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SHORT COMMUNICATIONS

Monoclonal antibodies distinguish between carboxylesterase isoenzymes in different tissues of rat and guinea pig

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Abstract—The carboxylesterase (CarbE) activity in the main tissues (lung, liver, plasma and small intestine) of both the rat and guinea pig was separated by chromatofocusing. The three CarbE isoenzymes in the small intestine from both species showed nearly identical pI values. Monoclonal antibodies (MAbs) raised against rat lung CarbE (pI 5.8) were used in enzyme-linked immunosorbent assays to distinguish between these closely related CarbE isoenzymes. None of the MAbs did bind to the active site as no inhibition of the enzyme was seen when the MAbs were added. The immunological study showed a strong relationship between lung CarbE (pI 5.8) and the rat liver CarbE (pI 6.0). The MAbs were also strongly bound to the high pI forms of the CarbE isoenzymes in plasma and small intestine from both rat and guinea pig, but not with the low pI forms. These results indicate that two immunochemically distinct categories of CarbE isoenzymes are present in the plasma and small intestine.

Carboxylesterases (CarbEs*) (EC 3.1.1.1) are a group of B-esterases which are characterized by a broad substrate specificity for aliphatic and aromatic esters as well as for aromatic amides. They are important in the hydrolytic transformation of many toxic pesticides, insecticides and drugs, and in the detoxification of organophosphorus compounds by covalent binding to the active sites of the enzymes. The unusual broad substrate specificity of the CarbEs is due, in part, to multiple CarbE isoenzymes. Many of these isoenzymes have now been partially purified, but the number of forms and the diversity of their structure are only beginning to be clarified.

Immunochemical approaches with polyclonal antisera have previously been used to reveal the distinct character of the liver CarbE isoenzymes [1], but the specificity of monoclonal antibodies might be more ideally suited to being used as probes of the structure and function of different forms of CarbE. In the present study we have raised murine monoclonal antibodies (MAbs) to rat lung CarbE isoenzyme (pI 5.8) by the hybridoma technology, and these MAbs have been used in enzyme-linked immunosorbent assays (ELISA) to distinguish between the different CarbE isoenzymes in the tissues (lung, liver, plasma and small intestine) of both the rat and guinea pig.

Materials and Methods

Chemicals. Sephadex G-25M, Ultrogel AcA 34, Polybuffer exchanger 94, Polybuffer 74, MAb trap G kit with a Protein G Sepharose 4 fast flow column were from Pharmacia LKB, Biotechnology Division (Uppsala, Sweden). 4-Nitrorophenyl butyrate, Freund's Complete and Incomplete Adjuvants, polyethylene glycol (M_r 1300–1600), 2,2′-azinodi-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ATBS) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Mouse monoclonal antibody isotyping kit was from Amersham International (Amersham, U.K.). Nunc Immuno plates were from Nunc (Roskilde, Denmark). Biotin-labeled goat anti-mouse IgG and horseradish peroxidase-labeled streptavidin were from Southern Biotechnology Associates (Birmingham, U.S.A.). Fetal bovine serum was from Hyclone Laboratories (Logan, U.S.A.). NSO/1 myeloma cells were kindly provided by Professor Z. Eshar, Weissman Institute, Rehovot, Israel. All other chemicals were of analytical-grade quality.

Animals. Male Wistar rats (200–300 g weight), guinea pigs (male albino within the weight range 200–400 g) and Balb/c mice (15–20 g weight) (from Møllegaard, Copenhagen, Denmark) were examined at the National Institute of Public Health, Oslo, Norway. The animals were given a standard laboratory diet and water *ad lib.*

Preparation of rat and guinea pig tissue fractions. Rat or guinea pig small intestine was rinsed with ice-cold 50 mM Tris, pH 7.5, containing 0.1 M NaCl and homogenized in 4 vol. of the same buffer with a Polytron instrument for 20 sec. The homogenate was filtered through glass wool to remove fat, followed by centrifugation at 10,000 *g* for 20 min at 4°. The sample buffer was exchanged with the starting buffer, 25 mM imidazole–HCl buffer, pH 7.4, on a Pharmacia PD-10 column. The preparation was chromatofocused at 4° as described in the chromatofocusing kit instruction (Pharmacia LKB). Rat and guinea pig liver, lung and plasma fractions were obtained as described previously [2].

Enzyme assay method. CarbE activity was measured spectrophotometrically with a Beckman DU-50 spectrophotometer and 4-nitrophenyl butyrate was used as substrate [3].

The rat lung CarbE (pI 5.8) was incubated with the different MAbs in 0.1 M sodium phosphate buffer, pH 7.8, 30° at molar ratios of IgG ($M_r \approx 160{,}000$):CarbE (pI 5.8, $M_r \approx 180{,}000$) of about 0.25, 1 and 6. After various times (5–20 min), the substrate, 4-nitrophenyl butyrate, was added (final concn 2 mM) and the residual activity determined as described above, but with a total volume of 0.2 mL. The absorbance of 4-nitrophenol at 400 nm was now followed with a Dynatech MR 700.

SDS–PAGE. SDS–PAGE was performed according to Laemmli [4] on the Phast Gel System from Pharmacia LKB. A polyacrylamide gradient gel, Phast Gel Gradient 8-25, was used with Phast Gel SDS buffer strips.

Preparation of anti-CarbE MAbs. MAbs were produced by the method described by Köhler and Milstein [5]. One of the carboxylesterase isoenzymes (pI 5.8) purified from rat lung was used in the MAb production [6].

The frozen positive subclones were thawed and transferred to growth medium. For large scale production of MAbs *in vitro* both low (2%) and high (10%) serum concentrations were used. MAbs were purified from culture supernatant by using affinity chromatography. A MAb trap G kit with a Protein G Sepharose column from Pharmacia LKB was used. The cell culture supernatant (~25 mL) was

* Abbreviations: CarbE, carboxylesterase; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assays; PBS, phosphate-buffered saline.

Table 1. ELISA for reactivity of MAbs with CarbEs

MAb	Ig subclass	Rat (pI)									Guinea pig (pI)								
		Liver				Plasma		Small intestine		Lung		Liver			Plasma		Small intestine		
		6.2	6.0	5.6	5.2	4.4	<4.0	5.4	4.75	<4.2	5.9	4.9	<4.2	5.6	5.0	4.6	6.2	5.2	<4.0	5.6	4.5	<4.2
2H6-1	IgG1, k	45 ± 8	82 ± 7	36 ± 5	50 ± 8	63 ± 5	<5	45 ± 7	73 ± 10	<5	30 ± 8	<5	<5	<5	<5	<5	<5	73 ± 3	<5	<5	36 ± 5	<5	<5
1C1-1	IgG1, k	21 ± 8	52 ± 2	<5	<5	20 ± 7	<5	58 ± 9	<5	<5	<5	<5	<5	<5	<5	<5	<5	66 ± 8	30 ± 5	<5	60 ± 6	21 ± 4	<5

Results are percentages of control, means ± SEM (N = 4).
The ELISA conditions for testing the binding of MAbs to CarbE isoenzymes are described in the text. The level of binding of MAbs to rat lung CarbE (pI 5.8) was set to 100%. A reaction of less than 5% was considered negative.

applied to the top fit and absorbed into the gel. Unbound protein was washed away with 20 mM sodium phosphate buffer, pH 7.0. A neutralizing buffer (1.0 M Tris–HCl, pH 9) was added to the collection tubes and IgG was eluted with a 0.1 M glysin–HCl buffer, pH 2.7.

ELISA. ELISA was used in these studies to analyse for antibody production and in the experiments analysing for antibody binding to CarbE isoenzymes from different tissues. Antigens to be tested against MAbs were diluted and titrated in phosphate-buffered saline (PBS) buffer (pH 7.4), and incubated overnight at 20° in Nunc Immuno plates. Plates were washed three times with PBS–Tween (PBS + 0.05% Tween 20) and the plates were incubated at 37° for 30 min between each step. Fetal bovine serum was used to block unreacted sites. Biotin-labeled goat anti-mouse IgG and horseradish peroxidase labeled streptavidin were used. The plates were then developed with a freshly made substrate solution (ATBS and H_2O_2). Plates were read after 10 to 30 min at 630 nm with a Dynatech MR 700. Controls were run simultaneously on the same plates.

Results

CarbE in rat and guinea pig small intestine. The CarbEs of the small intestine from both the rat and guinea pig were separated by chromatofocusing into three different isoenzymes. The small intestine preparation from the rat was separated into one main peak, pI 5.4, and a smaller one, pI 4.75. A third isoenzyme, pI <4.2, was detected after elution with 1 M NaCl solution. Similarly the small intestine preparation from the guinea pig was separated into two main peaks, pI 5.6 and 4.5, of CarbE activity and a small peak, pI <4.2, which could be detected after elution with 1 M NaCl solution.

Preparation and selection of MAb directed against rat lung CarbE (pI 5.8). The seven positive subclones, which were selected after an ELISA screening specific for CarbE (pI 5.8), were transferred to growth medium for large scale production. Only two of the cell cultures grew properly. The purified MAbs dissociated to only one heavy and one light chain in a SDS polyacrylamide gel, which supports the monoclonal nature of these antibodies.

ELISA in determination of immunological relationship. Non-competitive ELISA was used to assay for potential binding of two MAbs produced against CarbE (pI 5.8) toward the rat and guinea pig CarbE isoenzymes (Table 1). If binding of MAbs to the CarbE isoenzyme-coated microtest plates was less than 5% of control, the reaction was considered negative. For accurate results where cross-reactions were detected, it was essential to perform the ELISA under conditions where CarbE concentrations were proportional to each MAb. This was determined for each MAb and its reaction to purified rat lung CarbE (pI 5.8) before testing against other CarbEs. For each of the CarbE isoenzymes a sequence of dilutions was coated to microtest plates and tested against each MAb (see Fig. 1).

The results indicate the existence of common antigenic sites on rat CarbEs from lung (pI 5.8), liver (pI 6.2 and 6.0), plasma (pI 4.4) and small intestine (pI 5.4), and guinea pig CarbEs from plasma (pI 6.2) and small intestine (pI 5.6). Other CarbE isoenzymes reacted with only one of the MAbs and had therefore a weaker degree of similarity to the CarbE (pI 5.8). Table 1 also shows that MAb 1C1-1 reacted with two of the isoenzymes from the plasma and small intestine in the guinea pig, but the MAb 2H6-1 reacted only with the isoenzymes of highest pI. The results presented here indicate that 2H6-1 reacted better with the rat isoenzymes than with the guinea pig isoenzymes. The two MAbs did not bind to the CarbE isoenzymes purified from liver and lung in the guinea pig, except for one form (lung CarbE, pI 5.9).

Effect of MAbs on the catalytic activity of rat lung CarbE (pI 5.8). Each of the seven MAbs was tested for its ability to inhibit the hydrolysis of 4-nitrophenyl butyrate using rat

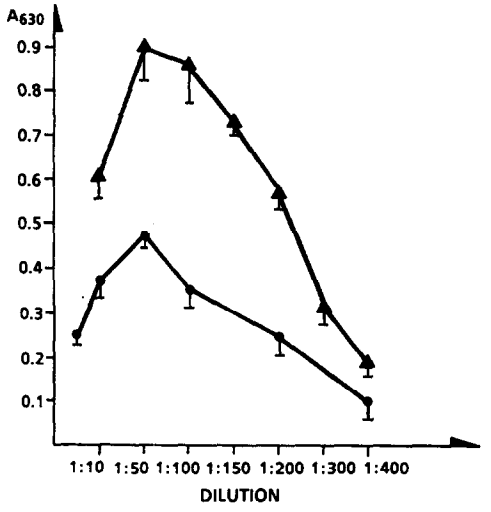

Fig. 1. ELISA for testing the linear range for different dilutions of CarbE (pI 6.0) from rat liver against two MAbs: 2H6-1 (▲) and 1C1-1 (●). Results are means ± SEM (N = 4).

lung CarbE (pI 5.8), but no inhibition was observed for these MAbs. These results indicate that the active site of the enzyme is not involved in the formation of the antigen–antibody complex.

Discussion

In rat liver four CarbE isoenzymes can be separated easily [7, 8]. These isoenzymes all behave as monomers with a molecular mass of about 60 kDa, except for CarbE (pI 6.0) which occurs as a stable trimer of about 180 kDa [7]. The CarbE (pI 6.0) reacted strongly with both MAbs (see Fig. 1 and Table 1). This isoenzyme also resembles rat lung CarbE (pI 5.8) in number of subunits, molecular mass and the N-terminus [6]. The other rat liver CarbE isoenzymes have at least one epitope in common, illustrated by the relatively high binding of the isoenzymes to the MAb 2H6-1.

Two different reports on the purification of CarbE from rat plasma have been published. Choudhury [9] reported a molecular mass of 43 kDa, but Hashinotsume *et al.* [10] reported a highly purified CarbE with a molecular mass of 84 kDa and pI 4.4. The discrepancy between the two molecular masses can be explained by the possibility that the enzyme was dissociated into subunits of 43 kDa, which were also active. Our immunological results show that there have to be two different CarbE isoenzymes with separate protein structures, as only CarbE (pI 4.4) was bound to the MAbs. The subunit masses of two of the guinea pig plasma CarbEs (pI 6.2 and pI <4.0) have been determined to be 58 and 80 kDa [11]. Both MAbs reacted strongly with guinea pig plasma CarbE (pI 6.2), but there were no significant reaction of the low pI form CarbE (pI <4.0) with the MAbs.

De Jong *et al.* [12] have shown that antiserum raised against a rat liver CarbE hydrolysing monoglyceride inhibits one of the three CarbEs from rat small intestine completely. These two CarbE isoenzymes, both showing monoacylglycerol hydrolase activity, were therefore suggested to be at least partially similar. Mentlein *et al.*[1] have demonstrated that an antiserum to rat liver CarbE (pI 6.0) gave a precipitation with rat serum. In agreement, our immunological results also indicate a relationship between CarbE from rat liver and the high pI forms of CarbE in the small intestine and plasma. However, the lack of response of the low pI forms indicates that two distinct categories of CarbE isoenzymes are present in both the rat and guinea pig, as shown earlier in the mouse [13].

Böcking and von Deimling [14] have suggested that one or two different CarbE isoenzymes from mouse jejenum enter the blood stream with the chylomicrons. Sterri [15] has proposed that intestinal CarbE may be a possible source of the enzyme in rat plasma, due to the oxime-induced reactivatibility and the low pI for enzyme in both tissues. Our immunological results could support such a proposal.

In summary, the immunological study showed a strong relationship between the rat lung CarbE (pI 5.8) and the rat liver CarbE (pI 6.0), but very little similarity of these CarbEs to the guinea pig liver and lung CarbE isoenzymes. The MAbs were strongly bound to the high pI forms of the CarbE isoenzymes in the plasma and small intestine from both the rat and guinea pig, but not with the low pI forms. These results indicate that two immunochemically distinct categories of CarbE isoenzymes are present in the plasma and small intestine.

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REFERENCES

1. Mentlein R, Schumann M and Heymann E, Comparative chemical and immunological characterization of five lipolytic enzymes (carboxylesterases) from rat liver microsomes. *Arch Biochem Biophys* **234**: 612–621, 1984.
2. Gaustad R, Johnsen H and Fonnum F, Carboxylesterases in guinea pig—a comparison of the different isoenzymes with regard to inhibition by organophosphorus compounds *in vivo* and *in vitro*. *Biochem Pharmacol* **42**: 1335–1343, 1991.
3. Sterri SH, Johnsen BA and Fonnum F, A radiochemical assay method for carboxylesterase, and comparison of enzyme activity towards the substrates methyl[1-^{14}C]butyrate and 4-nitrophenyl butyrate. *Biochem Pharmacol* **34**: 2779–2785, 1985.
4. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
5. Köhler G and Milstein C, Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**: 495–497, 1974.
6. Gaustad R, Sletten K, Løvhaug D and Fonnum F, Purification and characterization of carboxylesterases from rat lung. *Biochem J* **273**: 693–697, 1991.

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7. Mentlein R, Heiland S and Heymann E, Simultaneous purification and comparative characterization of six serine hydrolases from rat liver microsomes. *Arch Biochem Biophys* **200**: 547–559, 1980.
8. Robbi M and Beaufay H, Purification and characterization of various esterases from rat liver. *Eur J Biochem* **137**: 293–301, 1983.
9. Choudhury SR, Isolation and properties of an esterase isoenzyme of rat serum. *Histochem J* **6**: 369–382, 1974.
10. Hashinotsume M, Higashino K, Hada T and Yamamura Y, Purification and enzymatic properties of rat serum carboxylesterase. *J Biochem* **84**: 1325–1333, 1978.
11. Cain K, Reiner E and Williams DG, The identification and characterization of two separate carboxylesterases in guinea pig serum. *Biochem J* **215**: 91–99, 1983.
12. De Jong BJP, Kalkman C and Hulsmann WC, Partial purification and properties of monoacylglycerol lipase and two esterases from isolated rat small intestinal epithelial cells. *Biochim Biophys Acta* **530**: 56–66, 1978.
13. Ronai A, Wassmer B, Looze S de and Deimling O von, Immunochemical interrelationships between carboxylesterase isoenzymes of the house mouse, *Mus musculus*. *Comp Biochem Physiol* **82B**: 347–355, 1985.
14. Böcking A and Deimling O von, Dynamics of non-specific esterase during fat resorption in the jejunum of the house mouse, *Mus musculus*. *Histochemistry* **75**: 377–385, 1982.
15. Sterri SH, The importance of carboxylesterase detoxification of nerve agents. In: *3rd International Symposium on Protection Against Chemical Warfare Agent, Umeå, Sweden, 11–16 June, 1989*. FOA report C 40266-4.6, 4.7, pp. 235–240, 1989.

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Synergism in insulin-like effects of molybdate plus H_2O_2 or tungstate plus H_2O_2 on glucose transport by isolated rat adipocytes

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Abstract—The effect of molybdate, tungstate, molybdate plus H_2O_2 or tungstate plus H_2O_2 on 3-*O*-methylglucose (3-*O*-MG) uptake was studied in isolated rat adipocytes to investigate whether these agents possess an insulin-like action. High concentrations (10–30 mM) of molybdate or tungstate significantly stimulated the uptake of 3-*O*-MG while 1 mM of the metaloxides did not. The combination of 1 mM molybdate and 1 mM H_2O_2, or 1 mM tungstate and 1 mM H_2O_2 induced striking stimulation of the uptake of 3-*O*-MG in a synergistic manner, whereas 1 mM H_2O_2 alone showed only a small effect. The effect of metaloxides plus H_2O_2 (1 mM) and the effect of insulin (20 nM) were not additive, and both effects were ATP or energy dependent based on experiments using KCN. These results indicate that a weak insulin-like effect of molybdate or tungstate is potentiated synergistically with H_2O_2, presumably by producing peroxocompounds. Based on the present findings, these new agents may be useful for investigating the mechanism of insulin action and may indicate a new class of drugs for diabetes mellitus.

It is well established that vanadate [1–3], peroxovanadate [4–8] and selenate [9] have insulin-like actions. We found recently that molybdate and tungstate, which resemble vanadate and selenate, also had a weak insulin-like effect on glucose transport. This observation led us to investigate how this action could be potentiated at low concentrations and we performed further experiments based on the working hypothesis that the peroxocompounds possess more insulinomimetic activity than the parent compounds.

Materials and Methods

Materials. 3-*O*-[^{3}H]Methyl-D-glucose and L-[1-^{14}C]glucose were purchased from New England Nuclear (Boston, MA, U.S.A.); sodium molybdate from Nacalai tesque (Kyoto, Japan); sodium tungstate, sodium orthovanadate and H_2O_2 from Wako Pure Chemical Industries (Osaka, Japan); porcine insulin, phloretin and bovine serum albumin (RIA grade) from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and collagenase (CLS 1) from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.).

Glucose transport assay. Epididymal and perirenal adipose tissues were removed from male Wistar rats weighing 160–200 g under anesthesia induced by the intraperitoneal injection of 100 mg/kg sodium pentobarbital and isolated adipocytes were prepared by the collagenase method [10]. The cell suspension in Krebs–Henseleit HEPES buffer [11] supplemented with 20 mg/mL bovine serum albumin and 3 mM sodium pyruvate, pH 7.4, was adjusted to a cytocrit value of 21.3% (20.0% in the net cell volume as 6% of the packed cell volume is occupied by extracellular water [12]). The glucose transport activity was assessed by measuring the rate of specific uptake of 3-*O*-methylglucose(3-*O*-MG) for 3 sec at 37°, which was corrected for the non-specific uptake estimated using L-glucose. This method was based on the method of Toyoda *et al.* [13] and provided 52.3 ± 3.3 (N = 4) as the per cent of intracellular water space filled after 3 sec in the presence of 20 nM insulin. Unless otherwise stated, the metaloxides (sodium molybdate, sodium tungstate and sodium orthovanadate) and H_2O_2 were mixed immediately before use