

In vitro and *in vivo* cytotoxic activity of native and ricin conjugated monoclonal antibodies to HBs antigen for Alexander primary liver cell carcinoma cells and tumours

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SUMMARY In *in vitro* and *in vivo* systems, native or ricin conjugated monoclonal anti-HBs, are capable of inhibiting or slowing the growth of Alexander primary hepatocellular carcinoma cells. Failure of the immune response to this component of the hepatitis B virus may be one permissive factor in the development of some primary liver cell carcinoma in chronic HBV carriers.

Primary liver cell carcinoma occur predominantly in patients with chronic hepatitis B virus infection. Tumours occur late in the disease, usually after cessation of detectable viral replication and at a stage when the liver contains clones of cells containing integrated HBV DNA.¹ These cells display hepatitis B surface (HBs) antigen in the cytoplasm and in small amounts in association with the cell membrane.

Only a minority of patients with primary liver cell carcinoma are suitable for surgical resection and the remainder show a poor response rate to cytotoxic therapy including adriamycin.² The demonstration of virus coded and tumour specific antigens (in preparation) on these virus induced tumour cells has led investigators to believe that immunotherapy may be possible.

Initially, hetero-antisera to tumour specific antigens were used for cytotoxic therapy but more recently attempts have been made to raise monoclonal antibodies to these determinants and to conjugate these with plant toxins, thereby creating potent tumoricidal systems. In this paper, we have evaluated the effect of native and ricin conjugated monoclonal antibody to HBs antigen on the *in vitro* growth of the Alexander primary hepatocellular carcinoma cell line (PLC/PRF/5). Because this cell line can be implanted into athymic mice where it develops into a well differentiated tumour,^{3,4} we have also been able to study the effect of native and ricin conjugated anti-HBs on the *in vivo* growth of

these tumour cells. In order to mimic the situation existing in patients with primary liver cell carcinoma, we have allowed the Alexander tumour to grow to a size of 0.5-1 cm in diameter in the athymic mice, before starting immunotherapy.

Methods

ANIMALS

1. PRIMARY LIVER CELL CARCINOMA LINE (PLC/PRF/5)

These cells were routinely cultured in modified Eagle's medium with Earles salts supplemented with 10% V/V fetal calf serum, 50 u/ml each of penicillin and streptomycin and 2.9 µg/ml L-glutamine. The cultures were grown at 37°C in humidified 95% air, 5% CO₂ and received a complete medium change every three days. Subculturing was effected when the culture reached confluence at six to seven days. At this stage the cultures were trypsinised and split 1:4.

The cell line was implanted into male and female random inbred nu/nu (athymic) mice by injecting 10⁷ viable cells, subcutaneously into the neck region. The mice were maintained in isolation at a constant 25°C. They were housed in sterile cages, fed irradiated food pellets and given acidified water to avoid pseudomonas infection. Under these conditions the life span of the athymic mice was at least 18 months. Forty eight hours before inoculation with the primary liver carcinoma cells the mice received sublethal total body irradiation of 450 rads from a Cobalt 60 source.

Tumours appeared three to four weeks after

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inoculation of the cells. Treatment was commenced when the tumour mass had reached at least 5×5 mm in diameter. Tumour growth was assessed in two ways. Tumour size was determined by measuring the perpendicular diameters of the tumour with vernier calipers and the base area was calculated. Tumour development was also assessed by measuring the serum concentration of human alpha-fetoprotein. The primary liver carcinoma cell line has been shown to secrete alpha-fetoprotein when grown in athymic mice³ and the level of serum alpha-fetoprotein has been shown to be closely related to tumour size.⁴ Alpha-fetoprotein concentrations were measured by radioimmunoassay (Hoerst) at seven day intervals.

2. IMMUNOLOGICAL REAGENTS

A monoclonal antibody (RF-HBs-1) to hepatitis B surface antigen (HBs antigen), raised in our own laboratory, was used in these studies. This antibody, which is of murine IgG₁ sub-class, has been described in detail elsewhere.^{5,6} It is a high affinity antibody directed to an epitope present in high density on the 'a' determinant of all subtypes of hepatitis B surface antigen. The antibody was purified from ascites fluid on a staphylococcal A protein column.

Ricin was conjugated to the purified antibody (RF-HBs-1) using a mixed anhydride derivative of chlorambucil as the coupling agent.⁷ As a control, normal non-immune horse globulin (NHG) was conjugated to ricin by the same procedure. The globulin/ricin ratio was similar in both conjugates.

3. *IN VITRO* CYTOTOXICITY ASSAY

The cytotoxicity studies conducted *in vitro* utilised PLC/PRF/5 cells, harvested in logarithmic growth. The cells were suspended in normal growth medium at 2.5×10⁴ cells/ml and 200 μl aliquots were dispensed into flat bottomed microtitre plates. The cells were incubated for 22 hours at 37°C in a humidified 95% air, 5% CO₂ atmosphere and then 25 μl of either RF-HBs-1 ricin, NHG ricin or saline were added. The cells were incubated for a further 22 to 30 hours and 1 μCi of tritiated leucine added to each well. The cultures were then harvested at two, four, six or eight hours after addition of the leucine. The cells were washed and harvested by mild trypsinisation followed by aspiration on to glass fibre discs by means of a Titretrek cell harvester (Flow Laboratories Ltd). The discs were air dried and bound radioactivity counted.

4. *IN VIVO* CYTOTOXICITY ASSAY

After injection of 1×10⁷ primary liver carcinoma cells into the neck region of the nude mouse,

tumours appeared within three to four weeks. When they had reached a size of 5×5 mm in diameter, treatment with one of the following regimes was undertaken. Dosages were given per 25 gm weight. Group 1 received anti-HBs ricin weekly intravenously. The dosage given contained 500 μg of ricin and 1180 μg of anti-HBs (RF-HBs-1). Group 2 received anti-HBs weekly intravenously. The injection contained 1180 μg of antibody. This was the amount of antibody contained in the anti-HBs ricin conjugate. Group 3 received (non-immune) horse globulin ricin weekly intravenously. This injection contained 500 μg of ricin and 1100 μg of globulin. Group 4 received ricin weekly intravenously. Injection contained 500 μg of ricin. Group 5 received saline 0.5 cc weekly intravenously.

Each group consisted of five mice and the injections were continued until the mice died. Weekly blood specimens were taken for estimation of serum alpha-fetoprotein concentration and the diameters of the tumour were also assessed weekly by caliper measurements.

Results

EFFECTS OF ANTIBODY AND ANTIBODY-TOXIN CONJUGATES ON PRIMARY LIVER CARCINOMA CELLS *IN VITRO*

PLC/PRF/5 cells incubated under control conditions showed a linear uptake of tritiated leucine. Incubation of the cells with either native ricin, anti-HBs ricin or NHG ricin produced a marked inhibition of tritiated leucine uptake (Fig. 1). This effect was seen at 2 hours (Fig. 2). There was no change in leucine uptake in the presence of anti-HBs or saline. The inhibitory effect of the NHG ricin was considerably less than that of native ricin. The

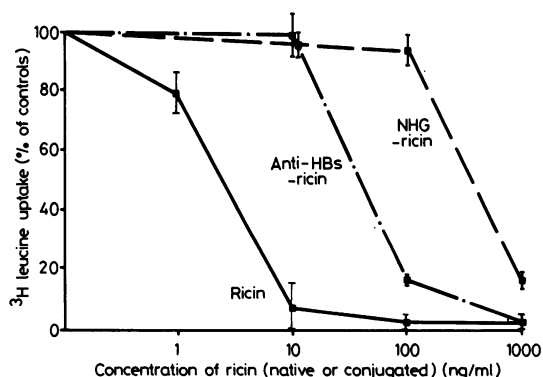


Fig. 1 Effect of ricin and ricin conjugates on ³H leucine uptake.

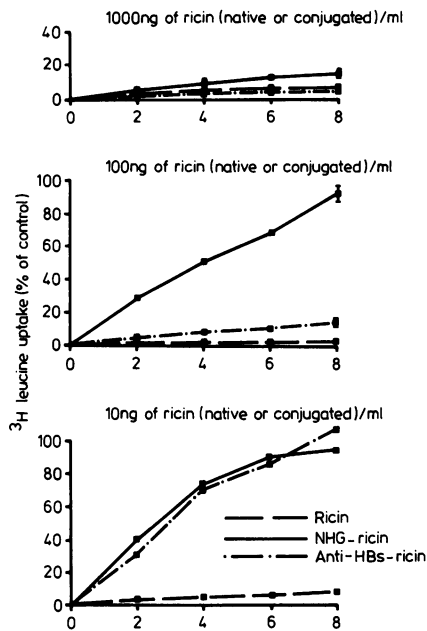


Fig. 2 Effect of ricin (native or conjugated) on ³H leucine uptake.

anti-HBs ricin conjugate, however, exhibited an increased cytotoxicity above that of the normal human globulin ricin conjugate.

The anti-HBs ricin and non-immune globulin ricin conjugates exhibited similar toxicity for peripheral blood mononuclear cells obtained from non-infected patients. In this system, ricin once again showed a greater level of toxicity (Fig. 3).

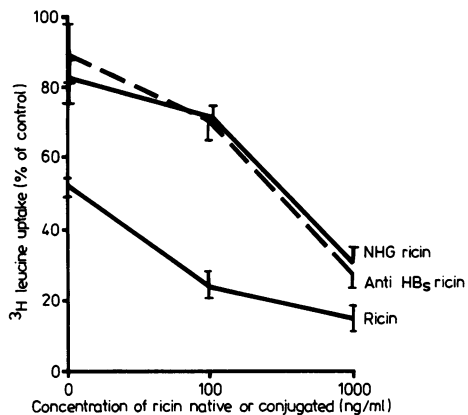


Fig. 3 Effect of ricin conjugates on ³H leucine uptake by lymphocytes.

EFFECTS OF ANTI-HBS AND ANTI-HBS RICIN CONJUGATES ON PRIMARY LIVER CARCINOMA GROWTH IN ATHYMIC MICE

Animals injected with 500 μg of ricin intravenously at weekly intervals died within three weeks. Animals receiving either normal horse globulin ricin (equivalent of 500 μg of ricin intravenously weekly) or saline, survived for between four and six weeks. Over this time the tumour increased in diameter and the serum alphafetoprotein concentrations rose exponentially (Figs. 4, 5). The animals given either native anti-HBs (1180 μg weekly) or anti-HBs ricin (500 μg intravenously of ricin weekly) showed a slower increase in diameter of the tumour (significant ($p < 0.05$) at two, three, four, and five weeks) and slower rise in alphafetoprotein (significant ($p < 0.05$) at three, four, and five weeks) than the saline and the normal horse globulin ricin injected groups. In the animals receiving native anti-HBs the tumours eventually achieved the same size and the same alphafetoprotein concentrations as the saline treated animals, whereas those animals receiving ricin conjugated anti-HBs continued to have smaller tumour masses with lower serum alphafetoprotein concentrations for a longer period of time (Figs 4, 5).

Discussion

In this study we have shown that the growth rate in athymic mice of a human primary liver cell cancer cell line which contains the hepatitis B virus genome^{8,9} and expresses hepatitis B surface antigen on the cell membrane, can be reduced by exposure to a monoclonal antibody to HBs antigen. These data are similar to those obtained by Shouval *et*

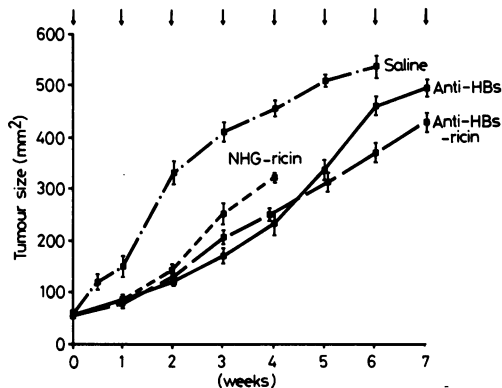


Fig. 4 Effect of anti HBs-ricin, NHG-ricin, anti HBs and saline on tumour growth.

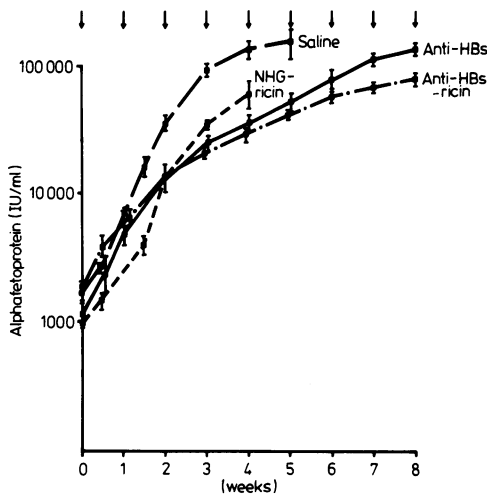


Fig. 5 Effect of anti Hbs-ricin, NHG-ricin, anti Hbs and saline on alphafetoprotein values in PLCC bearing nude mice.

al.^{10 11} In the present study, the monoclonal antibody RF-HBs-1 showed no cytotoxic activity *in vitro*. The antibody is of IgG 1 class and does not fix complement. It seems unlikely therefore that the activity of this antibody in the athymic mice, is related to complement dependent killing. It seems probable that the reduced rate of growth may be related to recruitment of antibody dependent cellular killing mechanisms.

Ricin is a potent toxin derived from the seeds of a plant *Ricinus communis*.¹² It is a glycoprotein of molecular weight 60–65 000 daltons which consists of two polypeptide chains, an A chain and a somewhat larger B chain, joined by a disulphide bridge. The B chain contains carbohydrate residues rich in mannose and glucose. This chain binds to receptors on cells resulting in internalisation of the dipeptide when the A chain becomes capable of inhibiting protein synthesis by inactivation of 60S ribosomal subunits. Ricin is an extremely potent toxin, one molecule being sufficient to kill a cell. The conjugation of the ricin molecule *via* its B chain to the monoclonal antibody, reduces the non-specific binding properties of the toxin and confers in their place specificity according to that of the antibody. In the studies reported, the ricin conjugated to anti-HBs exhibited greater toxicity for HBs antigen displaying primary liver carcinoma cells than NHG ricin, whereas both conjugates were equally toxic for non-HBs antigen displaying peripheral blood mononuclear cells. These *in vitro* studies establish the ability of this ricin conjugated

anti-HBs to express specific killing of the primary liver carcinoma cells. *In vivo*, the ricin conjugated anti-HBs produce similar slowing of the growth of the tumour as that produced by the native anti-HBs. After several weeks, however, whereas the animals given native anti-HBs showed a more rapid growth of the tumour, the animals receiving ricin conjugated anti-HBs continued to exhibit a slower rate of growth.

These studies raise some interesting possibilities regarding the pathogenesis of primary liver cell cancer. It now seems clear that hepatocytes containing integrated HBV-DNA but not replicating HBV particles, are present during the late phase of the infection.^{13 14} Recovery necessitates inhibition of viral replication and lysis of cells containing integrated HBV-DNA. It now seems probable that different immune mechanisms are responsible for each of these processes.¹⁵ In many patients with chronic HBV infection, virus replication ceases within the first few years of infection, presumably because of elimination of hepatocytes containing replicating virus. The majority of these patients remain HBs antigen positive because of the presence of residual clones of hepatocytes containing HBV-DNA and secreting HBs antigen. It is probable that malignant transformation occurs in this population of cells and gives rise to primary liver cell cancer. The PLC/PRF/5 cell line contains the integrated hepatitis B viral genome but does not replicate the intact virus. In this respect, it is similar to the clones of cells which give rise to the tumour in man. Our demonstration that the growth of these primary liver carcinoma cells may be inhibited by antibody to HBs suggests that their continuing growth *in vivo* may be related to a relative deficiency of the immune response to this particular antigen in those patients who develop HBs antigen secreting primary liver carcinoma. In this respect it is of interest that the fathers of patients with primary liver cell cancer show a defective immune response to HBs antigen suggesting a possible hereditary specific immune deficiency.¹⁶ Other mechanisms by which virus transformed cells may evade the immune surveillance system include failure to display a virus encoded antigen¹⁷ or non-association of viral antigens with the HLA determinants.¹⁸

In this study we have shown that anti-viral antibodies may inhibit the growth of a virus induced tumour. This effect is evident when the tumour is already well established and may offer some potential for the therapy of primary liver cell carcinoma.

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