Biochimica et Biophysica Acta, 367 (1974) 47–58

BBA 76743

SOLUBILIZATION OF MEMBRANE-ASSOCIATED TUMOUR-SPECIFIC ANTIGENS BY β-GLUCOSIDASE

R. W. BALDWIN, J. G. BOWEN and M. R. PRICE

Cancer Research Campaign Laboratories, University of Nottingham, University Park, Nottingham NG7 2RD (United Kingdom)

(Received April 2nd, 1974)

SUMMARY

The effects of various enzyme treatments on aminoazo dye-induced rat hepatoma cells or cell membranes, and the resulting degradation or solubilization of membrane-associated tumour-specific antigen are described. Both papain and β-glucosidase showed the capacity to liberate water-soluble antigenic products. Further fractionation by ion-exchange column chromatography of the soluble extract isolated by limited β-glucosidase digestion of hepatoma membranes, resolved the antigenic activity in a discrete region of the column eluate which was separated from the fractions displaying enzymic activity. This soluble antigenic material although heterogeneous by polyacrylamide-gel electrophoresis, retained the capacity both to inhibit the reaction of antibody in hepatoma-immune serum with cell surface-expressed antigens on viable hepatoma target cells in the membrane immunofluorescence test and also to elicit specific humoral antibody production in immunized animals. Tests were designed to analyse the mechanism of antigen solubilization by β-glucosidase and the results of these are discussed in view of the present understanding of the nature of tumour-specific antigens associated with aminoazo dye-induced rat hepatomas.

INTRODUCTION

Tumour-specific antigens expressed at the cell surface of aminoazo dye-induced rat hepatomas represent unique determinants of individual tumours [1, 2]. The antigenic determinants of these hepatomas are integrated plasma membrane components which upon tumour cell rupture, remain associated with tumour cell membrane fractions and not soluble cytoplasmic protein fractions [3, 4]. Furthermore, with one hepatoma (hepatoma D23), it has been found that antigen is susceptible to release from the tumour cell membrane in an immunologically active form following limited proteolytic treatment of tumour cell membrane fractions with papain [5–7]. This would indicate that the antigen consists, at least in part, of a protein component which is implicated in the structural presentation or organization of the antigen within the cell membrane. These findings, however, reveal little about the chemical nature of the antigenic determinant itself. The present communication reports the results of preliminary studies concerning this latter question. This has been approached by deter-

mining whether specific antigen expressed upon viable tumour cells or membrane fractions from one aminoazo dye-induced hepatoma (hepatoma D23) is susceptible to inactivation following treatment with a range of enzymes. Thus, under the conditions of these tests, enzyme-mediated inactivation of tumour-specific antigen may represent either enzymic degradation of the antigenic site (or residues in the immediate environment of the active site) or the release of antigen from the plasma membrane as a soluble entity. In some cases, it is conceivable that both mechanisms may, in fact, be operative. It is, however, possible to resolve whether enzymic treatment effects predominantly antigen solubilization or inactivation by examination of the products released from the tumour cell membrane.

MATERIALS AND METHODS

Rats and tumour

Hepatoma D23, originally induced in a male rat of Wistar strain by oral administration of 4-dimethylaminoazobenzene, was maintained by serial subcutaneous passage in syngeneic male rats. Hepatomas progressively growing in the peritoneal cavity of syngeneic rats were established by intraperitoneal injection of chopped tumour tissue suspended in phosphate-buffered saline, pH 7.3, containing 100 I.U. penicillin per ml and 100 μg streptomycin per ml.

Enzyme treatment of viable hepatoma D23 cells

Single cell suspensions of viable hepatoma D23 cells were prepared by trypsinization of tumour tissue fragments [8]. Cells were dispensed in aliquots of $5 \cdot 10^6$ cells, sedimented by centrifugation ($30 \times g$ for 3 min) and cell pellets were each resuspended in 1.0 ml in phosphate-buffered saline or enzyme solution in phosphate-buffered saline. Details of enzymes used and experimental conditions employed are summarized in Table I. All incubations were performed at 37 °C with regular agitation to maintain a dispersed cell suspension. Following incubation, cells were sedimented by centrifugation ($30 \times g$ for 3 min) and supernatants discarded. Cells were washed once with phosphate-buffered saline and once with Medium 199 by centrifugation

TABLE I

ENZYMES USED AND EXPERIMENTAL CONDITIONS EMPLOYED FOR THE ENZYMIC TREATMENT OF HEPATOMA D23 CELLS

Enzyme*	Enzyme–hepatoma D23 cell incubation	
	Concentration of enzyme (%, w/v)	Period of incubation (min)
α-Glucosidase	0.01–0.1	30
β-Glucosidase	0.01–0.1	30
β-Galactosidase	0.50	30
Hyaluronidase	0.05	30
Neuraminidase	0.02	30
Ficin	0.02	60

* Enzyme preparations were obtained from the Sigma Chemical Co. (Kingston-upon-Thames, England).

($30 \times g$ for 3 min). Enzyme-treated or untreated cells were then used as target cells in the membrane immunofluorescence test, and modification of antigenic determinants was detected by a reduction in staining in treated cell preparations as compared with untreated cells.

Enzyme treatment of membrane preparations

"Extra-nuclear" membrane fractions were isolated from hepatoma D23 tissue homogenates prepared by nitrogen pressure homogenization according to the method of Baldwin and Embleton [9]. The membrane suspension (in 1 mM $NaHCO_3$, 2 mM $CaCl_2$, pH 7.6, at a concentration of 30 mg membrane protein per ml) was dispensed in 1.0-ml aliquots, and 1.0-ml aliquots of enzyme solution (in phosphate-buffered saline or 1 mM $NaHCO_3$, 2 mM $CaCl_2$, pH 7.6) or in controls, buffer alone, were added to the membrane suspension. Details of the enzymes used and experimental conditions employed are summarized in Table II. All incubations were performed at room temperature for 60 min with gentle agitation. Following incubation, three 0.6-ml aliquots of the suspension in each test or control mixture were withdrawn and sedimented by centrifugation ($105\,000 \times g$ for 60 min). The supernatants were discarded and replaced by 0.1 ml aliquots of serum at dilutions between 1/2 to 1/6 in phosphate-buffered saline. For each group of three treated or untreated membrane samples, normal rat serum was added to one sample while hepatoma D23 immune serum at an equivalent dilution was added to the other two samples. The membranes were resuspended in the serum and absorption was performed for 18 h at 4 °C. After absorption, membranes were sedimented by centrifugation at $105\,000 \times g$ for 60 min and the serum collected. The absorbed serum was then tested using viable hepatoma D23 cells in the membrane immunofluorescence test, and modification of antigenic determinants was detected by the inability of enzyme-treated membranes to absorb antibody from immune serum as compared with untreated membrane preparations.

TABLE II

ENZYMES USED AND EXPERIMENTAL CONDITIONS EMPLOYED FOR THE ENZYMIC TREATMENT OF HEPATOMA D23 CELL MEMBRANES

Enzyme*	Enzyme-hepatoma D23 cell membrane incubation	
	Buffer used for preparing enzyme solution	Concentration of enzyme (%, w/v)
α-Glucosidase	phosphate-buffered saline, pH 7.3	0.05
β-Glucosidase	phosphate-buffered saline, pH 7.3	0.05
β-Galactosidase	phosphate-buffered saline, pH 7.3	0.50
Hyaluronidase	phosphate-buffered saline, pH 7.3	0.05
Neuraminidase	phosphate-buffered saline, pH 7.3	0.02
Trypsin	1 mM $NaHCO_3$, 2 mM $CaCl_2$, pH 7.6	0.25
Lysozyme	1 mM $NaHCO_3$, 2 mM $CaCl_2$, pH 7.6	0.02
Papain	1 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.1 M L-cysteine, pH 7.6	0.20

* Enzyme preparations were obtained from the Sigma Chemical Co. with the exception of trypsin which was supplied by Difco.

Liberation of soluble hepatoma D23 antigen by β-glucosidase treatment of hepatoma D23 membranes

Hepatoma D23 tissue was harvested from the peritoneal cavity and an "extra-nuclear" membrane fraction (the 78 000 × *g* sediment of the 600 × *g* supernatant from tumour homogenates) was prepared as previously described [6]. "Extra-nuclear" membranes were dispersed in 5 mM sodium phosphate buffer, pH 7.3, to a concentration of 30 mg of membrane protein per ml. β-Glucosidase (Sigma Chemical Co.) and reduced glutathione (Sigma Chemical Co.) were each added at 1 mg per 30 mg of membrane protein and the suspension was stirred for 60 min at 37 °C. Undegraded membrane material was sedimented by centrifugation at 78 000 × *g* for 90 min and the supernatant was dialysed for 16 h against 5 mM phosphate buffer, pH 7.3.

Column chromatography

The β-glucosidase-solubilized extract of hepatoma D23 "extra-nuclear" membranes was applied to a DEAE-cellulose column (2.5 cm × 40 cm) containing the anion exchanger (Whatman DE52) which was equilibrated with 5 mM phosphate buffer, pH 7.3. Fractions (10 ml) were eluted from the column by application of 100 ml 5 mM phosphate buffer, pH 7.3, followed by a 400-ml linear NaCl gradient (0–1.2 M NaCl, in 5 mM phosphate buffer, pH 7.3). The column was finally eluted with 50 ml of 1.5 M NaCl to remove any strongly bound protein. Fractions were dialysed against 5 mM phosphate buffer, pH 7.3, concentrated against Aquacide II (Calbiochem.) to 2 ml and dialysed against phosphate-buffered saline for 16 h. All procedures employed in the fractionation of β-glucosidase-liberated antigen were performed at 0–5 °C. Isolated fractions were stored at −20 °C.

Membrane immunofluorescence test

The indirect membrane immunofluorescence test was performed with viable hepatoma D23 or D30 cells in suspension using sera from rats immunized by implantation of γ-irradiated tumour grafts [8]. Fluorescence indices were calculated for absorbed or unabsorbed test sera by determining the percentage of cells unstained with normal rat serum minus the percentage unstained with test serum divided by the former figure. Serum fluorescence indices of 0.30 or greater were taken to represent significant staining of hepatoma target cells by tumour specific antibody.

Antigen assay

Individual soluble fractions, dialysed against phosphate-buffered saline, were assayed for antigenic activity by determining their capacity to neutralize the reaction of antibody in hepatoma D23-specific immune serum with tumour-specific cell surface antigen on viable hepatoma D23 cells as assessed using the membrane immunofluorescence test [5, 6]. Thus, antigenic activity associated with soluble fractions was denoted by a reduction of the fluorescent staining with absorbed immune serum as compared with immune serum diluted with equivalent volumes of phosphate-buffered saline. Reductions of the fluorescence index to below 0.30 were taken to represent a significant neutralization of tumour-specific antibody. The percentage inhibition of the fluorescence index with absorbed serum was defined as a measure of antigenic activity.

Enzyme assays

Individual or pooled fractions were assayed for β-glucosidase activity by mea-

suring spectrophotometrically at 400 nm the release of nitrophenol from *p*-nitrophenyl-β-D-glucoside [10]. The specificity of the reaction of the β-glucosidase preparation was also tested by determining the release of nitrophenol from the following substrates: *p*-nitrophenyl-β-D-galactoside, *o*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-β-D-mannoside and *o*-nitrophenyl-β-D-fucoside.

The β-glucosidase preparation was examined for proteolytic activity by determining its capacity to liberate trichloroacetic acid-soluble material, absorbing at 280 nm, from casein (BDH) according to method of Arnon [11]. Reagents used in enzyme studies were obtained from the Sigma Chemical Co. unless otherwise stated.

Protein analysis

Protein concentrations were determined by the method of Lowry et al. [12].

RESULTS

Enzyme treatment of hepatoma D23 cells

Viable hepatoma D23 cells were subjected to treatment with a range of enzymes and the resulting effects upon the expression of hepatoma D23-specific antigen were assessed using the membrane immunofluorescence test. As shown in Table III, treatment of hepatoma D23 cells with β-glucosidase modified the cell surface causing the loss of tumour specific antigenic activity so that the cells no longer reacted significantly with antibody from hepatoma D23 immune serum (fluorescence indices, F.I., 0.13–0.20). In contrast α-glucosidase was ineffective, and treated cells still reacted positively with immune serum. Also, β-galactosidase, hyaluronidase, neuraminidase and ficin under the conditions used in these tests had no effect upon cell surface-expressed antigenic activity.

TABLE III

EFFECT OF ENZYME TREATMENT OF HEPATOMA D23 CELLS ON THE EXPRESSION OF HEPATOMA D23 SPECIFIC ANTIGEN

Treatment of hepatoma D23 cells	Fluorescence index* of hepatoma D23-specific antiserum	
	Untreated cells	Treated cells
α-Glucosidase	0.58	0.66
	0.55	0.61
β-Glucosidase	0.58	0.13, 0.18
	0.55	0.13, 0.18
	0.50	0.20
β-Galactosidase	0.55	0.40
Hyaluronidase	0.54	0.30
Neuraminidase	0.54	0.56
Ficin	0.57	0.35
	0.54	0.50

* Fluorescence indices of 0.30 or greater represent significant membrane immunofluorescence staining of viable hepatoma D23 cells.

Enzyme treatment of hepatoma D23 membranes

An alternative method was adopted to confirm the effect of enzymic modification of hepatoma D23 antigenic determinants. In these studies, "extra-nuclear" membrane fractions were incubated with enzyme solution and the results showing the effect of various enzymes in modifying the capacity of hepatoma D23 membranes to absorb tumour specific antibody are summarized in Table IV. Treatment of membrane fractions with β-glucosidase resulted in the loss of antigenic activity and treated preparations were unable to neutralize antibody from immune serum. Papain-treated membranes similarly lacked the capacity to absorb tumour-specific antibody from immune serum, indicating the release or inactivation of antigen. Conversely, absorption of the antiserum with untreated membranes removed antibody so that absorbed sera were inactive. None of the other enzymes examined (α-glucosidase, β-galactosidase, hyaluronidase, neuraminidase, trypsin and lysozyme) were effective in modifying the membrane-expressed antigenic determinants and in all cases, treated membranes retained the capacity to neutralize tumour specific antibody in immune serum.

TABLE IV

EFFECT OF ENZYME TREATMENT OF HEPATOMA D23 MEMBRANES ON THEIR CAPACITY TO ABSORB HEPATOMA D23 SPECIFIC ANTIBODY FROM IMMUNE SERUM

Treatment of hepatoma D23 membranes	Fluorescence index* of hepatoma D23 immune serum		
	Unabsorbed	Absorbed with:	
		Untreated membranes	Treated membranes
α-Glucosidase	0.63	0.04; 0.06	0.24; 0.19
β-Glucosidase	0.63	0.04; 0.06	0.50; 0.56
β-Galactosidase	0.63	0.04; 0.06	0.19; 0.13
Hyaluronidase	0.60	0.08; 0.05	0.10; 0.23
Neuraminidase	0.63	0.04; 0.06	0.23; 0.29
Trypsin	0.58	0.08; 0.10	0.00; 0.00
Lysozyme	0.58	0.08; 0.10	0.03; 0.18
Papain	0.58	0.08; 0.10	0.50; 0.56

* Fluorescence indices of 0.30 or greater represent significant membrane immunofluorescence staining of viable hepatoma D23 cells.

The results of these studies on the enzymic modification of hepatoma D23 cell surface membrane, suggest that the antigenic determinant is susceptible to release from the tumour membrane or inactivation in situ by papain and β-glucosidase. Previous investigations have established that hepatoma D23-specific antigen is liberated in a soluble and immunologically active form following papain treatment of the tumour cell membrane fractions [5, 6] and the subject of present report has been to evaluate the products released by β-glucosidase treatment of hepatoma D23 membranes.

β-Glucosidase solubilization of hepatoma D23-specific antigen

Four large-scale β-glucosidase extractions of hepatoma D23 "extra-nuclear" membranes were performed. In each case, the soluble material released from the

TABLE V

β-GLUCOSIDASE SOLUBILIZATION OF HEPATOMA D23 ANTIGEN

Experiment	Wet weight of hepatoma D23 tissue (g)	"Extra-nuclear" membrane protein (g)	Protein displaying antigenic activity recovered from the DEAE-cellulose column (mg)
1	109	1.6	29
2	201	1.5	30
3	200	2.5	59
4	239	2.8	62

tumour membranes was applied to a DEAE-cellulose column and eluted with a linear NaCl ionic strength gradient in 5 mM phosphate buffer, pH 7.3. Details of the initial materials and recoveries are summarized in Table V. A representative $A_{280\,nm}$ profile given by the column eluate is shown in Fig. 1. The fractions isolated by column chromatography were examined by analytical polyacrylamide-gel electrophoresis and all fractions were complex, giving multiple bands of stained protein on the gel.

Hepatoma D23-specific antigenic activity was detected by the capacity of isolated fractions to neutralize the membrane immunofluorescence staining of viable hepatoma D23 cells by specific antibody in syngeneic immune serum. Two discrete peaks of material (fractions 11–15, and fractions 18 and 19, Fig. 1) were found to significantly reduce the fluorescence index of absorbed sera below the level of 0.30.

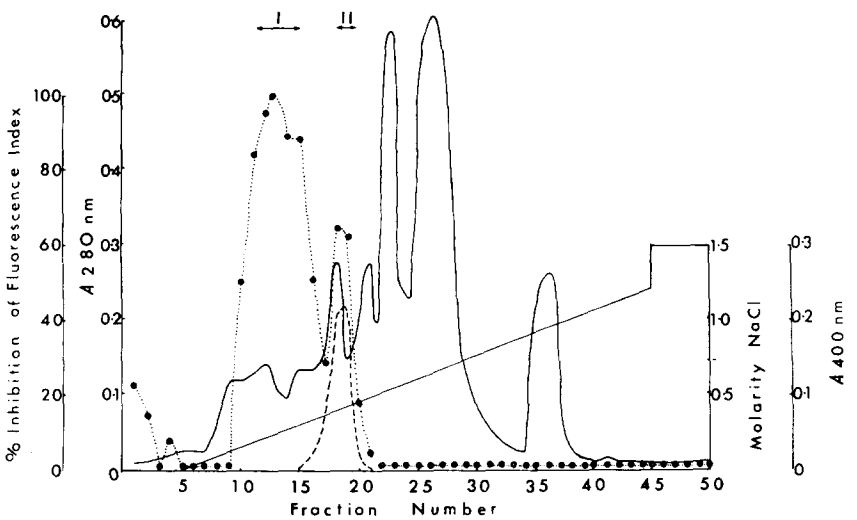

Fig. 1. DEAE-cellulose column chromatography of β-glucosidase-solubilized hepatoma D23 "extra-nuclear" membranes. Tumour antigen (● · · · ●) was determined from the capacity of fractions to neutralize antibody in a standard antiserum from syngeneic rats immunized against hepatoma D23 cells. This was expressed as the percentage inhibition of the serum fluorescence index compared with appropriately diluted untreated serum when tested against hepatoma D23 cells (see Materials and Methods). β-Glucosidase activity (– – –) was assayed by measuring spectrophotometrically at 400 nm the release of nitrophenol from *p*-nitrophenyl-β-D-glucoside.

The material isolated in fractions 11–15 and fractions 18 and 19 was designated Peak I and Peak II material respectively (Fig. 1). Two methods were initially adopted to determine whether two molecular species of hepatoma D23 antigen were fractionated by ion-exchange chromatography into Peaks I and II or whether one or both peaks of material contained the enzyme β-glucosidase which could effect the release or degradation of antigenic determinants expressed upon the viable hepatoma D23 cells used in the assay system for the detection of antigenic activity. Firstly column fractions were examined for β-glucosidase activity and it was determined that the enzyme was resolved into fractions 16–19 indicating its association with the soluble material in Peak II (Fig. 1). Secondly, material from Peaks I and II were absorbed with immune serum from syngeneic rats immunized against another immunologically distinct 4-dimethylaminoazobenzene-induced hepatoma (hepatoma D30). This serum, when absorbed with Peak I material, showed positive membrane immunofluorescence staining of viable hepatoma D30 cells (fluorescence index, F.I., 0.38–0.47) which was comparable to that exhibited by unabsorbed hepatoma D30 immune serum appropriately diluted with phosphate-buffered saline (F.I., 0.37–0.44). However, following absorption of immune serum with Peak II material containing the enzyme activity and subsequently testing this against viable hepatoma D30 cells in the membrane immunofluorescence assay, the fluorescence staining of the target cells was abolished (F.I., 0.00–0.07) indicating enzymic release or inactivation of hepatoma D30 cell surface-expressed antigen.

The immunogenicity and antigenic specificity of the material isolated in Peak I (Fig. 1) was further investigated. In these tests, a group of 11 syngeneic rats received three intraperitoneal injections at weekly intervals with 1 ml of the pooled antigenic fractions (Peak 1) at a concentration of 1 mg of protein per ml. With 6 of the 11 animals, the serum samples taken 5 days after the final injection were found to contain significant hepatoma D23-specific antibody demonstrable by the membrane immunofluorescence staining of viable hepatoma D23 target cells (F.I., 0.30–0.59). The sera, however, failed to react positively with hepatoma D30 target cells in the membrane immunofluorescence test (F.I., 00.0–0.07) although significant fluorescent staining of target cells was observed with serum from rats immune to hepatoma D30 (F.I., 0.44, 0.48). Seven days after the final injection, immunized rats received a subcutaneous challenge inoculum of $5 \cdot 10^3$ viable hepatoma D23 cells, this dose being sufficient to produce growth in untreated control recipients. No protection was, however, afforded to challenge since the outgrowth of tumours in immunized and untreated control animals was equivalent.

Having established that β-glucosidase treatment of hepatoma D23 "extranuclear" membranes effected the release of immunologically active antigenic determinants, several tests were designed to analyse the mechanism of solubilization. Firstly, the enzymic specificity of the β-glucosidase preparation used in the present studies was evaluated by its reaction with several chromophoric substances. The enzyme showed no activity towards *p*-nitrophenyl-β-D-galactoside, *p*-nitrophenyl-β-D-mannoside or *o*-nitrophenyl-β-D-xyloside although the preparation had the capacity to cleave nitrophenol from both *p*-nitrophenyl-β-D-glucoside and *o*-nitrophenyl-β-D-fucoside. The enzyme did not, however, display significant proteolytic activity as determined by its capacity to liberate trichloroacetic acid-soluble material from casein. When the fractions isolated by column chromatography of the β-glucosidase extract

TABLE VI

INHIBITION OF β-GLUCOSIDASE SOLUBILIZATION OF HEPATOMA D23 ANTIGEN BY ADDITION OF β-D-GLUCOSE

Absorption conditions: 30 mg "extra-nuclear" membrane protein per ml of hepatoma D23 immune serum. Serum samples were absorbed with soluble fractions as described in Materials and Methods. Fluorescence indices of 0.30 or greater represent significant membrane immunofluorescence staining of viable hepatoma D23 cells.

Test	Absorbing fraction	Fluorescence index of hepatoma D23 immune serum	
		Unabsorbed	Absorbed
1	Untreated extra-nuclear membranes	0.54 0.49	0.01 0.00
2	Extra-nuclear membranes extracted with β-glucosidase	0.54 0.63	0.62 0.59
3	Extra-nuclear membranes extracted with β-glucosidase in the presence of β-D-glucose (50 %, w/v)	0.54 0.69 0.67	0.06 0.11 0.12
4	Peak I from DEAE-cellulose column of β-glucosidase-extracted extra-nuclear membranes	0.61 0.58	0.06 0.01
5	Fraction corresponding to Peak I from DEAE-cellulose column of β-glucosidase extracted extra-nuclear membranes solubilized in the presence of β-D-glucose (50 %, w/v)	0.69 0.38 0.81	0.78 0.72 0.79

of hepatoma D23 membranes were assayed for β-fucosidase content, it was found that the activity was located only in those fractions displaying β-glucosidase activity (Fig. 1, Peak II). No enzymic activity was associated with any of those fractions retaining hepatoma D23 specific antigen (Fig. 1, Peak I). Glycolytic activity was further shown to be necessary for antigen release, since solubilization of hepatoma D23 antigen was prevented by terminal product (β-D-glucose) addition to the enzyme–membrane reaction mixture. Thus, as indicated in Table VI, "extra-nuclear" membranes extracted with β-glucosidase in the presence of β-D-glucose (Test 3) displayed an equivalent capacity to that of untreated membranes (Test 1) in their ability to absorb antibody from standard immune serum, whereas membranes treated with β-glucosidase alone (Test 2) were ineffective in this respect. In extension of these findings no antigenic activity was detected with material eluted in the region corresponding to Peak I (see Fig. 1) following chromatographic separation of the soluble extract obtained by β-glucosidase treatment of membrane fractions in the presence of the terminal product (Table VI, Test 5). However, when membrane extractions were performed in the presence of enzyme inhibitors (sodium iodoacetate and *p*-chloromercuriphenyl sulphonic acid) added to a concentration (0.05 M) which did not affect the glycolytic activity of β-glucosidase as assessed by hydrolysis of *p*-nitrophenyl-β-D-glucoside, the extracted membranes retained the capacity to neutralize antibody in immune serum (Table VII, Tests 1 and 3). Furthermore, fractions corresponding to the Peak I material (see Fig. 1) from ion-exchange columns of the proteins

TABLE VII

INHIBITION OF β-GLUCOSIDASE SOLUBILIZATION OF HEPATOMA D23 ANTIGEN IN THE PRESENCE OF IODOACETATE AND *p*-CHLOROMERCURIPHENYL SULPHONATE

Absorption conditions: 30 mg "extra-nuclear" membrane protein per ml of hepatoma D23 immune serum. Serum samples were absorbed with soluble fractions as described in Materials and Methods. Fluorescence indices of 0.30 or greater represent significant membrane immunofluorescence staining of viable hepatoma D23 cells.

Test	Absorbing fraction	Fluorescence index of hepatoma D23 immune serum	
		Unabsorbed	Absorbed
1	Extra-nuclear membranes extracted with β-glucosidase in the presence of 0.05 M iodoacetate	0.47 0.51 0.51 0.48 0.44	0.12 0.29 0.18 0.23 0.09
2	Material corresponding to Peak I from DEAE-cellulose column of extra-nuclear membranes extracted with β-glucosidase in the presence of 0.05 M iodoacetate	0.42 0.51 0.47	0.35 0.40 0.36
3	Extra-nuclear membranes extracted with β-glucosidase in the presence of *p*-chloromercuriphenyl sulphonate	0.44 0.42 0.39	0.14 0.18 0.00
4	Material corresponding to Peak I from DEAE-cellulose column of extra-nuclear membranes extracted with β-glucosidase in the presence of 0.05 M *p*-chloromercuriphenyl sulphonate	0.42 0.37 0.39	0.32 0.34 0.36

solubilized in the presence of enzyme inhibitors, showed no antigenic activity, since the fluorescence indices of absorbed sera were equivalent to those determined for unabsorbed immune serum (Table VII, Tests 2 and 4). These findings suggest that although the glycolytic activity of β-glucosidase is necessary for antigen solubilization, the involvement of a proteolytic enzyme system(s) may also play an essential role in the release of antigenically active macromolecules.

DISCUSSION

The present studies demonstrate that membrane-associated hepatoma D23-specific antigen is susceptible to release from the cell surface by treatment with β-glucosidase. Fractionation by ion-exchange chromatography of the solubilized extract allowed a discrete region of antigenically active material to be eluted from the columns although this material was heterogeneous as assessed by polyacrylamide gel electrophoresis. However, these soluble preparations displayed both a capacity to neutralize specific antibody in hepatoma D23 immune serum and to elicit humoral antibody production in immunized rats, indicating the retention of immunologically active antigenic determinants in the isolated fractions. Preliminary studies upon the further fractionation of β-glucosidase-solubilized antigen have already demonstrated that

antigenic activity is associated with material with a molecular weight of approximately 50 000–60 000, as determined by Sephadex G-200 gel filtration chromatography. It is worth noting that in previous investigations [6], the major antigenic fraction isolated following limited proteolytic digestion of hepatoma D23 cell membranes with papain, displayed a comparable molecular weight of approximately 55 000 suggesting that the two antigen preparations share some common characteristics. Hepatoma D23-specific antigen also shows some similarities to histocompatibility antigens, particularly those of the murine H-2 and human HL-A systems which have been most extensively characterized. For example, papain-solubilized H-2 and HL-A histocompatibility antigens are comparable to hepatoma D23 antigen isolated by similar procedures, in both their molecular weights [6] and amino acid composition (Baldwin, R. W. and Price, M. R., unpublished). Also, Muramatsu and Nathenson [13] have demonstrated that removal of sialic acid and galactose from purified, papain-solubilized H-2 alloantigens produced no loss of antigenic activity. Comparably, it would appear that these residues are not essential in the expression of hepatoma D23 specific antigen since neuraminidase and β-galactosidase were ineffective in modifying the reactivity of membrane-associated antigens towards antibody in syngeneic immune rat serum.

Purified papain-solubilized alloantigens have been shown to be glycoproteins containing 80–90 % protein [14]. Although characterization of the carbohydrate moieties of papain-solubilized hepatoma D23 antigen is incomplete, carbohydrate residues may be implicated in the structural presentation or organization of the antigen within the tumour cell surface membrane, since treatment of membrane fractions with β-glucosidase facilitated the release of soluble antigenic material. The finding that this release was also inhibited by β-D-glucose addition to the enzyme–membrane reaction mixture emphasizes that glycolytic degradation is operative in the mechanism of solubilization. It is considered unlikely that the β-D-fucosidase activity exhibited by the enzyme preparation contributed to antigen release since the L-isomer of fucose is most commonly found in mammalian cell surfaces [15] and antigen solubilization by the enzyme preparation was inhibited by addition of β-D-glucose.

The finding that hepatoma D23 antigen release from membrane fractions was prevented by iodoacetate and *p*-choromercuriphenyl sulphonic acid implies that proteolysis may also be required for solubilization. These tests were performed under conditions where the β-glucosidase activity was unaffected suggesting that antigen release may have been accomplished by a more complex mechanism than only glycolytic degradation. This mechanism is at present unresolved although several possibilities may be considered. Firstly, removal of glycosidic residues from the polypeptide backbone of the membrane-associated antigen may render the antigen more accessible to proteolysis. The origin of such a proteolytic system(s) is, however, not known and it remains to be determined whether under the conditions of the solubilization procedure, membrane-bound proteases may be activated or solublized to effect enzymic release of tumour antigen by a secondary interaction. Similar secondary proteolytic effects have been implicated in other studies upon the solubilization of membrane-associated antigens. For example, with 3 M KCl extraction of cells or cell homogenates, it was originally proposed that antigen solubilization was accomplished by a reduction of ordered structure of water molecules intimately associated with membrane protein, thus allowing hydrophobic regions of the protein to become

detached from their lipid environment and dispersing membrane molecules in the aqueous media [16, 17]. However, Mann [18] has suggested that products arising from 3 M KCl solubilization may in fact be released by the action of soluble intracellular and possibly membrane-bound proteolytic enzymes since proteolytic enzyme inhibitors prevented the solubilization of HL-A alloantigens by the action of 3 M KCl.

These studies emphasize the difficulties encountered when defining the mechanism of antigen release from its membrane location by treatment with various chemical or enzymic agents. However, the finding that β-glucosidase effectively solubilizes membrane-associated hepatoma D23 antigen is of significance since these preparations are suitable for further purification with a view to isolating homogeneous antigen fractions which may be subjected to critical biochemical analysis. The availability of well characterized tumour antigens both from experimental animal tumours and human tumours is considered to be of paramount importance at the present time since such preparations may prove to be valuable aids in immunodiagnosis and may allow the development of techniques, such as radioimmunoassays, to provide early cancer detection tests [19].

ACKNOWLEDGMENTS

This study was supported by the Cancer Research Campaign and also by a Government Equipment Grant through the Royal Society. One of us (J.G.B.) was supported by the Medical Research Council. The authors wish to acknowledge the skilful technical assistance of Mrs M. E. Marshall and Mrs J. E. Bullock.

REFERENCES

1 Baldwin, R. W. (1973) Adv. Cancer Res. 18, 1–75
2 Baldwin, R. W. and Barker, C. R. (1967) Int. J. Cancer 2, 355–364
3 Baldwin, R. W. and Moore, M. (1969) Int. J. Cancer 4, 753–760
4 Price, M. R. and Baldwin, R. W. (1974) Br. J. Cancer submitted for publication
5 Baldwin, R. W. and Glaves, D. (1972) Clin. Exp. Immunol. 11, 51–56
6 Baldwin, R. W., Harris, J. R. and Price, M. R. (1973) Int. J. Cancer 11, 385–397
7 Harris, J. R., Price, M. R. and Baldwin, R. W. (1973) Biochim. Biophys. Acta 311, 600–614
8 Baldwin, R. W. and Barker, C. R. (1967) Br. J. Cancer 21, 793–800
9 Baldwin, R. W. and Embleton, M. J. (1971) Int. J. Cancer 6, 373–382
10 Duerksen, J. D. and Halvorson, H. (1958) J. Biol. Chem. 233, 1113–1120
11 Arnon, R. (1970) in Methods in Enzymology, (Perlmann, G. E., and Lorand, L., eds) Vol. 19, pp. 226–244, Academic Press, New York
12 Lowry, O. H., Rosebrough, N. L., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
13 Muramatsu, T. and Nathenson, S. G. (1971) Fed. Proc. 30, 691 Abstr.
14 Muramatsu, T. and Nathenson, S. G. (1970) Biochem. Biophys. Res. Commun. 38, 1–8
15 Cook, G. M. W. and Stoddart, R. W. (1973) Surface Carbohydrates of the Eukaryotic Cell, Academic Press, London
16 Reisfeld, R. A. and Kahan, B. D. (1970) Fed. Proc. 29, 2034–2040
17 Reisfeld, R. A. and Kahan, B. D. (1972) Sci. Am. 226, No. 6, 28–37
18 Mann, D. L. (1972) Transplantation 14, 398–401
19 Price, M. R., Bowen, J. G. and Baldwin, R. W. (1974) Br. J. Cancer 29, 493–494