A highly sensitive e.l.i.s.a. for endopeptidase-24.11, the common acute-lymphoblastic-leukaemia antigen (CALLA, CD-10), applicable to material of porcine and human origin

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Endopeptidase-24.11 is a widely distributed cell-surface enzyme with a key role in the metabolism of neuropeptides. It is now known to be identical with CD-10, the common acute-lymphoblastic-leukaemia antigen (CALLA). An e.l.i.s.a. is described which utilizes two antibodies, one monoclonal, the other polyclonal, generated to pig endopeptidase-24.11. These antibodies cross-reacted with human endopeptidase-24.11, thus making the assay applicable to both species. By using optimum conditions for the e.l.i.s.a., as little as 25 pg of pure pig endopeptidase-24.11 could be quantified at 95 % confidence limits. E.l.i.s.a. of tissue homogenates from a variety of pig tissues and of human kidney correlated well with enzymic assays. However, the use of detergents to solubilize the antigen greatly decreased the sensitivity of the e.l.i.s.a. The e.l.i.s.a. is 1000-fold more sensitive than the immunoradiometric assay and has advantages in specificity over enzymic assays. Daudi cells, some leukaemic cells shown to be CALLA-positive, and Caco-2 cells, could also be assayed, but N_2 cavitation was necessary to fragment the cells, and only part of the total endopeptidase-24.11 activity in Daudi cells was recognized by the e.l.i.s.a.

INTRODUCTION

Endopeptidase-24.11 (E-24.11; EC 3.4.24.11) has a wide distribution in pig tissues (Gee *et al.*, 1985; Kenny *et al.*, 1987) and is very efficient in hydrolysing many biologically active peptides, its precise physiological function depending on its cellular location [for a review, see Kenny *et al.* (1987)]. In the central nervous system, there is good evidence to support the view that it is a key enzyme in the inactivation of some peptides, e.g. substance P, after release at the synapse [for a review, see Turner *et al.* (1987)]. It is also important in the inactivation of atrial and brain natriuretic peptides (Kenny & Stephenson, 1988; Bourne & Kenny, 1990).

The leucocyte cluster differentiation antigen CD-10 (CALLA, the common acute-lymphoblastic-leukaemia antigen) is a surface marker for pre-B-cells in the marrow, circulating neutrophils and the transformed cells in about 80 % of cases of acute lymphoblastic leukaemia (ALL) (Greaves *et al.*, 1980; Connelly *et al.*, 1985; Pesando *et al.*, 1979). It has been shown by the application of molecular cloning methods to be identical with E-24.11 (Devault *et al.*, 1987; Malfroy *et al.*, 1987; Shipp *et al.*, 1988, 1989; Letarte *et al.*, 1988; Jongeneel *et al.*, 1989). However, in such transformed cells, and indeed at many other sites, its role is at present a matter of speculation, but may be involved in modulating growth and differentiation (Kenny *et al.*, 1989).

At this stage of our knowledge of the function of this cell-surface endopeptidase, a sensitive quantitative assay will be important for a variety of future studies. There are several enzymic assays available, depending on fluorimetric, radiometric and h.p.l.c. methods (Turner *et al.*, 1989). In unpurified fractions, specificity needs to be demonstrated by the use of an inhibitor such as phosphoramidon (Kenny, 1977). An immunoradiometric assay (i.r.m.a.) overcame some of these problems and permitted an extensive survey of pig tissues (Gee *et al.*, 1985), but there were, however, some limitations in its sensitivity. In regard to

CALLA, an i.r.m.a. was developed using a radioiodinated monoclonal antibody (A12) (Carrel *et al.*, 1984). Binding of this to a CALLA-positive cell line (NALM-1) was inhibited by sera or culture fluid containing sequestered membrane fragments expressing the antigen (Losa *et al.*, 1986). In the present paper we describe the development of a new e.l.i.s.a. which is highly sensitive and applicable to both pig and human material.

EXPERIMENTAL

Materials

Antibodies. Monoclonal antibodies to pig E-24.11 were GK4A9 and GK7C2 (Gee & Kenny, 1985; Gee *et al.*, 1983) respectively. The monoclonal antibody A12, raised against human CD-10 (Carrel *et al.*, 1983), was kindly donated by Dr. S. Carrel, Ludwig Institute for Cancer Research, Epalinges, Switzerland. Polyclonal antibody RP161 was generated in a rabbit immunized with pig E-24.11. It was affinity-purified (1 mg of IgG/ml) as described previously (Matsas *et al.*, 1986) and shown to recognize a single 90 kDa band in an immunoblot of pig kidney microvillar membranes in tracks loaded with 10–100 µg of protein and subjected to SDS/PAGE. Donkey anti-rabbit Ig horseradish-peroxidase-linked F(ab')₂ fragment was obtained from Amersham International (Amersham, Bucks., U.K.).

Antigen. E-24.11 was purified from pig kidney cortex as described by Gee *et al.* (1983), except that GK4A9 was used in place of GK7C2 for its immunoaffinity purification.

Tissues. Piglets, 4 weeks old, were supplied by the University of Leeds Field Station. Human kidneys were obtained from the Department of Pathology, Leeds General Infirmary, Leeds, U.K., who obtained consent of the next-of-kin.

Cells. Daudi cells were originally derived from a Burkitts lymphoma (Klein *et al.*, 1968) and are known to be CALLA-positive (Ritz *et al.*, 1981), and leukaemic cells were from patients with ALL and chronic myelocytic leukaemia (CML). Caco-2

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid); ALL, acute lymphoblastic leukaemia; CALLA, common acute-lymphoblastic-leukaemia antigen; CML, chronic myelocytic leukaemia; E-24.11, endopeptidase-24.11; PBS, phosphate-buffered saline (for composition and pH, see the text); i.r.m.a., immunoradiometric assay.

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(human colonic carcinoma) cells were kindly donated by Dr. D. M. Swallow, MRC Human Biochemical Genetics Unit, Galton Laboratories, University College, London N.W. 1, U.K.

Other materials. 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was from Sigma, as were the detergents, except for Zwittergen 3-08, which was from Calbiochem, Nottingham, U.K.

E.i.s.a. protocol

The method used to quantify E-24.11 in cell and tissue extracts followed the general principles for the double-antibody sandwich e.i.s.a. described by Voller *et al.* (1980). Falcon Pro-Blind plates (Becton Dickinson and Co., Lincoln Park, NJ, U.S.A.) were coated with 100 μ l of GK4A9 at a concentration of 5 μ g/ml in $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH 9.6 at 4 °C overnight. Wells were then washed on a Denley Wellwash four-plate washer (Denley Instruments, Billingshurst, West Sussex, U.K.) three times with 250 μ l of phosphate-buffered saline (140 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4; PBS) containing 0.05% (v/v) Tween 20, and then blocked with 100 μ l of 0.1% (w/v) BSA in PBS, pH 7.4, for 30 min at 35 °C in a Thermocult incubator (Boehringer, Mannheim, Germany). Various dilutions of cell or tissue homogenates or E-24.11 at known concentrations were added (vol. 100 μ l) to the wells and incubated at 35 °C for 1 h. Plates were washed as described above. Polyclonal antibody RP161 diluted 1 in 1000 in PBS containing 0.1% (w/v) BSA (PBS/BSA) (100 μ l, i.e. 0.1 μ g of IgG) was added to each well and incubated at 35 °C for 30 min. Wells were again washed and incubated at 35 °C for 30 min with 100 μ l of a 1-in-1000 dilution in PBS/BSA of donkey anti-rabbit Ig, which was an F(ab')₂ fragment coupled to horseradish peroxidase. After washing, 100 μ l of freshly prepared substrate solution (0.55 mg of ABTS/ml and 0.001% H_2O_2 in 0.1 M-citrate buffer, pH 4.3) was added to each well and incubated at room temperature in the dark. After 30 min the reaction was stopped by adding 50 μ l of 0.1 M-citric acid to the wells. The absorbance at 405 nm was read with an Anthos 2001 plate reader (Anthos Labtec Instruments, Salzburg, Austria).

Preparation of tissue homogenates

Tissues were homogenized at 4 °C in 9 vol. of 50 mM-Tris/HCl/500 mM-NaCl, pH 8.0, with an Ultra-Turrax homogenizer (Janke and Kunkel KG, Staufen, Germany).

Cell culture and disruption of cell membranes

Daudi cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal-calf serum. Caco-2 cells were cultured in Dulbecco's modified Eagle medium with 20% foetal-calf serum (Pinto *et al.*, 1983). Cells (5×10^6) were centrifuged (100 g, 10 min) and washed by resuspension in 10 ml of PBS and pelleted as described above. The pellet was resuspended in 1.0 ml of PBS and loaded into a prechilled (0 °C) PARR Cell Disruption Bomb (Scientific and Medical Products, Manchester, U.K.). The bomb was pressurized to 5520 kPa (800 lbf/in²), maintained at 0 °C for 10 min and the cells then discharged. From the Caco-2 cells homogenate a P₄ membrane fraction was prepared as described previously (Booth & Kenny, 1974).

Other methods

The h.p.l.c. assay of E-24.11 used [D-Ala²,Leu⁵]enkephalin (0.5 mM) as substrate, as described by Matsas *et al.* (1983). To investigate the effects of detergent and salt on the e.i.s.a., E-24.11 was added to the plates in PBS/BSA containing the appropriate concentrations of detergent or salt. Protein was assayed by the Lowry method, with BSA as standard.

RESULTS

Optimization of the e.i.s.a.

Three monoclonal antibodies to E-24.11 were tested for maximal binding to pig E-24.11. All three bound the pig antigen, but the cross-reacting human antibody, A12, showed weakest binding, while both GK4A9 and GK7C2 had similar binding characteristics, as seen by their parallel absorbance curves (Fig. 1). GK4A9 was the antibody chosen for further development of the e.i.s.a. The effect of using different amounts of GK4A9 was investigated; the sensitivity of the assay was slightly greater at 0.5 μ g compared with 0.25 μ g, but decreased markedly at the higher amounts (results not shown).

Optimal dilutions of the polyclonal antibodies were determined by using a checkerboard titration. They were found to be 1/1000 for RP161 and 1/1000 for the donkey anti-rabbit Ig horseradish-peroxidase-linked F(ab')₂ fragment. The binding of E-24.11 to the coating antibody, GK4A9, was maximal in 1 h at 35 °C, as seen in Fig. 2(a). Lengthening the incubation time to 2 h did not affect the E-24.11 standard curve, whereas incubation times of less than 1 h resulted in lower absorbance values. RP161 polyclonal antibody bound to E-24.11 rapidly and maximum was reached after only 15 min; longer incubation times did not significantly alter absorbance values (Fig. 2b).

Effects of detergents and NaCl

The possibility of assaying E-24.11 in detergent extracts of Daudi cells was explored, using Triton X-100, β -octyl glucoside, CHAPS and Zwittergen 3-08, all at 1% (w/v). The standard curve could not be reproduced in the presence of the first three of these detergents, such that only amounts in excess of 1 ng of E-24.11 were detectable. In contrast, Zwittergen 3-08 did not perturb the standard curve. This result was, however, deceptive, in that 1% Zwittergen 3-08 was wholly ineffective in solubilizing the antigen from Daudi cells, assayed enzymically, a result consistent with the very high critical micelle concentration of this detergent (Gardas & Lewartowska, 1988).

The standard curve was not affected when the dilutions of E-24.11 were made in the presence of NaCl up to 0.5 M.

Species-specificity of the e.i.s.a. and correlation with enzymic assays

The conditions described above for the determination of the purified pig E-24.11 were then applied to homogenates of a

Fig. 1. Comparison of three monoclonal antibodies

Plates were coated overnight at 4 °C with 5 μ g (vol. 100 μ l) of GK4A9 (○), GK7C2 (△) and A12 (□) monoclonal antibodies/ml, and the e.i.s.a. was performed as described in the Experimental section.

Fig. 2. Optimization of times for binding of antigen and polyclonal antibody (RP161)

See the Experimental section for details. (a) Four incubation times were tested for the binding of E-24.11: ○, 15 min; △, 30 min; □, 60 min; ●, 120 min. (b) Four incubation times for the binding of RP161 were tested: ■, 5 min; ○, 15 min; △, 30 min; □, 60 min. All incubations were at 35 °C.

Fig. 3. Correlation of e.l.i.s.a. (abscissa) and enzymic assays of E-24.11 in pig and human tissues

●, Pig tissues: K, kidney cortex; L, lymph node; T, thyroid; SM, skeletal muscle; CM, cardiac muscle. ■, human kidney (whole). The enzymic assay values were converted into ng of E-24.11/mg of protein using the k_{cat} of 5645 min⁻¹ reported for this assay (Matsas *et al.*, 1984).

human kidney. Values of samples diluted from 25- to 125-fold were parallel with the standard curve obtained with the pig enzyme. The substitution of A12, the monoclonal antibody raised to human CALLA, for GK4A9 did not improve the absorbance values when human kidney was assayed. An immuno-

Table 1. E.l.i.s.a. of E-24.11 in homogenates of pig tissues (a) and in some human leukaemic cells (b)

See the Experimental section for details. Each tissue sample was homogenized in 9 vol. of buffer, and suitable dilutions were prepared. Known amounts of purified pig E-24.11 were run in conjunction with the tissue and cell homogenates to generate the standard curve. Results are means ± s.e.m. ($n = 3$, except for leukaemic cells, where only one assay was possible) and are expressed as ng of E-24.11/mg of protein. *, Result expressed as pg of E-24.11/10³ cells; abbreviations: n.d., not detectable; c-ALL, common acute lymphoblastic leukaemia; M1, M2, types 1 and 2 of myeloid leukaemia.

(a) Pig tissues

Tissue	E-24.11 (ng/mg of protein)
Kidney cortex	1480 ± 113
Lymph node	158 ± 27
Salivary gland	5.23 ± 0.87
Thyroid	1.02 ± 0.04
Aorta	1.01 ± 0.15
Skeletal muscle	0.439 ± 0.03
Striatum	0.307 ± 0.056
Pituitary	0.226 ± 0.094
Cardiac muscle	0.171 ± 0.02
Lung	0.070 ± 0.016
Liver	0.065 ± 0.009
Cerebellum	0.031 ± 0.001
Cerebral cortex	n.d.

(b) Human cells

Case no.	Leukaemia class	E-24.11 (ng/mg of protein)
881697	c-ALL	56.9
88812 (2)	c-ALL	77.2
893026	c-ALL	6.2
892676	c-ALL	n.d.
90985	M1	0.08
88651	M1	0.16
893108	M2	0.13
901058	M2	0.07
Daudi cells	—	0.35 ± 0.4*
Caco-2 cells	—	3.16 ± 0.28

affinity-purified preparation of human kidney E-24.11 also gave a parallel curve, but gave a value 3.6 % of that of the pig E-24.11 standard, consistent with the low enzyme activity of this particular preparation (about 5 % of that of the purified pig enzyme). Five different pig tissues were compared for their e.l.i.s.a. and enzymic assay values (Fig. 3). The values for pig kidney cortex, lymph node, thyroid, and skeletal and cardiac muscles showed an excellent correlation, as did the single sample of human kidney. Thus the assay developed for the pig antigen was suitable for detecting human enzyme and correlated well with an enzyme assay.

Distribution of E-24.11 in homogenates of pig tissues

Table 1 shows the amount of E-24.11 in various pig tissues expressed as ng of E-24.11/mg of protein. Kidney cortex was the major, and lymph node the second, location of E24.11, as previously noted in the i.r.m.a. survey (Gee *et al.*, 1985). Tissues with previously undetectable levels of E-24.11 by i.r.m.a., such as thyroid, aorta, lung, liver and cerebellum, were shown to contain

the antigen, albeit in low amounts. None was detectable in the cerebral cortex.

Assay of E-24.11 in human cells

Nitrogen cavitation yielded preparations of human cells suitable for e.l.i.s.a. The assay results for some leukaemic cells, of both lymphoblastic cells and of Caco-2 cells grown in monolayer are shown in Table 1. Caco-2 cells express E-24.11 (S. Howell, A. J. Turner & A. J. Kenny, unpublished work), and the e.l.i.s.a. value from P₄ fractions (3.16 ± 0.28 ng/mg of protein) agreed reasonably well with that by enzyme assay (4.66 ng/mg of protein). However, the e.l.i.s.a. value for Daudi-cell homogenates (0.35 ± 0.04 pg of E-24.11/10³ cells) appears to underestimate the E-24.11 content when compared with the total enzyme activity, determined after solubilization with 1% Triton X-100 (1.8 pg of E-24.11/10³ cells). Only about 20% of the antigen appeared to be available in the e.l.i.s.a. in this type of cell preparation.

DISCUSSION

Specificity of the e.l.i.s.a.

Specificity depends crucially on the two anti-E-24.11 antibodies employed. The hybridoma producing GK4A9, an IgG, was twice cloned and its antigen confirmed (Gee & Kenny, 1985) by immunoprecipitation from detergent-solubilized membranes, by i.r.m.a. screening against purified pig E-24.11 and by its repeated use in isolating pure, active, E-24.11 by immunoaffinity chromatography. By immunofluorescence, only brush borders were stained in cryostat sections of pig kidney. The epitope did not survive denaturation, since GK4A9 did not detect a band in Western blotting; in contrast, RP161 bound to a single polypeptide band of 90 kDa, even in tracks loaded with 100 µg of renal membrane protein. In any case, this antibody had been affinity-purified by elution from a column to which the pure enzyme was attached. The e.l.i.s.a. values of homogenates of pig tissues showed a good correlation with a specific enzyme assay depending on the phosphoramidon-sensitive hydrolysis of [D-Ala², Leu⁵]enkephalin. Indeed, it was superior in this respect to the previous i.r.m.a., in which good correlation was seen only with membrane fractions and was sometimes erratic in crude preparations (Gee *et al.*, 1985). An important observation was that the e.l.i.s.a. set up with antibodies to pig E-24.11 cross-reacted perfectly with the human antigen.

Sensitivity of the e.l.i.s.a.

Although some of the enzyme assays for E-24.11 can be modified to determine very low activities, e.g. by extending incubation times, it is often at the expense of lowering the signal-to-noise ratio. Furthermore, there is no truly specific substrate for E-24.11, and the specificity of such assays depends on the determination of the phosphoramidon-sensitive (or other specific inhibitor) component of the hydrolysis. In very crude extracts in which E-24.11 is the minor endopeptidase activity, this may be a very small proportion of the total and therefore liable to substantial error. Immunoassays overcame this problem, but the i.r.m.a. in the form which we described previously depended on the depletion by the antigen of a fixed quantity of a monoclonal antibody and the determination of the residual unbound antibody by a solid-phase i.r.m.a. Its detection limit was about 25 ng of E-24.11, 1000-fold less sensitive than the e.l.i.s.a. Any increase in sensitivity of the i.r.m.a. would require the use of a smaller amount of the monoclonal antibody, with a concomitant increase in the specific radioactivity of the ¹²⁵I-labelled second antibody. The highly sensitive e.l.i.s.a. described here overcomes these difficulties and, more importantly, avoids the use of ionizing radiation.

Potential uses of the e.l.i.s.a. in biology and medicine

E-24.11 is not ubiquitous in pig tissues, but it does have a very wide distribution and is expressed by a great diversity of cell types (Gee *et al.*, 1985; Kenny *et al.*, 1987). Precise determinations of E-24.11 in small samples of tissue and on cells in culture will be valuable in studying the factors controlling the expression of the antigen. The presence of E-24.11 on neutrophils (at least of human origin; Connelly *et al.*, 1985) might cause an elevation in the assay values of tissue samples not washed completely free of blood. However, it should be noted that E-24.11 was undetectable in pig cerebral cortex in three samples of tissue, suggesting that contamination was not a problem. However, we believe that human clinical applications may be important. In the adult respiratory distress syndrome ('ARDS') and in cases of septic pneumonia, serum E-24.11 is increased by a factor of 10–20 over normal values (Johnson *et al.*, 1985). Smaller elevations of serum E-24.11 have also been reported in cases of sarcoidosis (Almenoff *et al.*, 1984; Almenoff & Teirstein, 1987). Neither the cellular origin of this activity nor the physical state of the enzyme in serum was elucidated in these clinical studies. Serum CALLA in leukaemia patients was shown to be sedimentable at 100 000 g, indicating that it was membrane-bound (Carrel *et al.*, 1984). In leukaemia, the determination of E-24.11 is important in diagnosis and prognosis. CALLA-positive cells have been noted not only in ALL, but also in CML in lymphoid blast crisis, in acute undifferentiated leukaemia and in some non-Hodgkin's lymphomas [for a recent review, see LeBien & McCormack (1989)]. Our initial studies on leukaemic cells from ALL and CML patients suggest that the e.l.i.s.a. may be a useful alternative assay.

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