Cellular fluctuations, alpha-fetoprotein expression, and the question of stem cells in experimental liver regeneration and hepatocarcinogenesis

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Abstract

Hepatocytes and bile ductular cells possess stem-like potential in restoring liver tissue. This was derived from rat and mouse injury models such as (a) partial hepatectomy by surgery; (b) acute carbon tetrachloride intoxication; (c) injuries by D-galactosamine (GalN) or by N-nitrosomorpholine (NNM); (d) experimental hepatocarcinogenesis with NNM using different doses. Liver parenchyma was restored by adult hepatocytes after partial hepatectomy as well as after carbon tetrachloride intoxication. Concurrently, hepatocytes resumed synthesis of alpha-1-fetoprotein (AFP) which depended to a high degree on the animal species and strains used in that studies. In contrast to this way of regeneration, liver repair after heavy injuries by agents such as GalN and NNM was initiated by oval cell proliferation deriving from ductular epithelial cells of the canaliculi of HERING (canals of Hering, intrahepatic bile ductules) as sources of endogenous progenitors. Progenitor populations reach levels of differentiation with fetal gene reactivation and significant AFP expression. Proliferating oval cells operated as transit and amplification compartment during liver regeneration. Oval cell proliferation and concurrent AFP synthesis occurred also in the early stages of hepatocarcinogenesis when the carcinogen NNM was applied in toxic doses. The histologic features compared to those seen in GalN intoxication. NNM carcinogenicity was of concern for both oval cells and adult hepatocytes with risks of transformation and malignant development. At the cancer stage, carcinomas either synthesizing AFP or not can be found side by side in the same liver. AFP expression by cancer cells seems to be a special attribute to differentiation stages in carcinogenesis. Clonality, maturation arrest and retrodifferentiation feature cells with high autonomy.

Keywords

Alpha-fetoprotein, hepatocytes, oval cells, biliary epithelial cells, stem-like progenitor cells, liver regeneration, liver carcinoma

1 Introduction

The liver is an important organ with essential chemical functions for life. Cell production and cell loss are usually balanced, renewal of cells does not exceed cell loss. In restoring cell mass during physiological regeneration, adult hepatocytes switch from a quiescent state to a proliferative state. This capacity of normal adult hepatocytes can be blocked in certain situations, and other repair mechanisms become involved to replace lost liver mass [1, 2]. This leads to the all-moving question: are there stem cells in the adult liver for regeneration? In fact, the adult liver contains stem cells that can proliferate and differentiate

into different cell types. These cells are called hepatic stem cells or liver progenitor cells, they proliferate and differentiate into hepatocytes and bile duct epithelia, respectively.

Stem cell activation is an important mechanism for regeneration and is dependent on the severity of the damage. In some cases, liver regeneration may be impaired by progressive fibrosis, inflammation, or genetic factors, limiting the ability of cells to regenerate. Anyway, organ-specific stem cells are of importance in regenerative situations. Several studies have shown that stem-like cells can be found at the junction of the bile ducts and the hepatic cords. They reside in the terminal bile ductular system from which so-called oval cells derive [3, 4]. This cell population can act as progenitor cells and generate new lineages of liver epithelia.

In the search for stem-like liver cells it is reasonable to recall liver morphogenesis. In embryonic life, liver originates from the ventral foregut epithelium forming the hepatic diverticulum and the liver bud. In the early stages, growth factors and tissue interactions form endoderm and mesoderm, and they shape the process of liver development. During hepatogenesis, hepatic endoderm cells (hepatoblasts) differentiate and maturate gradually into functional hepatocytes and biliary epithelial cells. Other liver cells for example von Kupffer cells, stromal cells and blood vessels derive from the mesoderm. Many of the necessary genes and molecular pathways have been identified [5]. Endoderm patterning and pathways of hepatic maturation have an impact on liver regeneration and pathophysiology. In this context, differentiation markers are helpful to trace cellular fluctuation in repair situations. One of such marker molecules is alpha-1-fetoprotein (AFP).

Protein patterns vary in embryonic and postnatal life. They are signs of current gene activity in the developmental stages of histogenesis and organ differentiation. AFP has obtained some considerable relevance because of its association with cell differentiation in onco-developmental processes. Since its detection in embryonic life and in human diseases, this protein is studied in many experimental settings to unravel developmental pathways, regeneration, and carcinogenesis. Over the years, a lot of papers and opinions on liver physiology, liver diseases, regeneration and hepatocarcinogenesis were published [6-26]. Stem-like progenitor cells and their differentiation pathways are of special interest [27-32]. However, the properties of regenerative cells for tissue repair and their abilities such as differentiation, transdifferentiation, dedifferentiation and, most importantly, the possibility of reprogramming into certain cell types are not yet solved [33-36]. In this review we will show how alpha-1-fetoprotein (AFP) expression can mark cellular fluctuations in the liver under various pathophysiological conditions and highlight origin and fate of progenitor cells in the adult liver.

Historically, AFP became the first useful biomarker in liver research and diagnostics since G.I. ABELEV and YU TATARINOV described the occurrence of this protein in fetuses and in malignant liver tumors [37, 38]. Their observations confirmed the existence of specific fetal serum proteins that have been already postulated in previous reports [39, 40] and in reports regarding the relationship between embryonic and cancer tissues [41]. These observations have initiated new ideas in cancer research by linking carcinogenesis

and developmental biology [42], the era of oncodevelopmental aspects in tumor biology emerged.

AFP is a normal constituent of fetal serum and amniotic fluid of many species. Under physiological conditions its synthesis occurs in the yolk sac, the gastro-intestinal tract, and the liver of embryos in many species [43-45]. In late embryonic life, the liver is the main organ of AFP synthesis, then this protein disappears after birth [39, 44, 46]. AFP expression is strongly suppressed in adult life. However, AFP may reappear in substantial quantities during processes of liver repair and malignant transformation, and measurement of AFP has proved to be of value in the diagnosis of hepatocellular cancer and endodermal sinus tumors [33, 37, 38, 47, 48]. Elevated serum AFP levels also occur in non-malignant diseases of human liver, i.e., forms of regeneration associated with viral hepatitis, alcoholic liver cirrhosis and partial hepatectomy [49-51]. AFP expression has become a valuable marker in biomedical studies and in clinical medicine [33, 37, 47, 52].

In animal experiments, such as partial hepatectomy and ingestion of hepatotoxins (e.g., CCl_4 or galactosamine-HCl induced liver injury) or genotoxic carcinogens temporary elevation of AFP levels have been reported [53-66]. Interestingly, some studies have shown similarities between galactosamine-induced liver damage in the rat and acute human viral hepatitis [67, 68]. AFP elevation during the different stages of experimental hepatocarcinogenesis was supposed as being a response to the carcinogenic diet and was correlated either with carcinogenicity of the chemical agent or with subsequent liver alterations including oval cell proliferation [54, 57, 69-72]. Since elevated serum AFP levels without oval cell proliferation during and after feeding of very small quantities of N-2-fluorenylacetamide were observed, one can expect metabolic effects on liver cells with subsequent selective derepression of AFP synthesis [71, 73].

In any case and with respect to cellular fluctuations in the liver, AFP elevation depends largely on effects such as cell death, mitotic activity, and dynamics of cellular differentiation [58, 74]. Furthermore, regeneration patterns differ from one another in their ability to cope with damage types. Some injuries impair or even block proliferation of adult hepatocytes while other injuries do not so. Possibly, stem-like cells become engaged to renew lost tissue [1]. It is expected that models of induced liver injury by surgical and pharmacological interventions and differences in traumatism allow the view on inherent distinct processes of regeneration.

The adult liver harbors progenitor cells with the ability to differentiate either into hepatocytes or into cholangiocytes comparable to hepatoblast differentiation in embryonic life [75, 76]. Then, hepatic progenitor cells should behave like bipotential stem cells and expected to lie in the proximity to both hepatic and biliary compartments. This suggestion is supported by results of THEISE et al. [4] who studied normal livers as well as those with massive necrosis by immunostaining for cytokeratin 19 (CK19). They determined three-dimensional relationships by use of serial sections. From those studies it was concluded that the actual interface of hepatic parenchyma and the biliary tree is not the limiting plate, but rather the zone of hepatocytes adjoining the canal of HERING [77], radiating from the

portal tract. The canals of HERING are likely to be the source of ductular reactions in a variety of acute and chronic liver diseases and consist of facultative hepatic stem cells.

Re-expression of AFP in adult life occurs when hepatocytes proliferate. Thereby, this protein is a dedicated marker in studies on cell differentiation, tissue repair and hepatocarcinogenesis. AFP proved already useful in earlier studies with serology and immunofluorescent labelling techniques [46, 57, 64, 65, 78-85]. In the search for details of AFP synthesis and cellular fluctuations, immunohistology with peroxidase methods proved very promising and more suitable to detect histological changes in more detail than conventional techniques with immunofluorescence [58, 86, 87]. It is straight to note that other models including transgenic animals are equally useful to enable insights into the inherent ability of hepatocytes or bile ductular cells for hepatic regeneration [17, 34, 88-96].

Some major discussions pertained to the possibility of canalicular epithelial cells in hepatic restitution of damaged liver tissue with pros and contras for the population of so-called oval cells as progenitor or stem-like cells originating from Hering's canaliculi, equivalent in meaning with the canals of Hering, the intrahepatic bile ductules [77]. The nomenclature for one and the same cell structure as putative progenitor cells is sometimes confusing. Popular formulations for the term "oval cells" are notations such as canalicular epithelial cells, bile ductular cells, biliary epithelial cells, bile ductules, ductular oval cells, neoductules and ductular progenitor cells. Finally, they all together address the same cell type.

In our studies, we followed cellular fluctuations in various injury situations with focus on putative progenitor cells. The experimental approach included several injury models and the use of AFP as differentiation marker. Re-expression of AFP in postnatal life seemed to be a useful rationale since this oncodevelopmental protein is known to be associated with differentiation stages and pathophysiological developments. The models included (a) partial hepatectomy by surgery; (b) acute carbon tetrachloride intoxication; (c) hepatotoxicity by D-galactosamine (GalN) or by toxic doses of N-nitrosomorpholine (NNM) which inhibit hepatocytes to regenerate; and (d) carcinogenesis based on the use of the genotoxic NNM in both low and high doses, respectively. The necessary immunological and histological methods were adapted for this approach.

2 Materials and Methods

2.1 Animals

Injury models served to study progenitor cells in liver repair and hepatocarcinogenesis. Rats and mice are the most widely used animals for such purpose. Twelve-week-old rats and mice of male sex were segregated for the experiments. The well characterized BD X rat inbred strain was chosen. This rat strain proved to be appropriate in long-term studies on carcinogenesis because of its low incidence of spontaneous tumors [97, 98]. BD X rats were originally obtained from Prof. Druckrey (Forschergruppe für Präventivmedizin, Max Planck Institut für Immunbiologie Freiburg, Germany) and later bred in facilities of the Institut für Versuchstierkunde at the German Cancer Research Center (DKFZ Heidelberg). BALB/cJ mice were from the The Jackson Laboratory (Bar

Harbor, USA), and C3H/He mice from the Institut für Versuchstierkunde (Hannover, Germany). The inbred mouse strains BALB/cJ and C3H/He were chosen because both strains show significant differences in synthesizing the onco-developmental marker AFP. Several mechanisms of AFP gene control are mainly responsible for this [99, 100].

Parts of the experimental work were carried out at the German Cancer Research Center (DKFZ, Heidelberg, 1975-2006) and supported by research grants from the German Research Foundation (DFG, Deutsche Forschungsgemeinschaft). Mice and rats were held in the animal care facility of the DKFZ and maintained in air-conditioned rooms on a 12-hour day night cycle with free access to food and water. Studies were in accordance with German rulings to protect animals and according to guidelines and welfare assurance [101-103]. Animal studies were done under veterinarian surveillance by the Institut für Versuchstierkunde (DKFZ). Experimental and control groups were processed in parallel, the studies are summarized in Table 1.

In the case of surgical intervention such as partial hepatectomy or at autopsy for liver preservation and at the end of experiments, animals were maintained under anesthesia e.g., IP injection of ketamine and xylazine combinations or sodium pentobarbital or inhalation anesthesia with isoflurane [101-103]. Exsanguination was done by cardiac puncture under anesthesia, then animals were sacrificed by cervical dislocation.

Table 1: Experimental models in studies on hepatic repair and chemical hepatocarcinogenesis

Animal models	**Treatment**	**Study period, controls**
Normal controls — BALB/cJ mice — C3H/He mice — BD X rats	No treatment animals kept on standard diet and tap water	12-week-old animals no treatment
Partial hepatectomy — BALB/cJ mice — C3H/He mice — BD X rats	70% resection controls: sham operation	1-7 days post-op 5 animals per day
CCl_4 intoxication — BALB/cJ mice — C3H/He mice — BD X rats	100 µl CCl_4/100 g body weight controls: oral ingestion of liquid paraffin	1-7 days following injury 5 animals per day
GalN injury — BD X rats	375 mg/kg body weight (a) first dose at 8:30 a.m. (b) second dose between 8.00 and 9.00 p.m. controls: no treatment	1-10 days following injury 5 animals per day
Carcinogenesis by NNM — BD X rats	Chemical carcinogenesis (a) low dose feeding group 6 mg NNM/kg/day for 12 weeks (b) high dose feeding group 20 mg NNM/kg/day for 6 weeks (c) latent period	Carcinoma development (a) all animals bled at weekly intervals for serum AFP and 2 rats studied by histology (b) all animals bled at weekly intervals for serum AFP and 2 animals studied by histology (c) all animals bled at monthly intervals for serum AFP and 2 rats studied by histology

Abbreviations: CCl_4, carbon tetrachloride; GalN, D-galactosamine; NNM, N-nitrosomorpholine.

2.1.1 Partial hepatectomy

Surgical resection of liver lobes was essentially as described [104-106]. The use of magnifying operative loupes was useful in the mouse model. Intense regeneration will follow partial hepatectomy (about 70% liver weight) and proceeds fast within 6 to 8 days after operation. Histology is shaped by mitotic activity of the remaining mature hepatocytes [107]. Animals were bled 1 to 7 days after injury, livers were dissected out for histology.

2.1.2 Carbon tetrachloride

Carbon tetrachloride is a classical hepatotoxin leading to necrosis and apoptosis mainly in centrilobular areas [108]. Toxicity depends on a cytochrome P450 dependent monooxygenase system of the hepatocytes and on mechanisms generating toxic products such as free radicals. The latter are responsible for a peroxidative decomposition of microsomal and membrane lipids leading to structural damage and cell death [109, 110]. CCl_4 injuries were caused in rats and mice by the application of 100 µL CCl_4/100 g body weight (1 mL of 10% CCl_4 in liquid paraffin/100 g body weight). A single dose was directly applied into the esophagus by means of a trocar and syringe [66]. Animals were bled and livers dissected out between 1 to 7 days following injury.

2.1.3 Galactosamine

Galactosamine-HCl (GalN) leads to liver alterations similar in appearance to human viral hepatitis [67, 68, 111]. Liver injury by GalN is caused by its interference with the cellular uridine pool accompanied by reduced RNA synthesis and blocking of transcription [112, 113]. Hepatocyte necrosis and inflammatory reactions occur while the intrinsic hepatocyte regeneration is impaired. Toxic effects are dose dependent, the structural injuries are suggested to be secondary to the metabolic lesion. GalN was applied by IP injections of GalN in 0.9% NaCl, 375 mg/kg body weight [60, 84]. Livers and blood were taken 1 to 10 days after injury.

2.1.4 N-Nitrosomorpholine

The genotoxic carcinogen N-nitrosomorpholine (NNM) was synthesized by Dr. R. Preussmann (DKFZ, Division of Toxicology, Heidelberg). NNM was given in different doses in drinking water: (a) 6 mg/kg/day for 12 weeks or (b) 20 mg/kg/day for 6 weeks; all treatments were stop experiments [114]. Carcinogen intake was controlled by daily measurements of water drunk. NNM is known to produce liver tumors. Different malignant phenotypes may develop in the same liver. While low NNM doses have no significant toxic effects, high NNM doses are clearly toxic [98]. Hepatocellular carcinomas developed with either of the used NNM feeding schedules. Animals were bled at defined intervals for AFP detection and histological analysis.

2.1.5 DNA synthesis

Tritiated thymidine (^{3}H thymidine) was purchased from NEN Radiochemicals (Boston, USA). Thymidine incorporation in vivo was studied by intraperitoneal injections of [^{3}H] thymidine (spec. act. 6.7 Ci/mmole) according to these schemes: (a) 60-min pulse labelling by injecting 500 µCi/rat; (b) pulse labelling over 24 h by three successive injections of 250 µCi each; (c) pulse labelling for 60 min by injection of 500 µCi/rat followed by daily injections of unlabelled thymidine for 7 days [58]. Livers were dissected out and treated as described in section *2.7 Tissue fixation and processing for microscopy* and in section *2.11 Autoradiography*.

2.1.6 Control animals

Animals were kept on standard diet and tap water ad libitum. They were either untreated or sham operated or given oral ingestions of liquid paraffin.

2.2 Chemical reagents

Biochemicals and standard laboratory reagents of highest available purity were obtained from Merck (Darmstadt) unless noted otherwise. Horseradish peroxidase (HRP, EC 1.11.1.7, approx. 1000 units/mg solid using ABTS) and glucose oxidase (GOD, EC 1.1.3.4, 100,000-250,000 units/g solid using peroxidase as the coupling enzyme) were from Boehringer (Mannheim) and from Sigma-Aldrich (München).

Gel filtration methods were applied for molecular size sieving. Separation of biological macromolecules according to their molecular weight is a major application. Different particle size grades and degrees of concentration or cross-linking enable various fractionation ranges for globular proteins. Furthermore, gel media are used for the preparation of immunosorbents by covalent coupling of antigens or antibodies onto the gel matrix. For these purposes, media from dextran, acrylamide, agarose, or combinations of all of them were used. Inert gel media were obtained from Sigma-Aldrich (München) e.g., Sephadex and Sepharose, products from Pharmacia Uppsala Sweden), then further processed according to laboratory needs. A selection of gels for the separation of high molecular weight molecules is shown in Table 2.

Table 2: Gel filtration media for sieving of high molecular weight proteins

Filtration medium	Gel matrix	Fractionation (MW) [a]
Sephadex G-200	Dextran	$5 \times 10^3 \ldots 6 \times 10^5$
Sephacryl S-200	Dextran/acryl.	$5 \times 10^3 \ldots 2.5 \times 10^5$
Sephacryl S-300	Dextran/acryl.	$1 \times 10^4 \ldots 1.5 \times 10^6$
Sephacryl S-400	Dextran/acryl.	$2 \times 10^4 \ldots 8 \times 10^6$
Sepharose 2B/CL-2B	Agarose	$7 \times 10^4 \ldots 40 \times 10^6$
Sepharose 4B/CL-4B	Agarose	$6 \times 10^4 \ldots 20 \times 10^6$
Sepharose 6B/CL-6B	Agarose	$1 \times 10^4 \ldots 4 \times 10^6$

[a] Molecular weight (Da), fractionation range

2.3 Biochemical and serological methods

Reagents for serology and immunohistology such as immune sera, purified antigens, antibodies, and antibody-enzyme conjugates were prepared using selected methods. Qualitative and quantitative controls followed standard methods (Table 3). These methods covered protein determinations with the Folin-phenol reagent [115] and the quantitation of enzyme activities [116]. Furthermore, standard methods for the qualitative and quantitative evaluation of antigens and antibodies are based on electrophoresis in polyacrylamide gels [117, 118], immunoelectrophoretic analysis in agar or agarose gels [119]. Also, various single and radial immunodiffusion techniques as well as electro-immunodiffusion methods in agarose gel were used [120-123]. For enhanced sensitivity of the gel diffusion techniques, an enzyme amplification method using GOD labelled antibodies has been adapted for quantitation [124].

Enzyme-linked immunosorbent assays (ELISA) and technical adaptations of the original method to microtiter plates served to measure very low quantities of AFP in serum [125-127]. Antigen measurements were carried out by an antibody sandwich method [125]. The solid phase coated with rabbit anti-AFP antibodies acted as capture antibodies for AFP binding from serum specimens. After washings, detector antibodies (peroxidase labelled sheep anti-AFP) followed with the next incubation step. The formed sandwich complexes were revealed with peroxidase substrate. The colored reaction product was measured in a photometer. Assays were performed with serum dilutions and in duplicate. The test profile included control animals and standards with known AFP concentrations. AFP concentrations were determined by a curve fitting programme of the spectrophotometer. The detection limit of serum AFP was in the order of 5 ng/mL [114].

Table 3: Standard methods for antigen and antibody evaluation

Method	Main use, application	References
Electrophoresis polyacrylamide	Fractionation and analysis of biomolecules	Shapiro AL et al. 1967 Weber K, Osborn M 1969
Double radial immunodiffusion	Immuno-serological properties of antigens and antibodies	Ouchterlony Ö 1949
Single linear immunodiffusion	Analysis of antigen-antibody reactions for quantification	Oudin J 1946 Oudin J 1949
Single radial immunodiffusion	Quantification of antigens and antibodies	Mancini G et al. 1965
Immuno-electrophoresis	Immuno-serological properties of antigens and antibodies	Grabar P, Williams CA 1953
Electro-immunodiffusion rocket electrophoresis	Immuno-serological properties, quantification of antigens	Laurell CB 1965 Laurell CB 1966 Clarke HGM, Freeman T 1968 Kuhlmann WD 1984
Enzyme-linked immunosorbent assay	Quantification of antigens and antibodies	Engvall E, Perlmann P 1971 Schuurs AH, van Weemen BK 1977 Kuhlmann WD, Peschke P 2006

2.4 Purification of AFP

AFP was purified from amniotic fluids (taken from 14 days old mouse and rat embryos, respectively) using preparative electrophoretic and immunosorbent methods. In a first step, amniotic fluid was submitted to gel electrophoresis in 5% acrylamide-0.8% agarose plates [128]. AFP molecules migrating in the α1-zone were washed out from the supporting matrix by homogenization of the gel matrix followed by centrifugation. Purity of AFP was determined by SDS polyacrylamide gel electrophoresis [129, 130] and by various gel diffusion techniques (section *2.3 Biochemical and serological methods*) making use of cross-reactivity between mouse and rat AFP. For controls, a rat AFP immune serum was obtained from Nordic Immunological Laboratories (Netherlands).

The purified AFP molecules were used to immunize rabbits to prepare specific anti-AFP immune sera. The latter were absorbed with glutaraldehyde cross-linked normal mouse serum proteins (rat serum proteins, respectively) with the aim of eliminate possibly contaminating antibodies and to render them monospecific. Immunosorbent columns were finally prepared from those monospecific immune sera [58, 86].

2.4.1. Scaling of AFP preparation

Immuno-affinity chromatography is presumed to be one of the most powerful separation techniques because of the high specificity of the antigen-antibody reaction. For example, antigens or antibodies can be coupled to agarose beads and used for selective isolation of antibodies or antigens, respectively. In the case of AFP purification, immunosorbents were prepared by covalent binding of γ-globulin fractions of anti-AFP immune sera (γ-globulin fractions fractionated beforehand by DEAE ion exchange chromatography) to Sepharose 4B matrix (Pharmacia, Sweden). First, cyanogen bromide is reacted with agarose hydroxyl groups to form imidocarbonates and cyanate esters [131, 132]. These active groups will form isourea linkage with amino groups of the ligands to be added in the second step (specific antibodies). In the third step, the immobilized ligand is used as an immunosorbent that will bind the corresponding antigen in a reversible manner from added amniotic fluid. Unreactive substances are washed off from the immunosorbent by adding phosphate buffered saline (PBS) followed by washing with phosphate buffered saline (PBS) and with 2 M NaCl in 0.1 M NaOH-glycine buffer pH 9. Bound AFP molecules are finally eluted from the immunosorbent by treatment with a desorption buffer pH 7.4 containing 3 M chaotropic ions [133].

Eluted AFP fractions were filtrated on immunosorbent columns prepared by coupling γ-globulin fractions from immune sera against normal mouse (normal rat serum proteins, respectively) to Sepharose 4B beads. These immunosorbent columns served to bind traces of contaminating normal plasma proteins to secure the purity of AFP. Purification processes were monitored by immunological means and by SDS polyacrylamide gel electrophoresis (see above).

2.5 Immune sera, antibodies

Antibodies are produced by immunization of animals. Typical species are rabbits. Also, sheep, goats, donkeys, or horses are immunized when large volumes of immune sera are needed. We made antibodies usually in rabbits and sheep.

Immune sera against the bulk of serum proteins were obtained by immunization of rabbits with normal adult mouse and rat serum proteins (whole serum, respectively). Primary injections were done with whole serum mixed up with complete Freund's adjuvant. Booster injections followed at monthly intervals with the same antigens emulsified in incomplete Freund's adjuvant. Injections were intramuscularly and subcutaneously at different sites [134, 135].

Immune sera against antigens such as rat and mouse IgG, rat and mouse AFP and enzymes (GOD) were prepared by immunization of rabbits with the respective antigens. Injections were done with pure antigen in saline and mixed with complete Freund's adjuvant. Booster injections (intramuscularly and subcutaneously) followed one month later with antigen dissolved in saline and mixed with incomplete Freund's adjuvant. Further booster injections followed at monthly intervals. The blood was tested for antibodies two weeks after the fifth injection.

Antibodies were also raised in sheep by immunization with rat AFP, mouse AFP and rabbit IgG, respectively. In each case, primary injections were done with purified antigen in saline and emulsified with Freund's adjuvant. Animals received subcutaneous injections at different sites (shoulder and hip region). Booster injections were repeated at monthly intervals for 6-8 months.

Prior to use, immune sera and purified antibodies were submitted to specificity testing because minor contaminants may produce significant amounts of undesired antibodies. Immunological specificity was controlled by standard methods (see section *2.3 Biochemical and serological methods*). Immuno-staining of tissue arrays with known antigen targets and known histological pattern were included as validation tools.

Immune sera raised against purified antigens were routinely absorbed with insoluble immunosorbents prepared by glutaraldehyde cross-linking of unrelated proteins (normal adult serum proteins). This measure secured the specificity of immune sera as much as possible. The effect was controlled by standard methods (section *2.3 Biochemical and serological methods*). There exist several techniques of antibody preparation from whole immune sera with varying degrees of purification (Table 4). The needed degree of purification, however, depends to a great extent on the type of experimentation. For routine work, we used antibodies being prepared either by DEAE ion exchange chromatography or by gel filtration (Sephadex G 200). Immunosorbent methods proved in all applications most suitable [132, 133, 136]. Because immunoaffinity chromatography is quite elaborate, this method was mainly preferred for the preparation of enzyme-antibody conjugates in immunohistology and in enzyme-immunoassays of the ELISA type to quantify serum AFP [124].