Biochem. J. (1982) **204**, 377–381
Printed in Great Britain

A new Kupffer cell receptor mediating plasma clearance of carcinoembryonic antigen by the rat

Carol Ann TOTH, Peter THOMAS, Selwyn A. BROITMAN and Norman ZAMCHECK
Mallory Gastrointestinal Research Laboratory, Department of Medicine, Harvard Medical School, and Departments of Microbiology and Pathology, Boston University School of Medicine, Boston, MA 02118, U.S.A.

(*Received 16 September 1981/Accepted 14 January 1982*)

Native human carcinoembryonic antigen is rapidly removed from the circulation by the rat liver Kupffer cell after intravenous injection. The molecule is subsequently transferred to the hepatocyte in an immunologically identifiable form. Carcinoembryonic antigen has a circulatory half-life of 3.7 (±0.8) min, and cellular entry is by receptor-mediated endocytosis. Non-specific fluid pinocytosis and phagocytosis can be excluded as possible mechanisms by the kinetics of clearance and failure of colloidal carbon to inhibit uptake. Substances with known affinity for the hepatic receptors for mannose, *N*-acetylglucosamine, fucose and galactose all fail to inhibit carcinoembryonic antigen clearance. After two cycles of the Smith degradation, carcinoembryonic antigen is still able to inhibit clearance of the native molecule. Receptor specificity is apparently not dependent on those non-reducing terminal sugars of the native molecule. Performic acid-oxidized carcinoembryonic antigen also inhibits clearance of carcinoembryonic antigen *in vivo*. Receptor binding is not dependent on tertiary protein conformation. Non-specific cross-reacting antigen, a glycoprotein structurally similar to carcinoembryonic antigen, is cleared by the same mechanism.

Carcinoembryonic antigen (CEA) is a glycoprotein of approx. 200000 mol.wt. present in the human digestive system and in human colonic adenocarcinomas of endodermally derived epithelium (Gold & Freedman, 1965*a*,*b*; Go *et al.*, 1975). Carbohydrate comprises approx. 50% of the molecule, which is composed of galactose, fucose, *N*-acetylglucosamine (2-acetamido-2-deoxy-D-glucose), mannose and sialic acid. Branched oligosaccharide side chains are of the characteristic 'serum type', involving a *N*-glycosidic linkage between *N*-acetylglucosamine and asparagine (Westwood *et al.*, 1974). Carcinoembryonic antigen is monitored in plasma by radioimmunoassay as an aid to the diagnosis of many cancers of epithelial cell origin. Serial determinations of carcinoembryonic antigen can be useful in the determination of patient prognosis and the effectiveness of therapy, especially in the treatment of carcinoma of the colon, breast and lung (Skarin *et al.*, 1974; Sherlock & Zamcheck, 1976; Zamcheck & Martin, 1981). Increases in plasma carcinoembryonic antigen also occur in patients with benign liver disease (Loewenstein & Zamcheck, 1977), and experimental studies have shown that the liver is its major site of metabolism (Thomas & Hems, 1975).

Native carcinoembryonic antigen is removed from the circulation by the xenogenic rat liver both *in vivo* and in an isolated perfusion system (Thomas & Hems, 1975). Rapid circulatory clearance of carcinoembryonic antigen [$t_{\frac{1}{2}}$ (half-life) < 4 min] occurs in several species, including the dog, rabbit, hamster, mouse, rhesus monkey and baboon (Schuster *et al.*, 1973; Primus *et al.*, 1974; Thomas *et al.*, 1977, 1980). Within 15 min of injection most of the carcinoembryonic antigen is found in Kupffer cells, with little or none in the hepatocytes. After 1 h, carcinoembryonic antigen is present in both cell populations, and after 3 h it is exclusively in the hepatocytes (Thomas *et al.*, 1977). The nature of the specificity of the Kupffer cell receptor for carcinoembryonic antigen is the subject of the present paper.

Materials and methods

Chemicals

Carcinoembryonic antigen was isolated from a metastatic liver tumour of a patient with colorectal carcinoma, essentially by the method of Krupey *et al.* (1972). The preparation's activity was equivalent to or greater than that of the Roche standard.

0306-3283/82/050377-05$01.50/1

Carbohydrate analysis was performed by the method of Clamp *et al.* (1971). Carcinoembryonic antigen was radioiodinated by the chloramine-T method (Greenwood *et al.*, 1963).

Non-specific cross-reacting antigen (NCA) was prepared from the metastases of human colonic carcinoma by extraction with $HClO_4$ and purified by column chromatography on Sepharose 4B and Sephadex G-200. Additional purification was achieved by DEAE-cellulose chromatography and acetone precipitation from aq. 5% (v/v) acetic acid. Purified non-specific cross-reacting antigen ran as a single band on polyacrylamide-gel electrophoresis (in borate buffer, pH 8.6) (Westwood *et al.*, 1976).

β-Glucuronidase from bovine liver, yeast mannan and fetuin were purchased from Sigma Chemical Co. Agalacto-orosomucoid was prepared by treatment of orosomucoid with neuraminidase (*Vibrio cholerae*), followed by digestion with β-galactosidase (*Diplococcus pneumoniae*) (van Lenten & Ashwell, 1972) and was a gift from Dr. G. Ashwell (N.I.H., Bethesda, MD, U.S.A.). Fucoidin was purchased from ICN Pharmaceuticals.

Asialofetuin was prepared by incubating fetuin with neuraminidase (*Vibrio cholerae*) overnight at room temperature (Westwood *et al.*, 1974).

Immunohistochemistry

Balb/c mice were injected with 250μg of carcinoembryonic antigen via the tail vein, and killed by cervical dislocation at 15, 20, 120 and 180 min. The livers were removed and fixed in 10% formalin.

Histological localization of carcinoembryonic antigen in the liver was done by a triple-layer immunoperoxidase–peroxidase–antiperoxidase technique (PAP) as described by O'Brien *et al.* (1980).

Smith degradations of carcinoembryonic antigen

Removal of carbohydrate from carcinoembryonic antigen was accomplished by the Smith degradation. For the first degradation carcinoembryonic antigen (10 mg) was dissolved in 0.2 M-sodium aceate buffer (pH 3.8, 10 ml) containing sodium periodate (5 mM). The mixture was left in the dark at 20°C for 42 h. It was then dialysed against water for 7 h at 20°C. $Na_2CO_3/NaHCO_3$ buffer (0.05 M; pH 8.9; 10 ml) was added to the dialysed solution. $NaBH_4$ (20 mg) was added to the oxidized carcinoembryonic antigen and reduction proceeded at 20°C for 16 h. The mixture was dialysed against water, freeze-dried and redissolved in water. It was then applied to a Sephadex G-50 column (7.5 cm × 2.0 cm). The modified carcinoembryonic antigen (8 mg) was collected from the void volume. For the second degradation 5 mg of the material produced by the first was treated by the same protocol, resulting in a yield of 4 mg (Bessell *et al.*, 1975). Over-oxidation with periodate causes loss of immunological reactivity of carcinoembryonic antigen (Westwood & Thomas, 1975). The Smith-degraded carcinoembryonic antigens both showed an almost complete loss of activity in the Roche assay (Haagensen *et al.*, 1980) and thus did not interfere with the determination of carcinoembryonic antigen by this method.

Performic acid oxidation of carcinoembryonic antigen

Performic acid-oxidized carcinoembryonic antigen was prepared by dissolving 5 mg of carcinoembryonic antigen in 88% (v/v) formic acid (0.25 ml). H_2O_2 (30%) was then added (20μl) and the reaction mixture maintained at 0°C for 2 h, followed by the addition of iced water (2.0 ml). The preparation was freeze-dried, reconstituted in cold water (5 ml) and freeze-dried again. The effectiveness of the reaction was monitored by loss of immunological binding activity in the Roche carcinoembryonic antigen assay (Westwood & Thomas, 1975).

Clearance studies in vivo

CD male rats (Charles River) were used for the studies of clearance *in vivo*. Rats (250–350 g) were lightly anaesthetized with diethyl ether and then given intraperitoneally sodium pentabarbital (50 mg/ml; Elkins–Sinn). After an initial blood sample was taken, 250μg of carcinoembryonic antigen in 0.85% NaCl with or without the competitive inhibitor was injected intravenously via the femoral vein. Sequential blood samples (10–20μl) were drawn from the tail and placed in 0.037 M-EDTA buffer/0.17% NaN_3, pH 6.5 (Hoffman–La Roche), and vortex-mixed. The samples were assayed for carcinoembryonic antigen by radioimmunoassay by the Roche assay method as modified by Haagensen *et al.* (1980). In addition, the clearance of native carcinoembryonic antigen in the presence of modified carcinoembryonic antigens was measured by using ^{125}I-labelled carcinoembryonic antigen.

Results and discussion

The clearance of native carcinoembryonic antigen was followed histologically in the mouse by using the immunoperoxidase method. The molecule initially was taken up exclusively by the Kupffer cell. After 1 h immunologically identifiable carcinoembryonic antigen was present both in the hepatic macrophages and in the hepatocytes. Eventually carcinoembryonic antigen was present mainly in the parenchymal cells. These results confirm the cellular transfer of carcinoembryonic antigen described previously by using ^{125}I-labelled carcinoembryonic antigen and electron-microscopic radioautography (Thomas *et al.*, 1977). Immunoperoxidase staining

allows the technical advantage of detecting only undegraded carcinoembryonic antigen and excludes the detection of fragments and breakdown products.

Carcinoembryonic antigen was cleared from the circulation rapidly with $t_{\frac{1}{2}}$ (half-life) = 3.7 (±0.8) min (S.D., $n = 6$). Plots of log concentration against time showed that the rate of clearance was first-order (Fig. 1). This rapid disappearance implicates a mechanism with specific affinity for carcinoembryonic antigen. The hepatic uptake of ^{125}I-labelled polyvinylpyrrolidone by fluid pinocytosis has been reported to occur at a rate of 1.4 μl/min in a 300 g rat (Sinke *et al.*, 1979). From this rate, a dose of 250 μg of carcinoembryonic antigen in a 300 g rat would theoretically result in a blood concentration of approx. 10 ng/μl and a non-specific clearance rate of 14 ng/min. The experimentally determined clearance rate *in vivo* for carcinoembryonic antigen was 45 μg/min. Similarly, phagocytosis can be ruled out as a possible mechanism, since competition with colloidal carbon failed to inhibit carcinoembryonic antigen clearance (Table 1).

The kinetics of carcinoembryonic antigen uptake is consistent with a receptor-mediated hepatic uptake mechanism. The liver is known to have several receptors that recognize carbohydrates. A glycoprotein receptor with an affinity for mannose and *N*-acetylglucosamine is present on the Kupffer cells (Schlesinger *et al.*, 1978). Competitive-inhibition studies with yeast mannan (terminal mannose), agalacto-orosomucoid (terminal *N*-acetylglucosamine) and β-glucuronidase (terminal mannose) showed that these compounds failed to inhibit carcinoembryonic antigen uptake *in vivo* (Table 1). Asialo-(carcinoembryonic antigen) is cleared from the circulation by the hepatocyte receptor for galactose (Thomas & Jones, 1978). Since native carcinoembryonic antigen was taken up by the Kupffer cell exclusively, and competition *in vivo* with asialofetuin (terminal galactose) failed to inhibit this process (Table 1), galactose was also ruled out

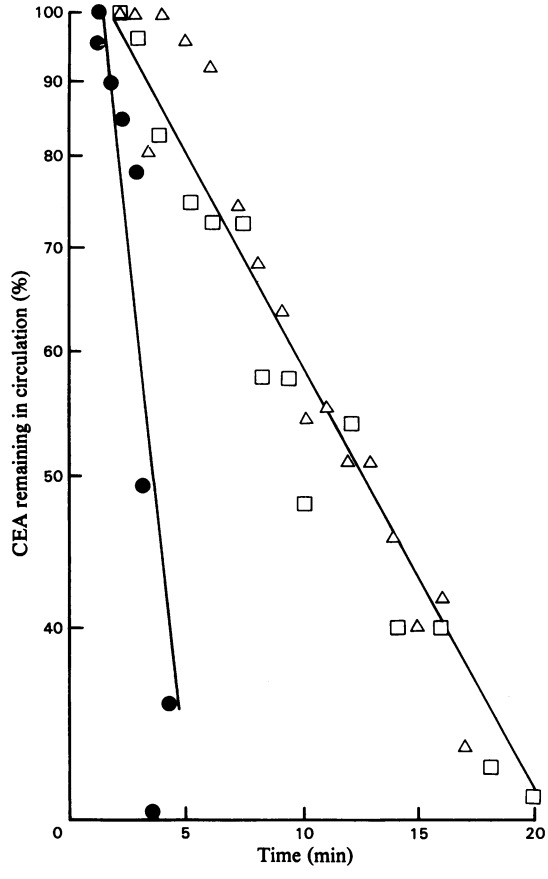

Fig. 1. *Inhibition of clearance of carcinoembryonic antigen (CEA) by Smith-degraded CEA*

CEA values were measured by radioimmunoassay of blood taken from the tail vein after intravenous injection of CEA and the inhibitory compound (see the Materials and methods section). $t_{\frac{1}{2}}$ values were calculated as the time when 50% of the CEA remained in the circulation. ●, CEA only ($t_{\frac{1}{2}} = 3.7$ min); □, CEA after first Smith degradation ($t_{\frac{1}{2}} = 12.5$ min); △, CEA after second Smith degradation ($t_{\frac{1}{2}} = 12.5$ min).

Table 1. *Potential inhibitors of clearance of carcinoembryonic antigen (CEA) from the circulation in the rat*

$t_{\frac{1}{2}}$ values were calculated as the time at which 50% of the CEA remained in the circulation from the time of injection. Blood concentrations of CEA were determined by radioimmunoassay (see the Materials and methods section).

Inhibitor	$t_{\frac{1}{2}}$ (min)	Concn. of inhibitor in excess of CEA (ratio by wt.)
None (CEA only)	3.7 (±0.8)	—
Colloidal carbon	4.9 (±0.7)	—
Yeast mannan	3.2 (±0.7)	9.7
Agalacto-orosomucoid	3.9	8
β-Glucuronidase	4.9	6.5
Fucoidin	4.3 (±1.1)	8
Asialofetuin	3.0	12
Non-specific cross-reacting antigen	21.5	17
Performic acid-oxidized CEA	12.1	10
CEA after first Smith degradation	12.5	8.3
CEA after second Smith degradation	12.5	7

as the receptor recognition site. Fucoidin (terminal fucose) was also unable to block circulatory clearance of carcinoembryonic antigen. Therefore mannose, galactose, *N*-acetylglucosamine and fucose as terminal sugars can be eliminated as the Kupffer cell recognition site. The two modifications of carcinoembryonic antigen produced by two sequential cycles of the Smith degradation both retain the ability to bind to the receptor and impede uptake of the native glycoprotein (Fig. 1). The Smith-degraded substances both show substantial loss of carbohydrate (Table 2), and, as periodate oxidation results in complete destruction of sugars in terminal non-reducing positions (Spiro, 1966), receptor-mediated endocytosis of carcinoembryonic antigen is not dependent on recognition of these terminal carbohydrate groupings. However, since 30% of the total carbohydrate is still intact after the second Smith degradation, the possibility remains that some internal sugars are recognized. This seems unlikely, however, unless the carbohydrate chains on carcinoembryonic antigen are looped over in some manner exposing internal sugar sequences at the surface of the molecule. A more credible possibility is that recognition is via part of the protein structure.

Performic acid-oxidized carcinoembryonic antigen retained its ability to be cleared from the circulation by the Kupffer cell receptor. In addition, it also inhibited the clearance of native carcinoembryonic antigen (Fig. 2). Studies of native carcinoembryonic antigen by circular dichroism showed that the intact molecule had a substantial amount of β-conformation, with some α-helix, and the remainder was random coil (Westwood *et al.*, 1978). Performic acid oxidation destroyed the structure, resulting in a molecule with only random coiling. Thus the receptor is not dependent for recognition on the presence of the ordered structure of the tertiary protein conformation of native carcinoembryonic antigen.

Non-specific cross-reacting antigen (NCA) is a glycoprotein of approx. 60000 mol.wt. which shares

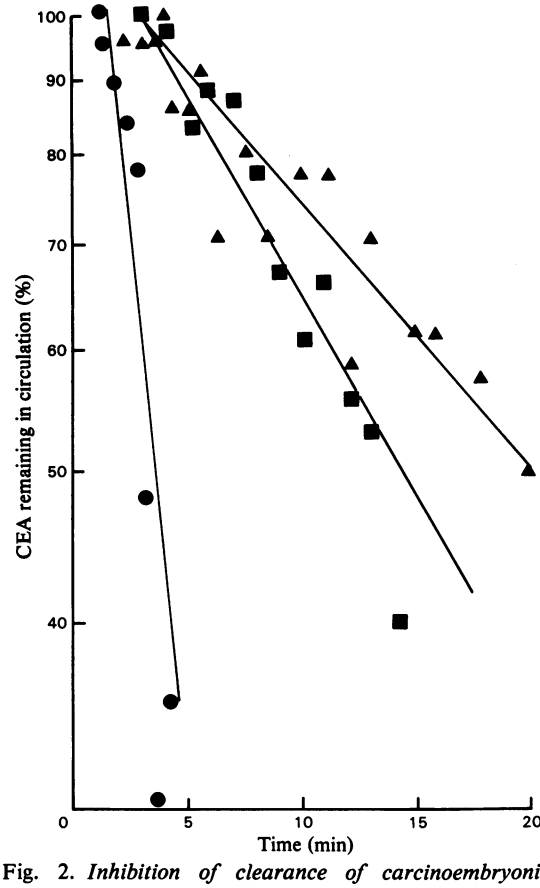

Fig. 2. *Inhibition of clearance of carcinoembryonic antigen (CEA) by non-specific cross-reacting antigen and by performic acid-oxidized CEA*

CEA values were measured by radioimmunoassay of blood taken from the tail vein after intravenous injection of CEA and the inhibitory compound (see the Materials and methods section). $t_{\frac{1}{2}}$ values were calculated as the time when 50% of the CEA remained in the circulation. ●, CEA only ($t_{\frac{1}{2}} = 3.7$ min); ■, performic acid-oxidized CEA ($t_{\frac{1}{2}} = 12.1$ min); ▲, non-specific cross-reacting antigen ($t_{\frac{1}{2}} = 21.5$ min).

Table 2. *Carbohydrate analysis of native and Smith-degraded carcinoembryonic antigen preparations*

Analysis was by g.l.c. (Clamp *et al.*, 1971)

	Carbohydrate (%)		
Carcinoembryonic antigen ...	Native	After first Smith degradation	After second Smith degradation
Fucose	5.0	—	—
Mannose	5.5	3.5	1.5
Galactose	15.1	8.7	4.0
N-Acetylglucosamine	18.8	20.6	9.5
Neuraminic acid	10.3	—	—
N-Acetylgalactosamine	4.8	4.8	3.4
Total carbohydrate	59.5	37.6	18.4

antigenic determinants and common structural features with carcinoembryonic antigen (Engvall *et al.*, 1978). Non-specific cross-reacting antigen has a circulatory half-life of approx. 5 min in the rat, with 80% of the glycoprotein accumulating in the liver (Thomas *et al.*, 1979). Non-specific cross-reacting antigen is capable of inhibiting the uptake of carcinoembryonic antigen by hepatic macrophages (Fig. 2), and it is possible that the Kupffer cell binds a protein sequence common to both glycoproteins. This common protein sequence is likely to protrude from the surface of the molecule in a random-coil conformation. The compounds effective as inhibitors, i.e. non-specific cross-reacting antigen, Smith-degraded carcinoembryonic antigen and performic acid-oxidized carcinoembryonic antigen, all show a lag phase in the clearance of carcinoembryonic antigen, which is typical for a competitive inhibitor of receptor binding. After this lag phase the decay of carcinoembryonic antigen in the circulation appears first-order.

In vivo, carcinoembryonic antigen is initially taken up by the Kupffer cell and transferred to the hepatocyte. The molecule is probably altered by the Kupffer cell, perhaps unmasking a cryptic receptor site with hepatocyte affinity. The process might involve the removal of sialic acid, exposing the penultimate galactose molecule. The theoretical alteration is not extensive, since carcinoembryonic antigen is still immunologically identifiable in the hepatocyte, and isolation of carcinoembryonic antigen from hepatocytes after uptake shows no change in molecular weight as determined by gel filtration on Sephadex G-200 (Thomas, 1980).

This investigation was supported by grant CA-04486 from the National Cancer Institute. Special thanks to Dr. R. Jeanloz for carbohydrate analysis, David V. Ives for technical assistance and Dr. G. Ashwell for his gift of agalacto-orosomucoid. We thank Hoffman–La Roche for providing the carcinoembryonic antigen assay reagents.

References

Bessell, E. M., Thomas, P. & Westwood, J. H. (1975) *Carbohydr. Res.* **45**, 257–268

Clamp, J. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–344

Engvall, E., Shirley, J. E. & Wrann, M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1670–1674

Go, V. L. W., Helmut, V. A., Holtermuller, K. H., Krag, E. & Phillips, S. F. (1975) *Cancer* **36**, 2346–2350

Gold, P. & Freedman, S. O. (1965*a*) *J. Exp. Med.* **121**, 439–462

Gold, P. & Freedman, S. O. (1965*b*) *J. Exp. Med.* **122**, 467–481

Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123

Haagensen, D. E., Cox, C. E., Dilley, W. G., Hensley, M., Murdoch, E., Newman, S. & Wells, S. A. (1980) *Clin. Chem.* **26**, 1787–1790

Krupey, J., Wilson, R., Freedman, S. O. & Gold, P. (1972) *Immunochemistry* **9**, 617–622

Loewenstein, M. S. & Zamcheck, N. (1977) *Gastroenterology* **72**, 161–167

O'Brien, M. J., Kirkham, S. E., Burke, B., Omerod, M., Saravis, C., Gottlieb, L. S., Neville, A. M. & Zamcheck, N. (1980) *Invest. Cell Pathol.* **3**, 251–258

Primus, F. J., Hansen, H. J. & Goldenberg, D. M. (1974) *Nature (London)* **249**, 837–838

Schlesinger, P. H., Doebber, T. W., Mandell, B. F., White, R., DeScheyver, C., Rodman, J. S., Miller, J. L. & Stahl, P. (1978) *Biochem. J.* **176**, 103–109

Schuster, J., Silverman, M. & Gold, P. (1973) *Cancer Res.* **33**, 65–68

Skarin, A. T., Delwiche, R., Zamcheck, N., Lokich, J. J. & Frei, E. (1974) *Cancer* **33**, 1239–1245

Sherlock, P. & Zamcheck, N. (1976) *Clinics in Gastroenterology*, pp. 625–638, W. B. Saunders Co., Philadelphia

Sinke, J., Bouma, J. M. W., Kooistra, T. & Gruber, M. (1979) *Biochem. J.* **180**, 1–9

Spiro, R. G. (1966) *Methods Enzymol.* **8**, 26–52

Thomas, P. (1980) *Biochem. J.* **192**, 837–843

Thomas, P. & Hems, D. A. (1975) *Biochem. Biophys. Res. Commun.* **67**, 1205–1209

Thomas, P. & Jones, M. (1978) *Biochem. J.* **173**, 981–983

Thomas, P., Birbeck, M. S. C. & Cartwright, P. (1977) *Biochem. Soc. Trans.* **5**, 312–313

Thomas, P., Edwards, R. G. & Westwood, J. H. (1979) *Biochem. Soc. Trans.* **7**, 699–701

Thomas, P., Rogers, A. E., Fox, F. G. & Zamcheck, N. (1980) *Clin. Res.* **28**, 632A

van Lenten, L. & Ashwell, G. (1972) *J. Biol. Chem.* **247**, 4633–4640

Westwood, J. H. & Thomas, P. (1975) *Br. J. Cancer*, **32**, 708–719

Westwood, J. H., Bessell, E. M., Bukhari, M. A., Thomas, P. & Walker, J. M. (1974) *Immunochemistry* **11**, 811–818

Westwood, J. H., Thomas, P., Edwards, R. G. & Neville, A. M. (1976) *Protides Biol. Fluids Proc. Colloq.* **24**, 413–418

Westwood, J. H., Thomas, P., Edwards, R. G., Scopes, P. M. & Barrett, M. W. (1978) *Br. J. Cancer* **37**, 183–189

Zamcheck, N. & Martin, E. W. (1981) *Cancer* **47**, 1620–1627