported for a number of tissues and tumor types (Abelev, 1968; Gold, 1971; Alexander, 1972). A typical protein of antigenic reversion during the processes of malignant conversion is alpha<sub>1</sub>-fetoprotein (AFP) which is of proven value in the diagnosis of hepatocellular cancer (Abelev *et al.*, 1963; Abelev, 1971; Laurence and Neville, 1972).

In a previous paper we published a comprehensive light microscopic study on cells producing AFP in experimental hepatocarcinogenesis by use of immunoperoxidase labelling techniques (Kuhlmann, 1978). In order to understand better the role of AFP under physiological and pathological conditions, it is necessary to localize cellular sites of AFP synthesis and secretion at the ultrastructural level. Because immunocytochemical methods in ultra-

structural research have proved useful for elucidation of relationships between biological structure and function in other tissues (for review see Kuhlmann, 1977), it was thought that analogous procedures might be useful too for the study of intracellular AFP under the electron microscope. For this purpose, however, reliable procedures had to be established. In studies which combine immunological and ultrastructural methods both satisfactory tissue conservation and immunological reactivity of the investigated material must be reconciled. The present paper is based on recent developments in peroxidase-labelling techniques (Kuhlmann, 1977). The aim was to obtain procedures by which intracellular AFP can be reproducibly stained. For this purpose, AFP was identified in solid rat hepatomas which were chemically induced (Kuhlmann, 1978) and maintained by serial transplantation. The influence of various fixatives and tissue sampling procedures upon quality of intracellular AFP staining was evaluated.

## MATERIAL AND METHODS

### *Induction and maintenance of hepatomas*

Inbred male rats (strain BD X) were used throughout. Hepatomas were induced in 12-week-old animals by feeding the hepatotropic carcinogen N-nitrosomorpholine (Druckrey *et al.*, 1967; Kuhlmann, 1978).

Nodules from an AFP-producing hepatoma of the primary host rat were taken and subcutaneously injected into syngeneic 12-week-old hosts. When the inocula reached 1.5 cm in diameter, these were transferred: transplanted tumors were freed of necrotic areas, minced with razor blades into blocks of 2-3 mm and subcutaneously inoculated into hosts. From the first generation, the hepatoma producing most AFP (from rat No. 9 and designated as hepatoma H 9) was selected for further transfers.

### *Immunological reagents and procedures*

Monospecific rabbit anti-rat AFP immune sera were prepared (Kuhlmann, 1975). Pure rabbit anti-AFP antibodies were isolated from whole immune sera by use of immuno-adsorbents, then IgG molecules were conjugated with horseradish peroxidase (HRP, RZ 3; Boehringer-Mannheim, Germany) and purified on a Sephadex G 200 column as described (Kuhlmann, 1975, 1977). HRP conjugates of rabbit anti-rat IgG antibodies and normal rabbit IgG globulins were prepared in the same way.

For immunocytochemical controls (see "Immunocytochemical techniques") purified conjugates were absorbed with an immuno-adsorbent which was prepared by coupling AFP to cyanogen-bromide-activated Sepharose 4B (Kuhlmann, 1978). AFP content in sera of transplanted rats was quantitated by electroimmunodiffusion (Laurell, 1966; Kuhlmann, 1978).

### *Tissue fixation and processing*

Hepatomas, free of necrotic areas, were chopped with razor blades into blocks of 2-3 mm in a drop of fixative. Formaldehyde freshly prepared from paraformaldehyde and vacuum-distilled glutaraldehyde with a purification index of PI = 0.1 to 0.2 were used in 0.2 M cacodylate buffer pH 7.2. All fixation procedures were carried out at 0° C with continuous agitation. In detail, fixation schedules were as follows: (1) 4% and 6% formaldehyde for 1, 6 and 12 h; (2) 6% formaldehyde plus 0.5% glutaraldehyde for 1 and 6 h; (3) 6% formaldehyde for 5 h, followed by 6% formaldehyde plus 0.25, 0.5 or 1 % glutaraldehyde respectively for 1 or 1

½ h. After fixation, tissue blocks were washed at 0° C for 18 h with frequent changes of the buffer solution.

Prior to immunocytochemical incubations, tissue blocks were reduced with razor blades to small fragments. Furthermore, tissue blocks were treated with 10% dimethylsulfoxide (DMSO), rapidly frozen in liquid nitrogen-cooled isopentane and cut at 10-40 µm in a Dittes-Duspiva cryostat (Kuhlmann and Miller, 1971). Sections were dropped straight into cacodylate-buffered 10% DMSO.

For microscopical control of AFP staining, both fresh tissue blocks from the same hepatoma and blocks of aldehyde-fixed and cacodylate-washed tissues were fixed in 96% ethanol-1 % acetic acid for 12-15 h at 4° C, dehydrated in absolute ethanol, cleared in benzene and embedded in paraffin (Kuhlmann, 1975).

#### *Immunocytochemical techniques*

All procedures were carried out at laboratory temperature. Tissue fragments and frozen-cut sections were floated in peroxidase-labelled anti-AFP antibodies (0.1 mg/ml cacodylate buffer; higher concentrations did not result in stronger AFP staining) for 2 h. Non-reacted conjugates were eliminated by three successive rinsings followed by three washings of 10 min each in cacodylate buffer.

Peroxidase activity was revealed by incubation for 20 min in 3,3'-diaminobenzidine (Graham and Karnovsky, 1966), 0.5 mg/ml 0.2 m TrisHCl buffer containing 0.01 % hydrogen peroxide. After washing in cacodylate buffer, sections were postfixed for 30 min in cacodylate-buffered 1 % OsO<sub>4</sub>.

Parallel to frozen sections, paraffin-embedded tissues were prepared for light microscopical immunocytochemistry as described (Kuhlmann, 1975).

Specificity of the reactions was examined on tissue sections which were incubated in HRP conjugates of (1) normal rabbit IgG globulins; (2) rabbit anti-rat IgG antibodies; (3) rabbit anti-AFP antibodies absorbed with an immunoabsorbent of rat AFP. Also, tissue sections were treated with 1 % H<sub>2</sub>O<sub>2</sub>/ml cacodylate buffer for 1 ½ h prior to these control reactions in order to abolish endogenous peroxidase activities (Kuhlmann, 1975). Finally, either procedure was followed by enzyme substrate.

#### *Electron microscopy*

Tissues were dehydrated in an ascending alcohol series and frozen sections were flat embedded in Epon (Kuhlmann and Miller, 1971). Ultrathin sections were cut with an ultramicrotome and mounted on Formvar-coated 200 mesh copper grids. Ultrathin sections were examined either unstained or after further staining with lead citrate (Reynolds, 1963) for 30-60 sec in a Siemens Elmiskop IA operating at 80 kV and with a 50-µm objective aperture.

## **RESULTS**

#### *Transplantable hepatomas*

Transplanted hepatomas showed increasing growth rates (76 days in the first generation) up to 10 transfers; afterwards, the transfer intervals averaged about 30 days. At that time,

donor tumors measured at least 1.5 cm in diameter, calculated from length and width, and AFP concentration in the host rat was in the range of 0.8 to 1.5 mg AFP/ml serum. Transplanted hepatomas grow in an encapsulated form and are at present in the 19th generation (designated as hepatoma H 9/19).

#### *Immunological reagents and control reactions*

Qualitative and quantitative data on preparation of monospecific immune sera, isolation of specific antibodies and labelling with HRP were reported previously (Kuhlmann *et al.*, 1974; Kuhlmann, 1977). HRP conjugates of rabbit anti-rat AFP antibodies (Kuhlmann, 1975), anti-rat IgG antibodies and normal rabbit IgG globulins were obtained by the described procedures.

In the light microscope, anti-AFP conjugates showed good specificity and strong staining in the cytoplasm of hepatoma cells (Fig. 1). When tested on sections of normal liver and AFP-negative hepatoma, cells did not stain. No cross-reacting antibodies were observed. No staining was seen in AFP-positive hepatomas upon incubation in conjugates of normal rabbit IgG globulins and specifically absorbed anti-AFP conjugates. Furthermore, HRP-labelled anti-rat IgG antibodies did not react with intracellular components of hepatoma cells. Extracellular staining of rat IgG was a normal feature due to synthesis and excretion of IgG globulins by immunocompetent cells of the host rat.

#### *Detection of AFP in the light microscope*

Immunocytochemical studies were performed with transplanted hepatomas of the 14th to 19th generations. In routine histology the cytoplasm of hepatoma cells was strongly basophilic. In ethanol/acetic-acid-fixed preparations cellular localization of AFP and preservation of tissue were excellent (Fig. 1a). More than 70% of the cells were actually engaged in AFP production. The intensity of AFP staining by this method was compared with that after the various aldehyde fixations. Details are shown in Table I.

Fixation times as short as 1 h resulted only in faint AFP staining; 4-6% formaldehyde for 6-12 h gave strong AFP reactions. Also, combination of 6% formaldehyde and 0.25 to 0.5% glutaraldehyde was successfully employed (Fig. 1b-c). Fixation with glutaraldehyde concentrations higher than 0.5% and for periods longer than 1 h led to a significant decrease in the immunocytochemical AFP reaction.

#### *Ultrastructural localization of AFP*

In the case of hand-cut small tissue fragments, HRP conjugates of anti-AFP antibodies did not penetrate intracellular spaces and AFP-positive cells were not detected. In contrast, AFP-staining cells were found in cryostat sections of prefixed hepatoma tissue blocks. Results are summarized in Table I.

After fixation in formaldehyde alone, cell conservation was not sufficient because cell surface membranes and intracellular organelles were damaged and diffusion artifacts occurred. Ultrastructural detail was sufficiently preserved for immunocytochemical work with cryostat sections when hepatomas were fixed in (1) formaldehyde-glutaraldehyde or (2) formaldehyde followed by mixture of formaldehyde plus glutaraldehyde. For ultrastructural preservation and detection of AFP, fixation in 6% formaldehyde for 5 h followed by 6% formaldehyde plus 0.25-0.5% glutaraldehyde for 60-90 min was preferred. Fixation in 6% formaldehyde plus 0.5% glutaraldehyde for 60 min proved only useful when tissue blocks were smaller than 2 mm in diameter.

The intracellular localization of AFP was observed in subcellular structures described as the perinuclear space (PNS), the rough-surfaced endoplasmic reticulum (RER) and the Golgi apparatus. Non-membrane-bound ribosomes in the cytoplasm did not stain for AFP. AFP was also observed in extracellular spaces and delineating adjacent hepatoma cells (Fig. 2-5).

Hepatoma cells were irregular in shape and possessed abundant cytoplasm; cellular filiations were often difficult to appreciate. The abundance of free cytoplasmic ribosomes was typical for the hepatoma cells. Compared with normal adult hepatocytes, the RER was only moderately developed and consisted of flat, elongated lamellae which were not specially arranged with respect to the nucleus and cell surface membrane. Within a given cell, AFP staining occurred in all regions of the RER or only in some compartments. In all cases, the perinuclear space was labelled. The Golgi complex appeared small and could also stain for AFP. The cell nucleus was irregularly formed, containing deep invaginations. In the compact nucleoplasm, one, two, or more prominent nucleoli were distinguished.

#### *Controls at the ultrastructural level*

Tissue preparations did not stain upon incubation in absorbed anti-AFP antibodies and HRP conjugates of normal rabbit IgG globulins followed by Graham and Karnovsky's substrate medium. No intracellular labelling was observed when organ preparations were incubated in HRP conjugates of anti-rat IgG antibodies (for extracellular staining see "Immunological reagents"). Only the known endogenous peroxidase activities in macrophages and cells of hemopoietic and myeloid origin were seen, but these were readily distinguished from immunocytological reactions by their topological arrangement. Endogenous peroxidase activity was completely inhibited by incubation of the hepatoma sections in 1 % hydrogen peroxide for 1 ½ h. Then, upon incubation in the various control reagents, immunocytological specificity was confirmed and tissues did not stain (Fig. 6). For artifactual stainings described as false positive and false negative reactions, see Discussion ("Detection of AFP at the ultrastructural level").

TABLE I  
INFLUENCE OF TISSUE FIXATION ON AFP DETECTION

Tissue fixation	Light microscopy (paraffin sections) intracellular AFP	Electron microscopy (cryostat sections)	
		Intracellular AFP	Cell conservation
96% ethanol/1% acetic acid 12-15 h	+ <sup>1</sup>	—	—
4% or 6% formaldehyde 1 h	(+)	(+) <sup>2</sup>	Not sufficient
4% formaldehyde 6-12 h	+	(+)/+ <sup>2</sup>	Not sufficient
6% formaldehyde 6-12 h	+	(+)/+ <sup>2</sup>	Not sufficient
6% form.—0.5% glutaraldehyde 60 min	(+)	(+)/+	Sufficient
6% form.—0.5% glut. 6 h	∅	∅	Excellent
6% formaldehyde/5 h 6% form.—0.25% glut. 60-90 min	+	+	Good
6% formaldehyde/5 h 6% form.—0.5% glut. 60-90 min	(+)	(+)/+	Good
6% formaldehyde/5 h 6% form.—1% glut. 60-90 min	∅/(+)	∅/(+)	Good

<sup>1</sup> Intensity of specific reactions: ∅, none; (+), faint; +, strong; —, not examined. —<sup>2</sup> Diffusion artifacts.

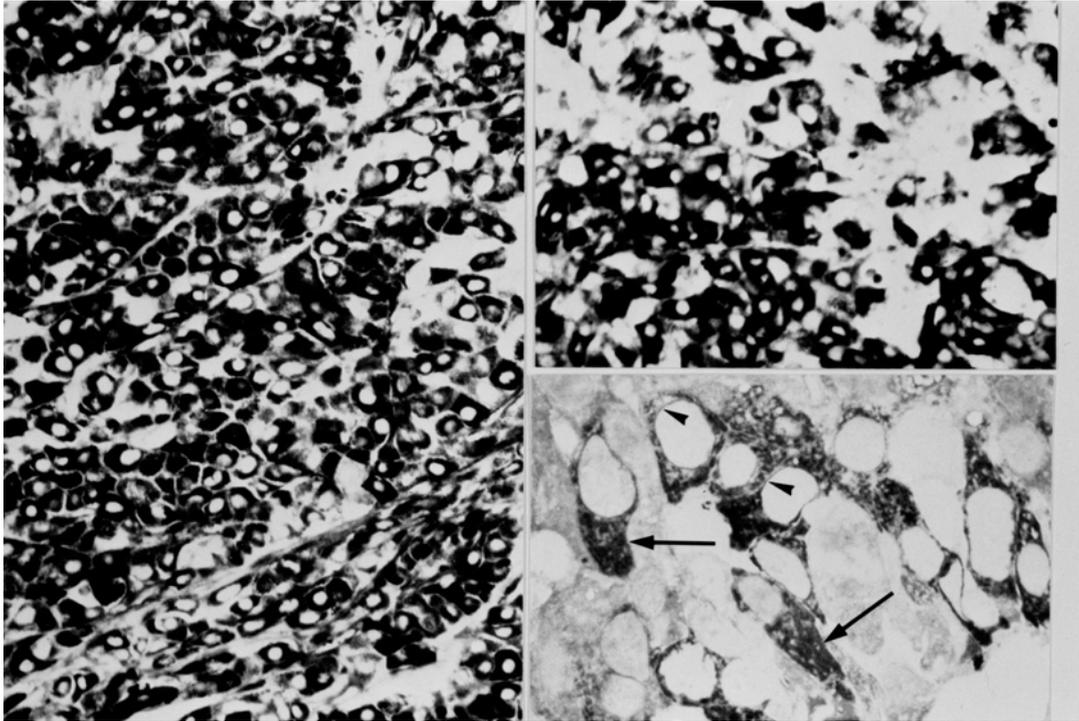


FIGURE 1 - Detection of AFP in the cytoplasm of hepatoma cells. (a) Control tissue was fixed in ethanol/acetic acid; AFP staining of paraffin section. (b) Fixation was in 6% formaldehyde for 5 h followed by 6% formaldehyde plus 0.25% glutaraldehyde for 1 h; AFP staining of paraffin section. (c) Same fixation as in (b), but tissue block was processed for ultrastructural immunocytochemistry (preembedding technique); semi-thin section in the light microscope.

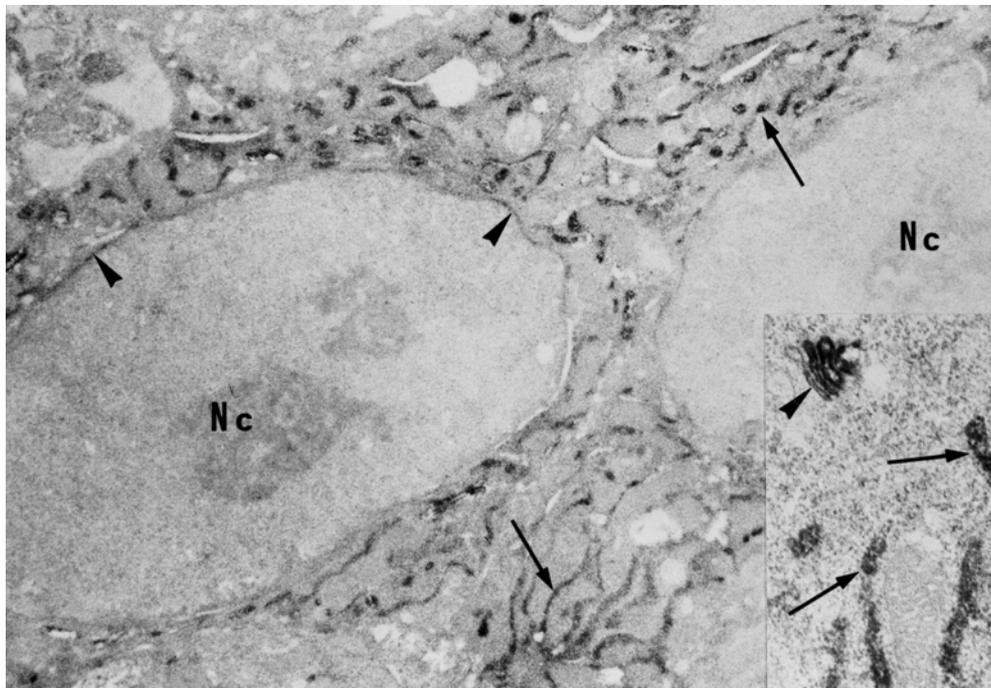


FIGURE 2 - Low magnification view of hepatoma cells. Note localization of AFP in RER lamellae (←) and PNS (arrowhead), fixation was in 6% formaldehyde/5 h followed by 6% formaldehyde plus 0.25% glutaraldehyde/60 min; nucleoli (Nc); lead citrate staining 30 sec. Inset: Same preparation; AFP staining in RER (←) and Golgi apparatus (arrowhead).

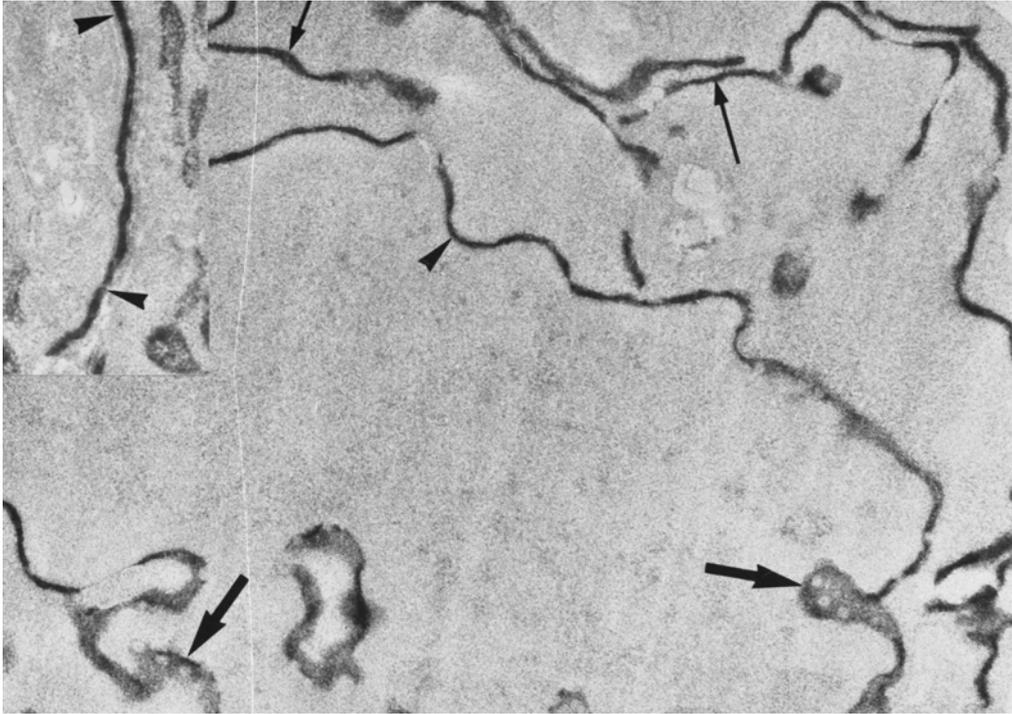


FIGURE 3 - Hepatoma cell synthesizing AFP in RER lamellae and PNS. Invaginations of the positive PNS are cut tangentially, note nuclear pores. Inset: high magnification view of AFP containing Golgi apparatus.

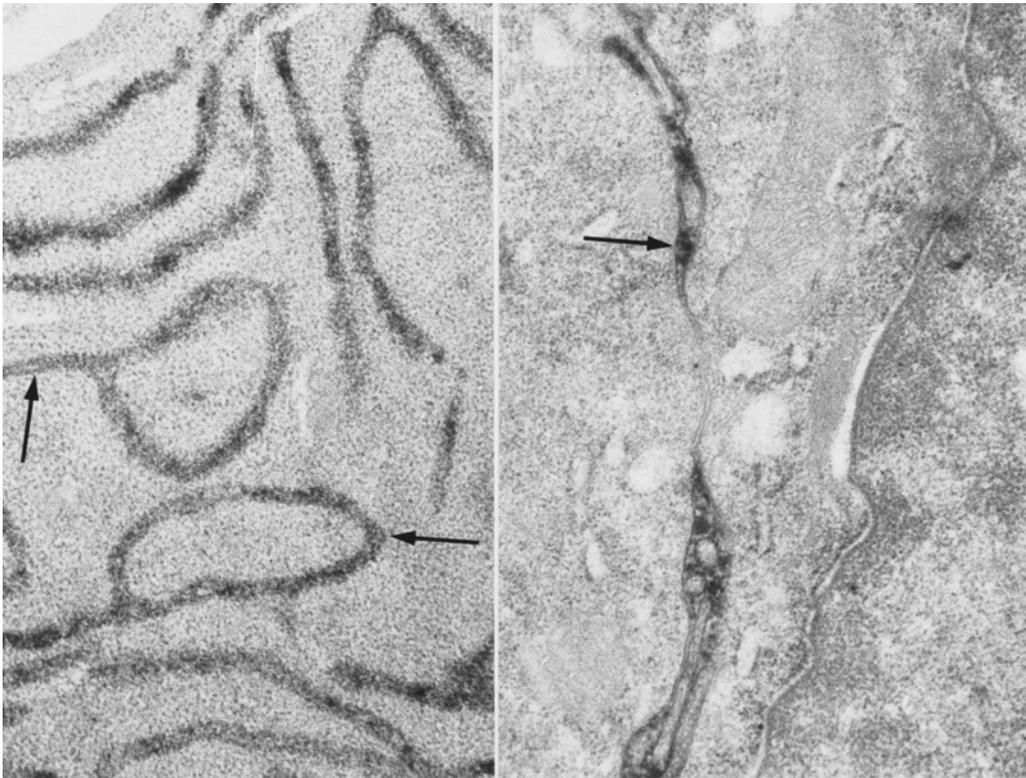


FIGURE 4 - Localization of AFP in RER lamellae (←). Non-membrane-bound ribosomes did not stain for AFP. Fixation as in Figure 3; lead citrate counterstain for 60 sec.

FIGURE 5 - Detection of AFP between adjacent hepatoma cells (←). Lead citrate staining for 60 sec.

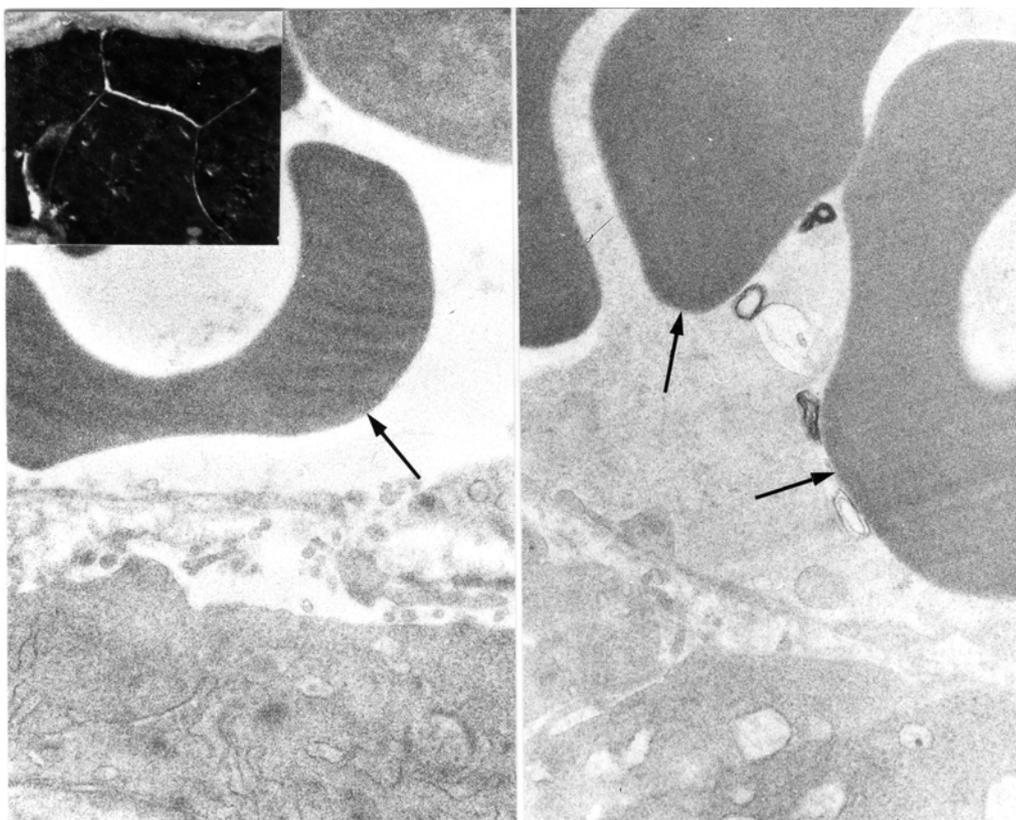


FIGURE 6 – Control reactions. (a) Cryostat section was treated with 1%  $H_2O_2$  prior to incubation in immunologically absorbed anti-AFP peroxidase conjugate and finally stained by DAB/ $H_2O_2$  substrate. No intracellular and extracellular stainings, absence of endogenous peroxidase activities in erythrocytes ( $\leftarrow$ ). Not counterstained. Inset: Detection of endogenous peroxidases in erythrocytes by routine DAB/ $H_2O_2$  technique. (b) Cryostat section from formaldehyde-fixed hepatoma. No treatment and only post-fixation in  $OsO_4$ . Note same natural contrast of erythrocytes ( $\leftarrow$ ) in Figures 6a and 6b. Not counterstained.

## DISCUSSION

Ultrastructural studies on intracellular sites of AFP production and the description of histogenesis of AFP-producing hepatoma cells are still lacking because adequate methodology was hitherto not available. Only rare attempts were made to show AFP at the fine-structural level (Shikata, 1975), but localization of AFP was not conclusive. From previous studies we know that appropriate tissue fixation, tissue sampling and the use of pure immunocytochemical reagents are unequivocally a prerequisite for specific and reproducible results (for review see Kuhlmann, 1977), otherwise cellular detection of antigen, here AFP, might not be convincing. The necessary steps for isolation of specific antibody molecules, its conjugation with HRP and purification of the active conjugates were described elsewhere (Kuhlmann, 1975, 1977, 1978).

The present paper was initiated to explore procedures with which intracellular AFP can be reliably detected under the electron microscope. The use of an established transplantable hepatoma instead of chemically induced hepatomas proved to be of great advantage: transplanted hepatomas were mainly composed of AFP-synthesizing cells, whereas in experimental liver cancer AFP-producing and non-AFP-producing nodules were found side by side

(Kuhlmann, 1978). In the latter, the probability of selecting AFP-negative hepatoma nodules instead of AFP-positive ones was high.

#### *Effect of aldehyde fixation on AFP staining*

In the light microscope, both localization of AFP and organ preservation were excellent when tissue blocks were fixed with an ethanol/acetic acid mixture (Kuhlmann, 1975). Unfortunately, such a fixation procedure cannot be employed for electron microscopic studies, but intensity of AFP reactions in paraffin sections of those preparations proved useful for control and comparison of AFP reactions after the various aldehyde fixations. For this purpose, aldehyde-fixed and cacodylate-washed tissue blocks were treated in the same way as the control tissues, i.e. they were further fixed in ethanol/ acetic acid, dehydrated in absolute ethanol and finally embedded in paraffin. From light microscopic studies it became clear that fixations with 4 to 6% formaldehyde up to 12 h and in combination with 0.25 to 0.5% glutaraldehyde for 60-90 min did not essentially inhibit subsequent immunocytological reactions. Yet, higher concentrations of glutaraldehyde were deleterious for AFP localization (Table I).

Generally, ultrastructural immunocytochemistry requires individual tissue preparation in order to avoid false negative and false positive antigen staining. After single formaldehyde fixation poor AFP staining was obtained and also expected because stabilization of cell components, especially the soluble ones, cannot be as efficient as after glutaraldehyde fixation (for review see Kuhlmann, 1977). On the other hand, glutaraldehyde was shown to alter specific biological activity of the studied material by intra- and intermolecular cross-linkages, thus a compromise between ultrastructural conservation and retention of biological activity was necessary. Therefore, hepatomas were processed by combined light and electron microscopic examinations and, finally, we decided to employ 6% formaldehyde for 5 h followed by 6% formaldehyde plus 0.25-0.5 % glutaraldehyde for 60-90 min as fixatives. Under these conditions ultrastructural detail was sufficiently preserved for intracellular localization of AFP.

Our fixations were followed by immersion of tissue blocks into the fixatives. The first step (6% formaldehyde for 5 h) of the recommended schedule may be omitted when organ perfusion can be performed. In this case, perfusion with the second-step fixative (6% formaldehyde plus 0.25 to 0.5% glutaraldehyde for 60-90 min) may be shorter than described for immersion fixation. In the latter technique, effectiveness of fixation of cells within the tissue block depends largely on the penetrability of fixatives, which is a well-known fact. Hence, fixation of tissue blocks in 6% formaldehyde plus 0.5% glutaraldehyde for 60 min (see Table I) proved useful only when hepatomas were cut into blocks of less than 2 mm in diameter.

#### *Detection of AFP at the ultrastructural level*

Experiments with different fixation procedures have shown that localization of intracellular AFP with peroxidase conjugates of anti-AFP antibodies was governed by the penetration of the immunological reagents and not only by the aldehyde fixation used. Thus, we were unable to stain intracellular AFP in hand-cut small tissue fragments (false negative staining). From a previous study we know that techniques which disrupt cell membranes enhanced penetration of conjugates into intracellular spaces (Kuhlmann et al., 1974). Then, satisfactory AFP localization was obtained with cryostat sections from well-fixed hepatoma blocks, e.g. reproducible reactions were found after fixation in 6 % formaldehyde for 5 h followed by 6 % formaldehyde plus 0.25-0.5% glutaraldehyde for 60-90 min. The fragility of 10- $\mu$ m thick frozen sections made them difficult to manipulate, hence 20-40- $\mu$ m thick sections were used in routine.

Numerous papers concerning hepatoma cells have been published and will not be referred to here (for references see Bannasch, 1975). In such studies hepatomas were described according to their structure but functional aspects could not be shown by classical histology and electron microscopy. In the present work, attention was mainly paid to the methodology of AFP localization by ultrastructural immunoperoxidase cytochemistry. Detailed description of cytology and phenomena of AFP resurgence during experimental hepatocarcinogenesis is now in preparation. Here, we have concentrated on major cytologic features of transplanted hepatomas. These were, firstly, strongly basophilic due to ribosome abundance in the cytoplasm which brought about the high natural contrast at the finestructural level. The localization of AFP in either the whole RER including the PNS or only in some RER lamellae or on parts of RER membranes and their attached ribosomes indicated the production of this embryo-characteristic substance on the rough-surface endoplasmic reticulum of hepatoma cells and not on free cytoplasmic ribosomes.

The importance of the above described staining pattern is not clear and may reflect a switching on and off of the ribosome function by some kind of intracellular regulation. The observation that sometimes only a few RER lamellae stained for AFP raises the question as to what the other lamellae contained. It could be that AFP was present in these lamellae too, but at quantities too low to be detected with the method employed.

The mechanisms by which newly formed AFP pass from the polysome through the membrane into the lumen of the RER are virtually unknown and were not elucidated by our method. RER channels merely have a transport function for synthesized products (Palade *et al.*, 1962) which then accumulate in the Golgi complex. Here, posttranslational control of glycoprotein synthesis occurs at the level of carbohydrate attachment (Schachter *et al.*, 1970). Because AFP molecules are known to possess variable amounts of carbohydrate moieties (Zimmerman and Madappally, 1973), AFP-positive reactions within the Golgi apparatus are readily explained as an usual feature. Heavy AFP staining in extracellular spaces and high serum AFP levels in the host animals indicated continuous release of synthesized AFP by the producer cells. Since the moderately developed Golgi apparatus was regularly stained for AFP, secretion of this protein via that organelle was suggested to be a typical pathway.

#### *Immunocytochemical specificity*

Endogenous peroxidase activity in macrophages and cells of erythropoietic, myeloid origin was easily distinguished from specific AFP label in hepatoma cells by morphological criteria. Moreover, staining of endogenous peroxidases was quite different from the typical and specific AFP label (where PNS, RER and Golgi complex have reacted) and no confusion of sites of AFP localization with sites of endogenous peroxidases occurred. In any case, endogenous peroxidase activities could be readily abolished in our transplanted hepatomas by treatment of tissues with hydrogen peroxide prior to the above described immunocytological incubations. This procedure has already proved useful for immunocytochemical localization of AFP in the light microscope (Kuhlmann, 1975, 1978). Inhibition of endogenous peroxidases was not an essential step for the ultrastructural detection of AFP in our material for the reason mentioned above. However, inhibition of endogenous peroxidases might be important in other cell systems.

Diffuse labelling of cytoplasmic matrix was observed in hepatoma cells of necrotic areas (diagnosed as such already by macroscopic inspection). Under the microscope, cell surface and intracellular membranes appeared heavily damaged and this reaction was judged as artifactual, most probably due to diffusion before fixation was started. Diffuse labelling of cytoplasm could also occur in cryostat sections of non-necrotic areas after single fixation in

formaldehyde. Furthermore, after formaldehyde fixation for a very short time (1 h), extremely faint AFP stainings were observed, which could not be attributed to too strong a fixation. In these cases, membranes were regularly damaged, and AFP localization was also taken to be a diffusion artifact (insufficient stabilization; false positive and false negative stainings).

Finally, membrane damage, obviously due to a freeze-thaw cycle, was observed in cryostat sections after combined formaldehyde-glutaraldehyde fixation. However, in contrast to the observations above, no diffusion artifacts were observed when tissues were stabilized by appropriate fixation. Comparable results were obtained with other tissue models in which leakage of insufficiently fixed material was reported (Kuhlmann *et al.*, 1974; Kuhlmann, 1977). These results are readily understood when the different cross-linking qualities of formaldehyde and glutaraldehyde are considered. However, extensive formation of cross-linkages by glutaraldehyde may prevent conjugates from reaching antigenic sites, and negative results (false negative staining, too) must be expected. The extent of this phenomenon could be explored by combined light and electron microscopic examinations, and is recommended for any immunocytochemical work. Under our conditions, intracellular detection of AFP was most reliable when frozen sections from well-fixed specimens were used.

#### ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 136, publ. no. 25) Bonn, Germany.

I wish to thank Mrs. M. Tiedemann for excellent assistance.

#### REFERENCES

- ABELEV, G. I., Production of embryonal serum  $\alpha$ -globulin by hepatomas: review of experimental and clinical data. *Cancer Res.*, **28**, 1344-1350 (1968).
- 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., and IRLIN, I. S., Production of embryonal  $\alpha$ -globulin by transplantable mouse hepatomas. *Transplantation*, **1**, 174-180 (1963).
- ALEXANDER, P., Foetal " antigens" in cancer. *Nature (Lond.)*, **235**, 137-140 (1972).
- BANNASCH, P., Die Cytologie der Hepatocarcinogenese. In: H. W. Altmann, F. Büchner, H. Cottier, E. Grundmann, G. Holle, E. Letterer, W. Masshoff, H. Meesen, F. Roulet, G. Seifert and G. Siebert (ed.), *Handbuch der allgemeinen Pathologie*, Vol. 6, pp. 123-276, Springer-Verlag, Berlin-Heidelberg-New York (1975).
- DRUCKREY, H., PREUSSMANN, R., IVANCOVIC, S., and SCHMÄHL, D., Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.*, **69**, 103-201 (1967).

GOLD, P., Antigenic reversion in human cancer. *Ann. Rev. Med.*, **22**, 85-94 (1971).

GRAHAM, R. C, and 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).

KUHLMANN, W. D., Purification of mouse alpha<sub>1</sub>-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization. *Histochemistry*, **44**,155-167 (1975).

KUHLMANN, W. D., Ultrastructural immunoperoxidase cytochemistry. *Progr. Histochem. Cytochem.*, **10**, 1-57 (1977).

KUHLMANN, W. D., Localization of alpha<sub>1</sub>-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats. *Int. J. Cancer*, **21**, 368-380 (1978).

KUHLMANN, W. D., AVRAMEAS, S., and TERNYNCK, T., A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates. *J. Immunol. Meth.*, **5**, 33-48 (1974).

KUHLMANN, W. D., and MILLER, H. R. P., A comparative study of the techniques for ultrastructural localization of antienzyme antibodies. *J. Ultrastruct. Res.*, **35**, 370-385 (1971).

LAURELL, C. B., Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.*, **15**, 45-52 (1966).

LAURENCE, D. J. R., and NEVILLE, A. M., Foetal antigens and their role in the diagnosis and clinical management of human neoplasms: a review. *Brit. J. Cancer*, **26**, 335-355 (1972).

PALADE, G. E., SIEKEVITZ, P., and CARO, L. G., Structure, chemistry and function of the pancreatic exocrine cell. In: A. V. S. Reuck and M. P. Cameron (ed.), *CIBA Foundation Symposium on the exocrine pancreas*, pp. 23-55, Churchill, London (1962).

REYNOLDS, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.*, **17**, 208-212 (1963).

SCHACHTER, H., JABBAL, I., HUDGIN, R. L., and PINTERIC, L., Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. *J. Biol. Chem.*, **245**, 1090-1100 (1970).

SHIKATA, T., Immunoelectronmicroscopic study of  $\alpha$ -fetoprotein synthesis in hepatoma cells. *Ann. N. Y. Acad. Sci.*, **259**,211-216(1975).

ZIMMERMAN, E. F., and MADAPPALLY, M. M., Sialyltransferases: regulation of  $\alpha$ -foetoprotein microheterogeneity during development. *Biochem. J.*, **134**, 807-810 (1973).