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PURIFICATION OF SPECIFIC ANTIBODY TO α-FOETOPROTEIN AND ITS IMMUNOLOGICAL EFFECT ON CANCER CELLS

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SUMMARY

Human or rat α-foetoprotein (AFP) was highly purified from ascitic fluid or serum of hepatoma bearers. The purification was carried out mainly by means of immunoadsorbent chromatography using Sepharose coupled to specific anti-AFP antibody with BrCN activation, and by Sephadex gel filtration.

Horses were immunized with the purified AFP and the specific antibody was isolated from the antiserum by means of an immunoadsorbent coupled to purified AFP. The specific antibody was found to bind specifically with AFP-producing tumour cells.

The antibody was applied for radio immunodetection of the tumour. ^{125}I-labelled antibody was administered to patients or rats with hepatoma, and radioactivity localized in the tumours was scintiscanned with a scintillation camera. In this way, the location of the tumour was detected in about 50% of the hepatoma bearers.

The cytotoxicity of the antibody was clearly demonstrated both *in vitro* and *in vivo* in animal experiments. The antibody was administered to patients with advanced hepatoma. Although no improvement of the disease was demonstrated, serum AFP levels decreased greatly and in some cases low AFP levels were maintained for long periods suggesting that the antibody suppressed AFP-producing hepatoma cells.

No significant side effects were observed in patients who had been administered with the horse antibody.

INTRODUCTION

Trials of antiserum therapy for cancer have been made over a long period, but none has been successful. The principle of antiserum therapy for cancer is the presumption that cancer-specific antigens are present in cancer cells and the antibodies to these antigens are cytotoxic to cancer cells but not to normal cells. The presence of cancer-specific antigens, however, has not yet been clearly demonstrated.

α-Foetoprotein (AFP) is a foetal serum protein in mammals, but after birth its

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production ceases and the serum level in human adults is less than 20 ng/ml. However, when hepatoma develops the production of this protein recommences; serum AFP levels are in the range $5 \cdot 10^2$–$1 \cdot 10^7$ ng/ml in over 80% of hepatoma patients. Increased serum AFP levels are seen also in patients with yolk sac tumours which develop most frequently in the testes and ovaries. In the foetus AFP is produced mostly in the yolk sac in early stages of embryogenesis, and in later stages in the liver. In adults, AFP is produced only in liver cancer and yolk sac tumours. Therefore, AFP may be said to be a cancer-specific protein in older children and adults.

Animals easily produce serum antibodies to AFP when immunized. In this study, AFP and its antibody have been purified by affinity chromatography using immunoadsorbents and by gel filtration. Some immunological applications of the purified antibody were attempted in animal experiments, *e.g.*, for the radioimmuno detection of cancer localization and for the therapy of AFP-producing cancer. This paper describes some results obtained so far.

PURIFICATION OF AFP AND ITS ANTIBODY

Purification of AFP by immunochemical methods

Sources of AFP

Human foetal blood plus tissues obtained by artificial abortion, rat foetal blood from 12–15 weeks' gestation, serum or ascitic fluids of hepatoma patients and ascitic fluids or serum of rats transplanted with AH66 rat hepatoma were the sources of AFP. AH66 is a rat ascites hepatoma cell line which is a high AFP producer. The concentration of AFP in ascitic fluids is about 50–100 μm/ml in the third week after transplantation. Rats died in 3–4 weeks as a result of tumour growth and about 50 ml of ascitic fluid had accumulated intraperitoneally by the time of death.

The first antiserum

Animals, such as rabbits, horses and goats, were immunized with the above materials containing AFP. Freund's complete adjuvant was used with an effective aid. The antisera obtained were absorbed with normal adult serum. The absorbed antisera reacted only with AFP. Fig. 1 shows the monospecificity of an absorbed antiserum which reacts only with AFP and not with other serum proteins.

The specific antisera were used for the immunochemical purification of AFP. The purification was carried out in the following two ways: (1) by gel filtration from dissociated antigen–antibody complex, and (2) by affinity chromatography with immunoadsorbent.

Isolation of AFP by gel filtration from dissociated antigen–antibody complex (batch method)[1]. The monospecific antiserum was added to materials containing AFP in the optimal proportions and the mixture was allowed to stand overnight to complete the precipitin reaction. The antigen–antibody complex formed was spun out and washed repeatedly with a large amount of saline. The precipitate was then dissolved in 0.1 *M* glycine–HCl buffer (pH 1.8) as a result of which the antigen–antibody complex dissociated into the individual components, which were separated by molecular sieving with Sephadex[2]. A Sephadex G-200* column was equilibrated with the

* Sephadex and Sepharose were purchased from Pharmacia (Uppsala, Sweden).

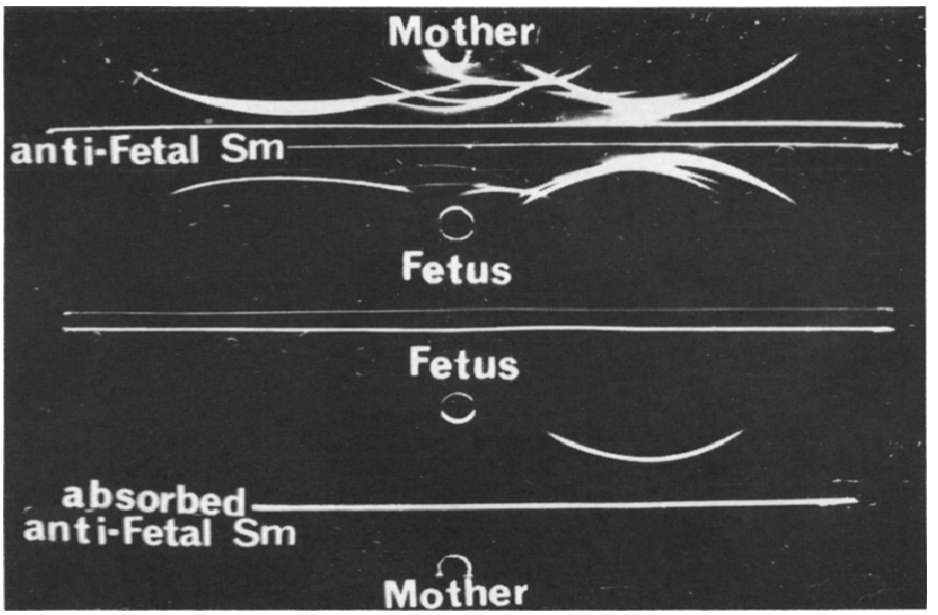

Fig. 1. Immunoelectrophoresis of antisera to human AFP. Mother: serum of an adult serum. Foetus: serum of a 5-month-old foetus. Anti-foetal serum: antiserum of rabbits immunized with human foetal serum. Absorbed anti-foetal serum: rabbit antiserum absorbed with adult human serum.

buffer as described above, and the dissociated complex was applied and eluted with the same buffer. Three peaks were eluted: the first, small peak was shown to consist of aggregates of denatured antibody and antigen–antibody complex; the second, large peak, eluting at the position of molecular weight 150,000, was demonstrated to contain anti-AFP antibody; the third peak was found to consist of AFP. The third peak fraction was collected and dialyzed against 0.1 *M* phosphate buffer (pH 7.0) and concentrated. This fraction was re-chromatographed on a Sephadex G-100 column equilibrated with the same phosphate buffer and eluted. A small fraction eluted at the void volume was antigen–antibody complex which was soluble at pH 7.0. The main peak, eluting at the position of molecular weight 70,000, was taken as purified AFP. The yield was approximately 70–80 % of that in the original serum or ascitic fluid.

Isolation of AFP by affinity chromatography with immunoadsorbent[3]. The IgG fractions obtained from the antisera to AFP were coupled to Sepharose 4B by the cyanogen bromide method devised by Axén *et al.*[4]. Approximately 5–10 mg of IgG fraction were coupled per millilitre settled volume of Sepharose. This IgG-coupled agarose was placed in a column and washed with saline buffered with 0.01 *M* phosphate (pH 7.0). The materials containing AFP dialyzed against the buffered saline were applied to the immunoadsorbent column. The column was washed thoroughly with the buffered saline and then AFP bound to the immunoadsorbent was eluted with 0.1 *M* glycine–HCl buffer (pH 1.8). An eluted fraction containing AFP was immediately neutralized with 0.2 *M* K_2HPO_4 and passed through a Sephadex G-200 column equilibrated with 0.1 *M* phosphate buffer (pH 7.0). The main peak, eluting at molecular weight 70,000, was collected as the purified AFP.

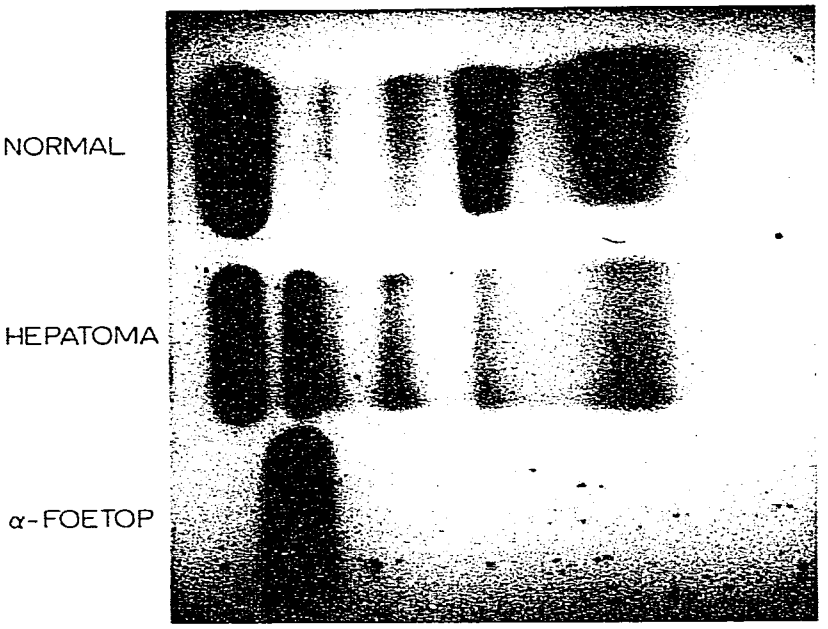

Fig. 2. Electrophoresis of purified human AFP on a cellulose acetate membrane. Serum from a hepatoma patient shows a clear band at a position slightly ahead of α_1-globulin. This serum contained AFP at an extremely high concentration. Purified AFP shows a single band.

Some properties of purified AFP. The purified human AFP showed a single band on electrophoresis either on a cellulose acetate membrane or on polyacrylamide gel. The purified rat AFP gave two bands on disc electrophoresis. Fig. 2 shows the electrophoretic pattern of human AFP on a cellulose acetate membrane. The purified AFP did not give any precipitin line on immunoelectrophoresis against antisera to normal adult human or rat serum proteins. AFPs from various animals were purified from the foetal serum, taking advantage of the fact that the antiserum to human AFP cross-reacts with AFPs of the other mammalian species tested (horse, cow, pig, goat, sheep, dog, cat, rabbit, rat and mouse)[3]. The electrophoretic mobilities of these AFPs differed according to the species.

The purified AFP was crystallized by the gradual addition of saturated ammonium sulphate solution or from pentanediol solution (Fig. 3).

The physico-chemical properties and amino acid composition of human and rat AFP isolated from the serum of hepatoma patients and from foetal serum have been reported previously[5]. No differences between the AFPs of the foetus and hepatoma bearers could be detected in their immunochemical and physico-chemical properties (Figs. 4 and 5).

The purified AFP was a good antigen. Animals such as horses, rabbits and goats produced antibody by immunization with purified AFP suspended in Freund's complete adjuvant. Amounts of AFP from 100 μg to 2 mg, depending on the size of the animal, were sufficient to produce antisera with reciprocal antibody titres of 32 to 128. The antisera were monospecific, as shown in Figs. 4 and 5.

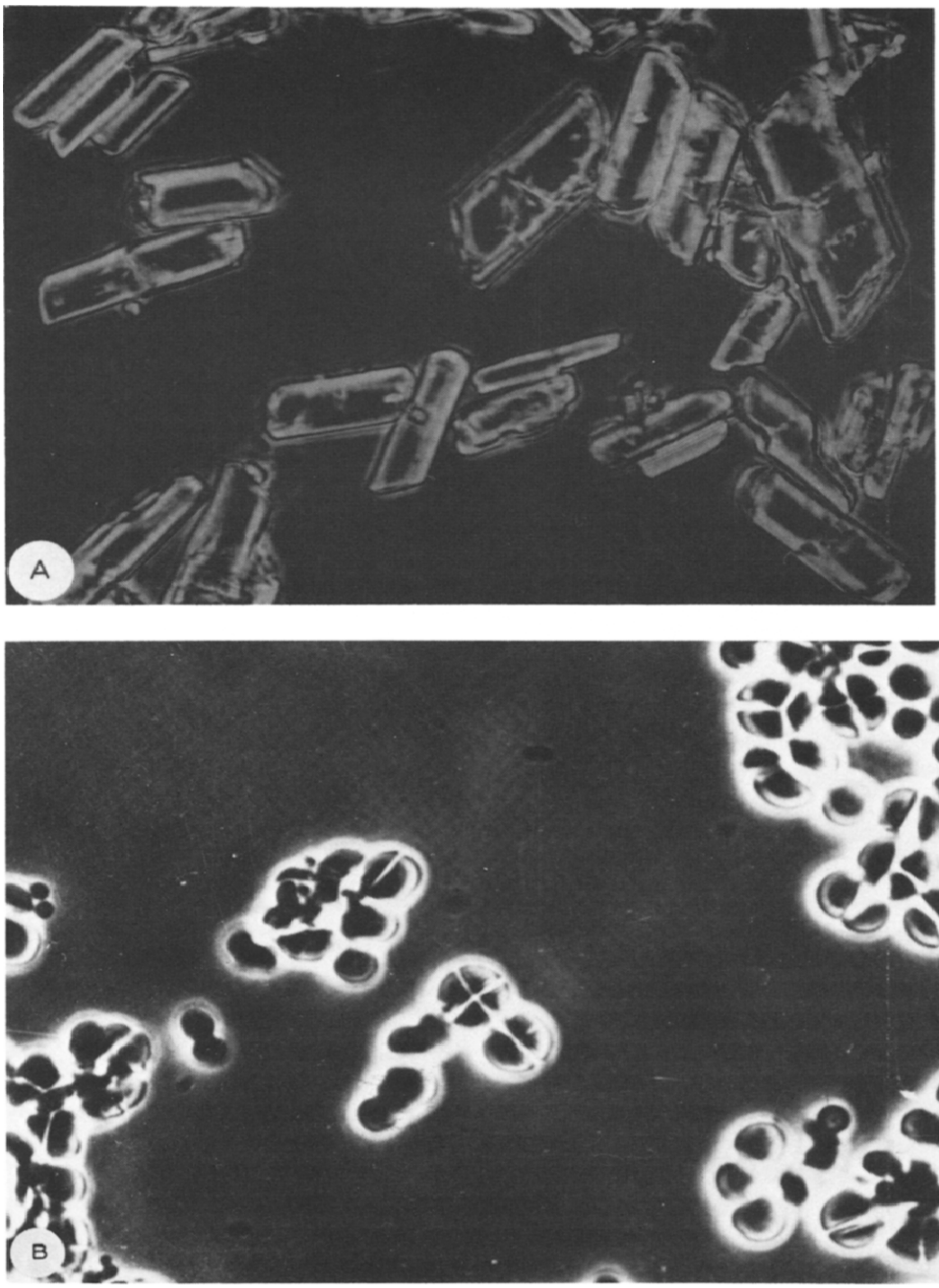

Fig. 3. (A) Crystals of human AFP obtained using ammonium sulphate solution. (B) Crystals of human AFP obtained by the pentanediol method: about 1% of AFP was dissolved in 10% pentanediol in a small dish, which was placed together with another dish filled with 50% pentane diol in a tightly closed chamber. The vapour of pentanediol from the latter dish penetrated the AFP dish, gradually increasing the concentration of pentanediol. Crystals were obtained within 2 days.

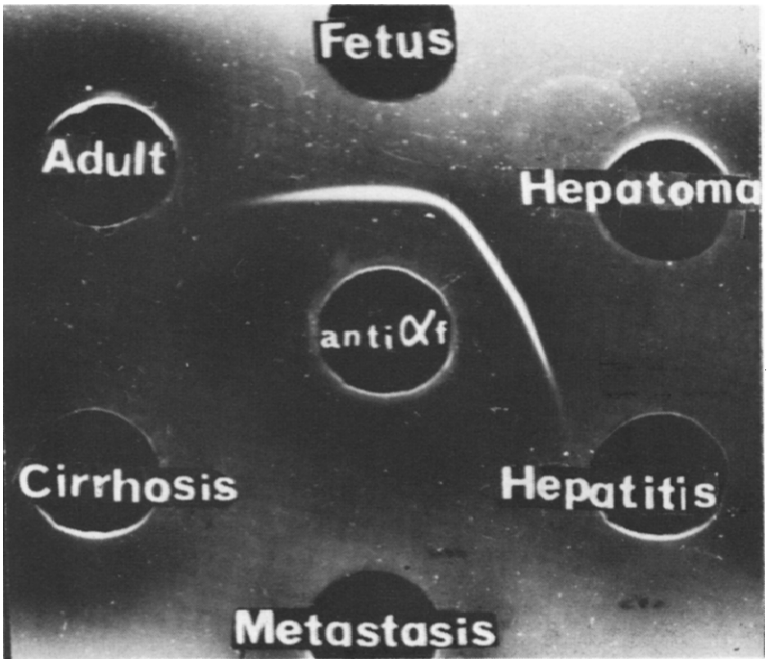

Fig. 4. Specificity of anti-AFP serum. Anti-αf: horse antiserum against human foetal AFP. Hepatoma: serum of a patient with hepatoma. Hepatitis: serum of patient with hepatitis. Metastasis: serum of a patient with metastatic liver cancer (original: gastric cancer). Cirrhosis: serum of a patient with liver cirrhosis. Adult: serum of a healthy adult man. Foetus: serum of a human foetus, 5 months gestation.

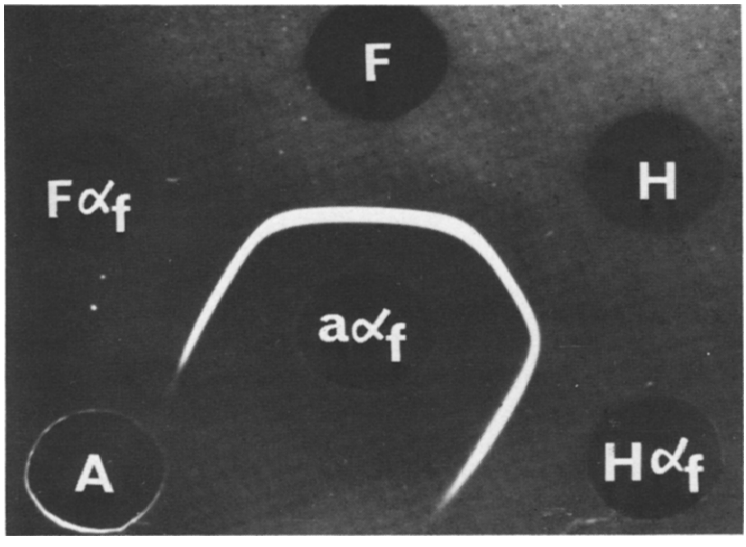

Fig. 5. Specificity of anti-AFP serum. aαf: a horse antiserum to human AFP isolated from serum of a hepatoma patient. F: serum of a 5-month-old human foetus. H: serum of a hepatoma patient. A: pooled serum of normal adult human. Fαf: AFP purified from serum of a foetus. Hαf: AFP purified from ascitic fluid of a hepatoma patient.

Purification of antibody and its properties

Reversed immunoadsorbent column chromatography was used for the purification of antibody. Purified human or rat AFP was coupled to Sepharose 4B by the cyanogen bromide technique[4]. Approximately 5–10 mg of AFP were bound per millilitre settled volume of Sepharose. A typical experiment was carried out as follows. About 200 ml of AFP-coupled Sepharose were placed in a 50 × 5 cm I.D. column and about 1 l of the horse antiserum was applied. This column is capable of binding about 1.4 g of the antibody. After thorough washing with saline, 8 *M* urea solution buffered with 0.1 *M* phosphate (pH 7) was passed through the column. The antibody eluted in tubes 26–41, as shown in Fig. 6, was collected and further purified by gel filtration using Sephadex G-200. The first small fraction eluting at the void volume was discarded and was probably an aggregate of denatured antibody. The main peak component, with a molecular weight of about 150,000, was collected as a purified antibody preparation.

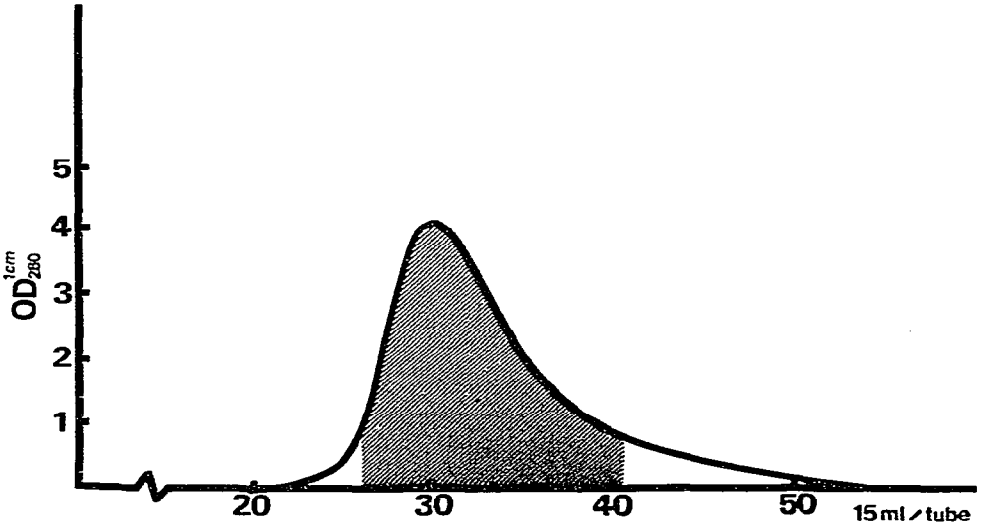

Fig. 6. Elution pattern of horse antibody to human AFP. The column was filled with human AFP-coupled Sepharose and horse antiserum was applied. The antibody bound to the immunoadsorbent was then eluted with 8.0 *M* urea solution. Fraction volume: 15 ml. The fractions in the shaded area were collected.

Some immunochemical properties of the purified horse antibody to human AFP

Fig. 7 shows an immunoelectrophoresis pattern of a purified horse antibody to human AFP. Two precipitin lines were distinguished. A slower fraction is located in the IgG position, and a faster fraction is located in the position of IgA. The latter fraction was considered to be the T-globulin [IgG(T)], which is an immunoglobulin specifically found in horse antiserum[6,7]. IgG(T) was identified with a specific antiserum to IgG(T), although the data are not shown in Fig. 7. These two fractions, IgG and IgG(T), could easily be separated by DEAE-cellulose chromatography because of the difference in their electric charges, although the specific antibody activities were identical.

The antibody activity was determined by the quantitative precipitin reaction,

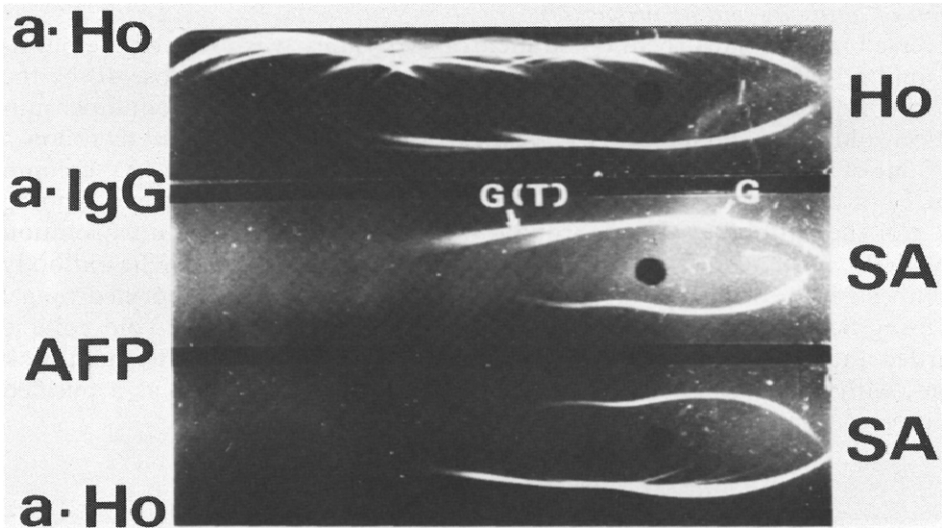

Fig. 7. Immunoelectrophoresis of a purified horse antibody to human AFP. a-Ho: rabbit antiserum to horse serum proteins. a-IgG: rabbit antiserum to horse IgG. Ho: horse serum. SA: purified specific antibody. G(T): IgG(T). G: IgG.

and it was found that 1 mg of the antibody could bind about 150 μg of AFP. The molar ratio of binding of antibody to antigen was about 3:1 at the optimal proportions.

APPLICATION OF AFP ANTIBODY IN CANCER RESEARCH

AFP is a protein that is produced in the adult only by hepatoma and yolk sac tumour cells, production by normal cells being almost negligible. AFP is found not only inside but also on the surface of the tumour cells. The binding of the AFP antibody to these cells has been clearly demonstrated by conventional immunohistochemical techniques. Recently, in our laboratory, the presence of AFP was demonstrated directly in the cell membrane fraction of rat ascites hepatoma isolated by cell component fractionation. The property of the antibody which binds to AFP-producing cells is applicable to the immuno detection of the tumor *in vivo*. On the other hand, AFP antibody reveals cytotoxicity when it binds to the AFP-producing cells. This cytotoxic effect may be applicable to therapy for cancer. Some experimental data are presented below.

Radioimmuno detection of hepatoma in rats

Transplanted hepatoma

About $1 \cdot 10^5$ cells of AH7974 rat ascites hepatoma were transplanted subcutaneously in the thighs of rats. This tumour produces AFP to a certain extent and grows well both intraperitoneally and subcutaneously. Fig. 8 shows a tumour grown to a diameter of about 2 cm in 2 weeks. Serum AFP levels at this stage were several hundred nanograms per millilitre.

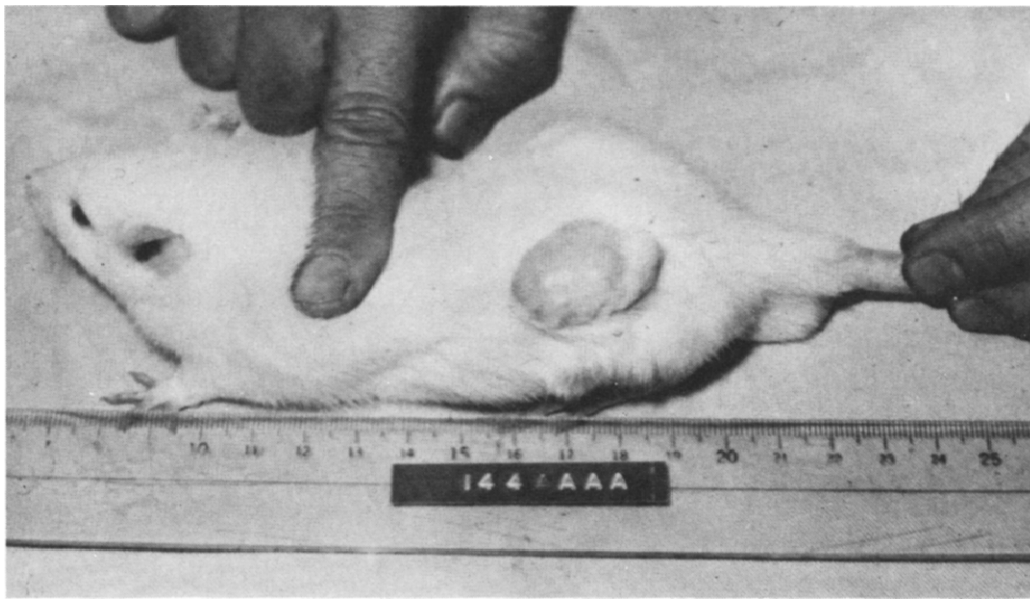

Fig. 8. Transplanted hepatoma in a rat. AH7974, an ascites hepatoma cell line, was transplanted subcutaneously in the right thigh. About 2 weeks after transplantation the tumour grew to the size shown.

Radioactive labelling of the horse antibody was carried out by the Chloramine-T method using ^{131}I or ^{125}I. The specific radioactivity was about 150 μCi per 100 μg of protein, corresponding to about 0.5 mole of iodine per mole of the antibody. About 100 μCi of the labelled antibody were administered to rats bearing hepatoma as above and scintigrammed every day for a week. The tumour image was demonstrated as shown in Fig. 9. The images after 24 and 48 h were not clear because the radioactivity was distributed over a wide area, but the tumour image became more clearer after the third day. Fig. 9B shows the images on days 5 and 7. Ten rats were examined by the same procedure and six showed positive images.

Normal horse IgG was labelled in the same way and administered to rats bearing tumours. A slight uptake of radioactivity was observed in some rats. This uptake may have resulted in localization of labelled antibody due to endophagocytic activity of the tumour cells for foreign substances.

Hepatoma developed in the liver of rats fed a carcinogen

Rats were fed with a diet containing 0.06% of *p*-dimethylaminoazobenzene, a hepatocarcinogen of rats. Hepatoma developed approximately 12 weeks later. 100 μCi of ^{125}I-labelled antibody was given and the animals were scintiscanned. Fig. 10 shows a positive image of the tumour taken at 72 h after administration of the radioantibody. After scintigraphy, the rat was autopsied on day 7 and the organs were taken out as shown in Fig. 11. These organs were scintiscanned directly. Fig. 12 shows the images at the same sites corresponding to the location of the hepatoma shown in Fig. 11.

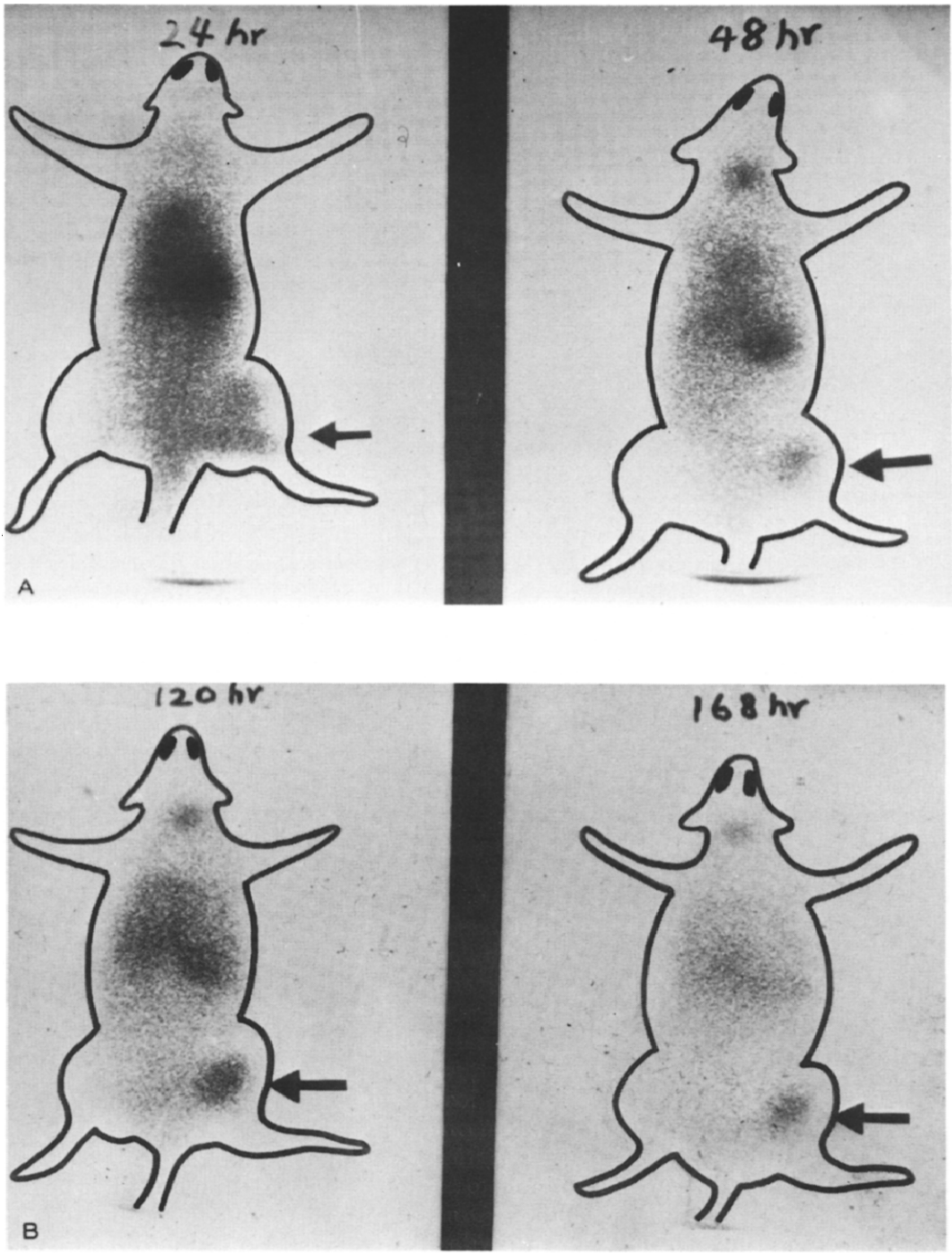

Fig. 9. (A) Scintigrams of rat hepatoma. Rats as shown in Fig. 8 were given i.v. about 100 μCi of ^{125}I-labelled anti-AFP antibody and scintigrammed 24 and 48 h after injection. The arrows indicate the tumour localization. (B) Scintigrams on days 5 and 7.

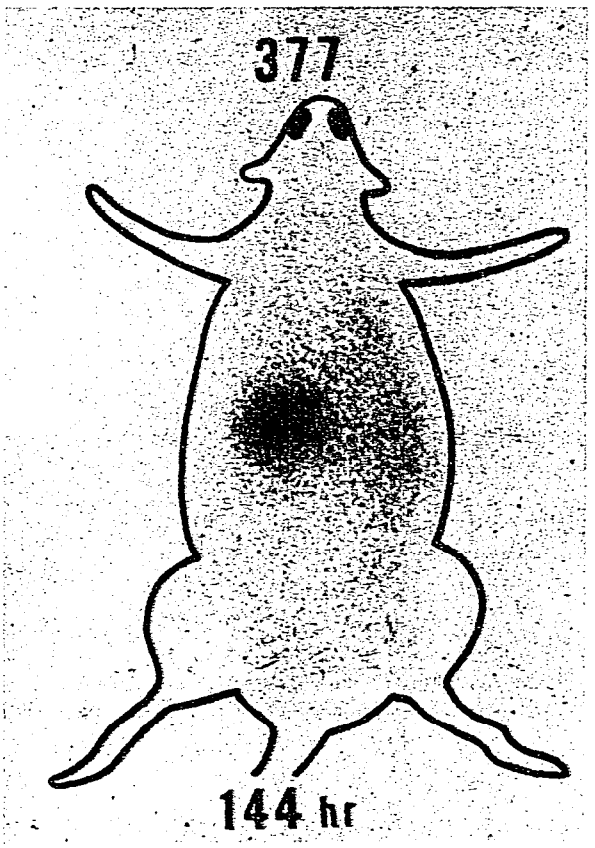

Fig. 10. Scintigram of hepatoma developed in the liver of a rat by DAB feeding. Scintiscanned on day 6 after administration of 100 μCi of the radioantibody.

Effect of AFP antibody on tumour cells

Cytotoxicity

AFP antibody has been demonstrated to be cytotoxic[8]. The cytotoxicity test was carried out as follows. The rat ascites containing AH66 tumour cells was drawn and cells were collected by slow centrifugation after washing with saline three times; $1 \cdot 10^4$ cells were suspended in 0.1 ml of saline. Then 0.1 ml of antibody solution of various concentrations was added to the cell suspension and incubated for 2 h at 37°C. The number of dead cells was counted by the Trypan blue exclusion method.

Fig. 13 shows the result of an experiment with a horse antiserum and a purified antibody. Normal horse serum and its IgG were used as controls. An obvious cytotoxicity was observed.

Inhibitory effect of the antibody on growth of tumour cells in culture

AH66 tumour cells were cultured in Eagle's medium containing 20% of foetal calf serum. Anti-rat AFP horse serum, or its purified antibody preparations, were

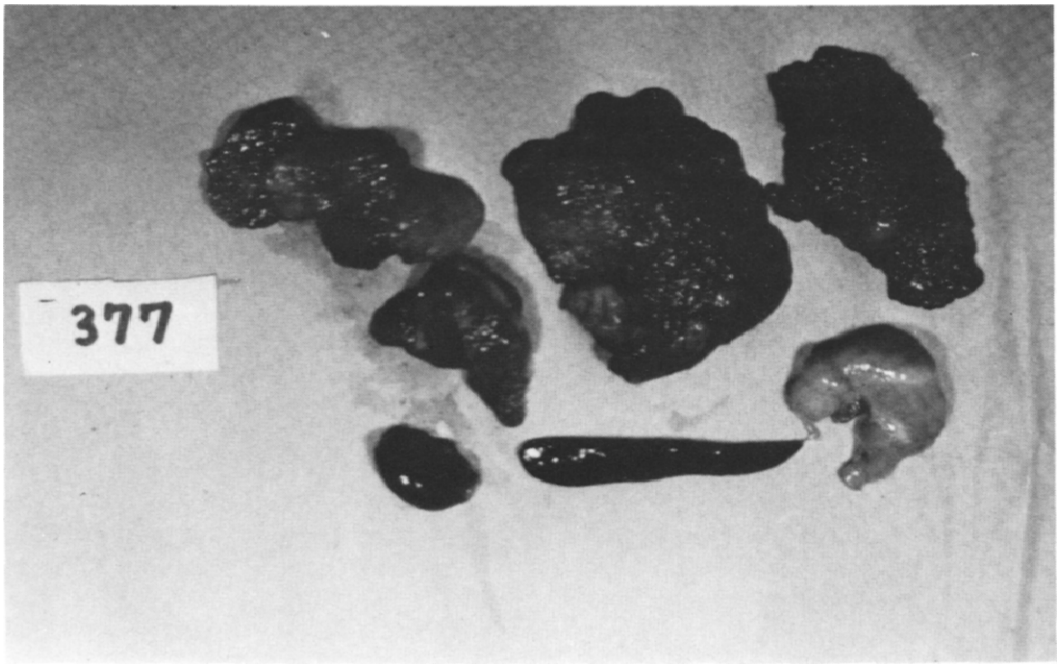

Fig. 11. Organs of the rat fed DAB. The rat in Fig. 10 was autopsied and the organs were taken out. The liver was cut into four pieces (upper row). Lower row: stomach, spleen and kidney. Hepatoma can be seen in two pieces of the liver.

added at various concentrations and the growth curves were compared. Normal horse serum and normal horse IgG were tested as controls. Fig. 14 shows the result of some experiments. An obvious inhibitory effect of antiserum and purified antibody was observed.

Cytotoxicity of the antibody upon tumour cells in Millipore chambers

AH66 cells ($5 \cdot 10^4$) were confined in Millipore chambers and inoculated into the peritoneal cavity of rats. One day after inoculation 1.2 mg of a purified antibody in 1 ml of saline was administered i.p. to the rats. The chambers were removed 48 h later and the number of dead cells was counted by the Trypan blue exclusion method. Normal horse IgG was used as a control. About 34% of the cells were dead in the antibody sample, but only 9.4% in the control group (average figures for five experiments).

Anti-tumour effect of the antibody in vivo

Rats were inoculated with $1 \cdot 10^4$ AH66 cells i.p. and 400 μg of the purified antibody were administered i.p. on days 3, 5 and 7 after the tumour cell inoculation. Saline and normal horse IgG were used as controls. Survival rates of the rats are shown in Table I. The rats of the saline group died within 16 days but their lifespan approximately doubled after treatment with the antibody.

Fig. 12. Scintigram of hepatoma in liver. The liver samples in Fig. 11 were directly scintigrammed. The radioactivity was detected in the same position corresponding to the site of hepatoma.

DISCUSSION

In spite of the efforts of many investigators, the isolation of cancer-specific antigen has not been successful although its presence has been suggested indirectly.

AFP is present as the main component of foetal serum but in the healthy adult this protein is synthesized only in trace amounts. When some tumours, such as hepatoma or yolk sac tumour, occur, the production of this protein commences again in tumour cells and the serum levels increase. AFP may be said to be a cancer-specific antigen, and the demonstration of its elevated serum levels can be used for the diagnosis of these tumours.

Serum AFP levels also increase in women with malformed pregnancy, *e.g.*, neutral tube defects, such as anencephalia and spina bifida. The determination of AFP is therefore of great importance at present for the antenatal diagnosis of such foetal malformations.

The determination of AFP in body fluids and tissues is possible only by im-

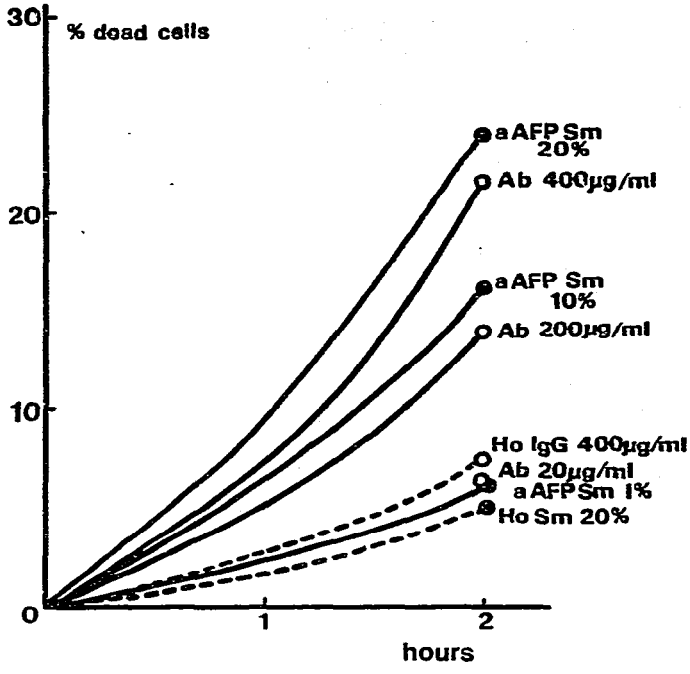

Fig. 13. Cytotoxicity of AFP antibody. AH66 rat hepatoma cells were incubated at 37°C for 2 h in a medium containing the specimens listed below and the cytotoxicity was examined by the Trypan blue exclusion method. aAFPSm: a horse antiserum to rat AFP. Ab: a purified specific antibody from aAFPSm. HoIgG: IgG fraction of normal horse serum (control to Ab). HoSm: serum of normal horses (control to a AFPSm). The cytotoxicity index (*CI*) was calculated by the equation $CI = [(b - a)/(T - a)] \cdot 100\%$, where T = total number of tumour cells, a = number of dead cells at time zero and b = number of dead cells at a certain time after the incubation. Dead cells = cells stained with Trypan blue.

munological methods at present. Pure AFP is required, which can be isolated most efficiently from the complex of AFP and its specific antibody. The serum or ascites of patients or animals with AFP-producing tumours are good sources of AFP. The concentrations of AFP in these materials differ in individual cases but the mean level in hepatoma patient serum is a few milligrams per 100 ml.

On the other hand, these materials contain albumin in high concentrations. The physico-chemical properties,such as the solubility, isoelectric point and molecular size, of albumin are very similar to those of AFP, so that the isolation of AFP from these materials is very difficult if conventional physico-chemical methods are used. We have used two immunological methods successfully for this purification: a batch method and affinity chromatography using AFP antibody. A problem is the method used for the dissociation of the antigen–antibody complex. We used a low pH buffer (pH 1.8–2.0) or 8 *M* urea to dissociate the complex into AFP and its antibody. These conditions are drastic and not favourable for the protein, but we could not find milder conditions for splitting the strong link between the antigen and antibody.

Immunoadsorbent affinity chromatography is more suitable than the batch method. The period of exposure to low pH or concentrated urea is much shorter, and the immunoadsorbent column can be used repeatedly, possibly 10–20 times, although

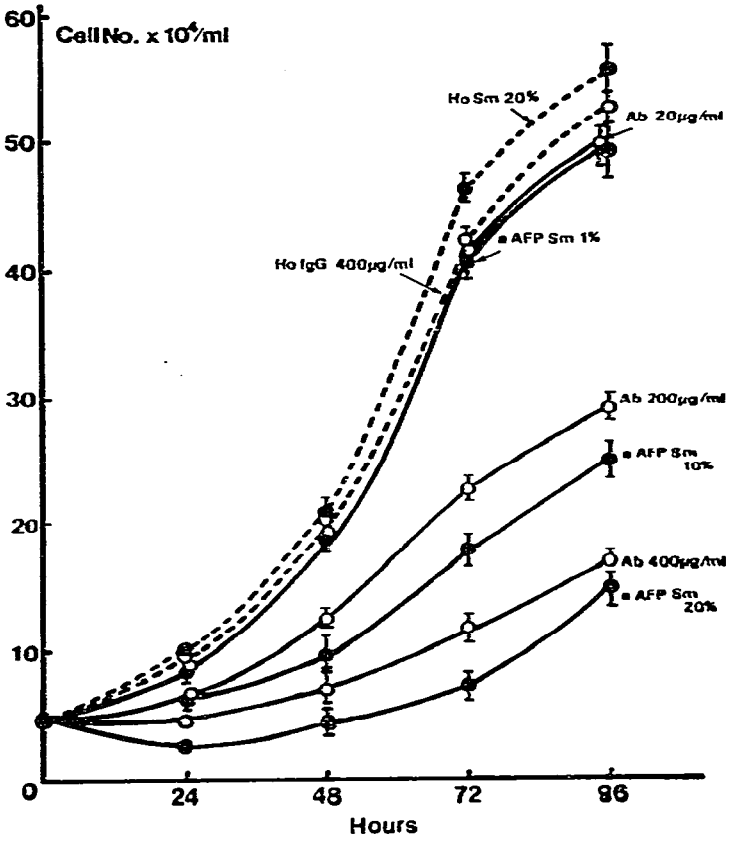

Fig. 14. Growth inhibition of tumour cells by anti-rat AFP antibody. AH66 cells were cultured in Eagle's medium containing 10% calf serum added to the specimens indicated. The change in cell number was counted for 4 days. Abbreviations as in Fig. 13.

the affinity of the column decreases gradually. The antibody separated by the batch system using a low pH buffer could not be re-used because of the considerable decrease in antibody capacity.

For the elution of either AFP or AFP antibody, 8 *M* urea solution appeared to be slightly better than the low pH buffer, over 90% of the applied material being recovered.

In reversed affinity chromatography the immobilized antigen was used for the purification of the AFP antibody. Fortunately, the antigenecity of AFP and the

TABLE I

EFFECT OF THE ANTIBODY ON SURVIVAL TIME OF TUMOUR-BEARING RATS

A 400-μg amount of a purified anti-rat AFP antibody was injected into rats on days 3, 5 and 7 after transplantation of the tumour cells.

Treatment	*Mean survival time of 10 rats (days)*
Saline	16.2 ± 1.0
Normal horse IgG	20.4 ± 1.2
Purified anti-rat AFP antibody	33.3 ± 2.0

antibody activity of AFP antibody were resistant to these drastic conditions and we could obtain purified materials without much loss of activity. Examination of the purified antibody by the quantitative precipitin reaction showed about 10–20% less activity compared with the natural antibody in the antiserum.

Large amounts of the purified antibody are now produced in this laboratory for clinical use. The cytotoxic effect of anti-AFP antibody towards hepatoma cells was demonstrated in rat experiments. The therapy of patients with hepatoma or yolk sac tumours is now being attempted. The patients have been given several hundred milligrams of purified horse antibody. The serum AFP level decreased immediately almost to zero in all cases. The antibody is a foreign protein to the patients and disappeared from the blood within 2 weeks; however, the lowered AFP level was maintained for a long period in some cases. One case has been reported previously[9]. We have not been able to observe any clinical improvements so far, perhaps because for this study we accepted only patients with very advanced hepatoma.

Conjugates of AFP antibody with anti-tumour drugs are also under investigation: recent studies using conjugates of anti-rat AFP antibody with daunomycin or adriamycin have indicated a remarkable anti-tumour effect.

The radioimmuno detection of tumour localization using radio-labelled anti-AFP antibody was successful to some extent as far as animal experiments are concerned. High AFP levels in blood seem to not interfere with the accumulation of radio-labelled antibody at the target tumour. Successful imaging was shown in cases of DAB hepatoma, in which serum AFP levels are very high (of the order of several hundred micrograms per millilitre) compared with the levels in rats with the transplanted hepatoma (less than 1 μg/ml). Goldenberg *et al.*[10] studied radioimmuno detection of cancer with anti-CEA antibody and reported very good positive imaging by using the so-called subtraction method for scintigraphy. We have also used this technique but the positivity has not yet improved. Further studies are in progress.

Radioimmunoassay of AFP is now commonly available, which is essential for the diagnosis of AFP-producing tumours. The assay method was established in this laboratory in 1972 in connection with the accomplishment of the purification of AFP.

The purification of both AFP and its antibody could be performed by gel filtration and affinity chromatography using an antigen- or antibody-coupled immunoadsorbent. Without these excellent methods our research on AFP would not be possible.

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