Table 1 Specific Coprecipitation of θ Antigen from ^{125}I-Iodide Labelled Cell Surface Proteins

Cell type	% ^{125}I-iodide labelled surface proteins precipitated with AKR anti-$C_3H\theta$	Normal AKR	FγG+rabbit anti-FγG
CBA/H/Wehi thymus cells	6.7±0.5	0.1±0.02	0.06±0.03
nu/nu spleen cells	0.2±0.01	0.15±0.03	0.07±0.03

For cell surface iodination 1×10^7 cells from mouse spleen and thymus were suspended in 50 μl. PBS containing 20 μg lactoperoxidase prepared as described previously[4]. 1–2 mCi carrier-free K^{125}I-iodide (Radiochemical Centre, Amersham) was added followed by 10 μl. 0.03% H_2O_2, diluted in PBS immediately before addition. Iodination was initiated by agitation and the cells were incubated for 5 min at 30° C. Five to ten aliquots of 1×10^7 cells were iodinated. After one wash the cells were pooled and washed once with PBS. Approximately 40% of the ^{125}I-iodide was incorporated. The cells were disrupted by the addition of 10 M urea, 1.5 M acetic acid (1 ml./$1–2\times10^7$ cells) and incubation for 2 h at 37° C. Insoluble material was removed by centrifugation at 500*g*. Approximately 97% of ^{125}I-iodide labelled cell surface proteins were soluble in urea-acetic acid. The urea-acetic acid soluble surface proteins were dialysed against 500–1,000 volumes of 0.05 M Tris HCl, *p*H 8.0, which was 0.15 M in NaCl. Approximately 15–20% of the ^{125}I-iodide labelled surface components were retained after dialysis. Identification of θ antigen in the non-dialysable fraction of cell surface proteins was obtained by specific coprecipitation in two steps. Cell surface immunoglobulin was removed by coprecipitating the Tris-NaCl soluble surface proteins with mouse gamma globulin and rabbit antiserum to mouse IgG (RAMIg). Rabbit antiserum was prepared[15] which reacted with mouse immunoglobulin light chains and γ-heavy chains. Coprecipitation conditions were such that 90% of mouse IgG was precipitated. Two hundred μl. of ^{125}I-iodide labelled cell surface proteins were mixed with 50 μl. of mouse γ globulin (5 μg) and 50 μl. of RAMIg. The mixtures were incubated at 37° C for 2 h and overnight at 4° C. As observed previously[10], approximately 5% of the ^{125}I-iodide labelled proteins were specifically precipitated with mouse IgG and RAMIg. The supernatants of the precipitation described above (Ig-depleted) were pooled and 200 μl. aliquots were then mixed with 25 μl. of a 1 : 10 dilution of AKR anti-$C_3H\theta$ antiserum and 50 μl. of RAMIg. The anti-θ serum was prepared by repeated injection of AKR mice with CBA/H/Wehi thymus cells. To control for non-specific precipitation ^{125}I-iodide labelled cell surface proteins were precipitated with normal AKR antiserum or with fowl gamma globulin and rabbit antiserum to fowl gamma globulin in the conditions described above. Data represents the arithmetic means and standard errors of results obtained in three experiments with quadruplicate samples precipitated in each experiment.

with a molecular weight of approximately 60,000. This suggests that this molecule may be somewhat larger than that of 40,000–50,000 reported for H-2 antigens or TL antigens which have also been isolated and analysed using similar techniques[12]. That all these molecular weights were determined by gel electrophoresis in dissociating buffers containing SDS raises the possibility that considerable uncertainty may exist in the measurements. The anionic detergent binds less to polypeptides which contain carbohydrate moieties than it does to pure polypeptide[13]. If, therefore, the surface antigens contain appreciable carbohydrate, their mobilities relative to the standards probably would not be a true reflexion of their mass[14]. Detailed investigations of the structure of θ antigen and its relationship to other cell surface components are in progress.

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Note added in proof. After this letter was submitted, Jones[16] reported the use of lactoperoxidase-catalysed iodination in the isolation of θ antigen and H-2 antigens. His study did not include physicochemical characterization of the isolated molecules.

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Radioimmunoassay for Substance P

IN 1931, Von Euler and Gaddum demonstrated the presence of a substance in extracts of brain and of intestine that causes contraction of isolated intestinal muscle *in vitro*, and vasodilatation and hypotension *in vivo*[1]. These effects occur in the presence of atropine and hence are independent of acetylcholine. The active principle became known as substance P (P for preparation). Chang and Leeman recently isolated an undecapeptide from bovine hypothalami, and showed that it was substance P by multiple criteria[2]. The amino-acid sequence was determined[3], and the peptide was synthesized by the solid-phase procedure[4]. The synthetic product was found to possess equipotent activity in multiple bioassays when compared to natural bovine substance P[4]. We now describe the development and application of a sensitive and specific radioimmunoassay for substance P.

Synthetic substance P[4] was coupled to bovine gamma globulin using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide[5]. Rabbits and guinea-pigs immunized with the conjugate[6] were bled 50 days later, and their sera tested for ability to bind ^{125}I-labelled substance P.

To permit labelling with radioactive iodine, a substance P derivative was synthesized with a tyrosyl residue substituted for the phenylalanine at position 8 (tyrosine occurs at position 8 in physalaemin, a structurally related compound with similar biological activities[7]).

Tyr-8 substance P (5 μg) was labelled to a specific activity of 100–150 μCi/μg (ref. 8), using high specific-activity ^{125}I, phosphate buffer (0.5 M; *p*H 7.4) and 50 μg of 'Chloramine T'. The reaction was terminated after 20 s by addition of 150 μg of sodium meta-bisulphite, followed by 1 ml. of blood-bank plasma. The radioactive peptide was separated from free iodine by adsorption to micro-fine silica[9] and elution with 20% acetone–1% acetic acid.

The assay was carried out in 10×75 mm disposable flint glass tubes. Sodium barbital (0.05 M) containing 5% blood-bank plasma at *p*H 8.6 was used as diluent. Although all antisera tested exhibited some binding to tracer, antiserum GP-7 at a final dilution of 1 : 8,000 gave the most sensitive standard curves. The final incubation volume was 0.5 ml.

After incubation of antiserum and synthetic substance P standard (or unknown sample) at 4° C for 24 h, label was added and incubation continued for another 48 h. Antibody-bound label was then separated from label free in the incubate by adding 200 µl. of 1.5% charcoal slurry coated with 0.15% dextran T 20 in barbital buffer to each tube[10]. Appropriate control tubes containing diluent (and sample) and label without added antibody were included in the assay to correct for apparent bound label in the absence of antibody (so-called "damaged" label). The correction factor was usually about 5%.

Characteristics of the assay dose-response curve are shown in Fig. 1. The minimum detection limit is 0.3 ng of peptide per incubate; 3 ng causes displacement of 50% of the bound tracer. Dose-response curves for synthetic and natural bovine substance P are parallel, and when corrected for equivalent biological activity, can be superimposed. Thus, evidence of immunochemical structural similarity can be added to existing evidence for equipotent biological activities between the compounds[4], evidence which altogether suggests identity between the synthetic and the natural bovine substances. Identical standard curves were obtained using either the charcoal or the double antibody[11] phase separation techniques. Extracts of human brain and of rat brain gave dose-response curves in the assay parallel to those for the bovine peptide. Bovine parathyroid hormone, and the synthetic peptides representing residues 1–12 and 1–34 of bovine parathyroid hormone; human calcitonin, beef insulin, and bovine growth hormone, in amounts of from 5–100 ng all failed to displace bound tracer. Interestingly, two compounds with biological activities very similar to substance P, physalaemin and eledoisin (30 ng per incubate), also failed to displace tracer from antibody. Thus the immunoassay distinguishes substance P even from these related peptides, although structural identity occurs among these compounds at their carboxy-terminal ends. A direct linear relationship between quantities measured by bioassay (rat sialogogic activity[12]) and by immunoassay was found when bovine hypothalamic extracts containing natural substance P at various stages of purity were compared over a 600-fold concentration range (Fig. 2).

Preliminary results of the concentrations of substance P found in crude extracts of human brain tissue carried to the step before chromatography are shown in Table 1. When subcellular fractions of rat hypothalamic tissue[13] were analysed for relative contents of substance P, immunoreactive substance P was absent from the myelin layer, was found in intermediate amounts in the mitochondrial fraction, and was present in highest concentration in the nerve ending particles. Immunoreactive substance P was undetectable in unextracted human plasma from six normal subjects and in cerebrospinal fluid from six patients suspected of having various neurological disorders. However, substance P standard added to plasma or to cerebrospinal fluid could be quantitatively recovered, with a minimum detection of 2 ng/ml.

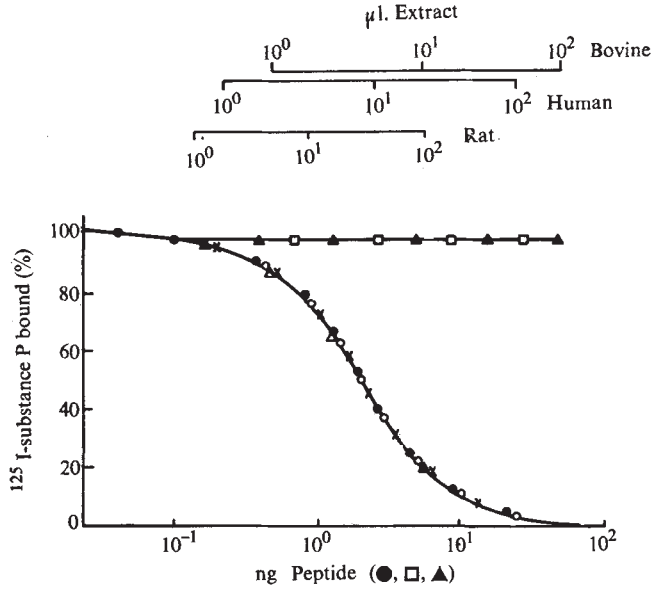

Fig. 1 The radioimmunoassay dose-response curves for synthetic (●) bovine substance P, and for crude extracts of bovine hypothalami (○), rat hypothalami (△) and human substantia nigra (×). Lack of response by physalaemin (▲) and eledoisin (□) is also shown.

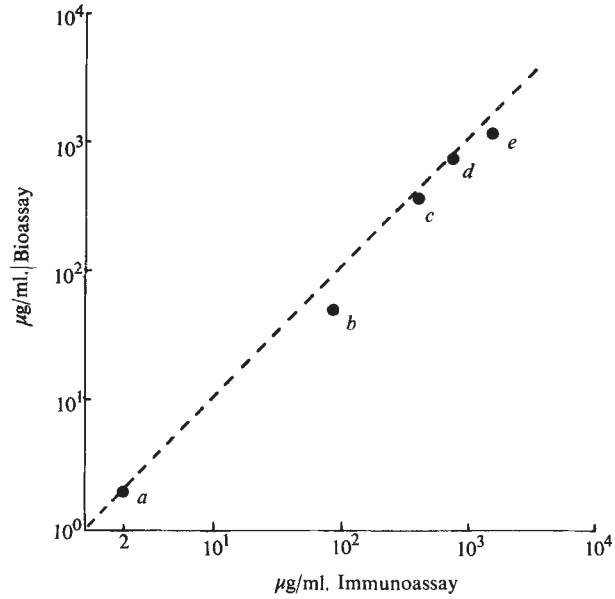

Fig. 2 Comparison between quantities of substance P measured by bioassay and by radioimmunoassay in bovine brain extracts at various stages of purification as described previously[3]. The theoretical relationship is given by the dashed line. *a*, Crude extract; *b*, post 'Sephadex' (dilute); *c*, post 'Sephadex' (conc.); *d*, post CMC; *e*, post paper electrophoresis.

In spite of its multiple potent biological actions, the physiological role of substance P is unknown. Extracts of dorsal nerve roots are known to contain much more bioassayable substance P than ventral roots[16]. A motoneurone-depolarizing peptide has been extracted from dorsal roots of bovine spinal nerves by methods used to extract substance P[14], and physalaemin has been shown to produce strong excitatory depolarization of frog spinal motorneurones *in vitro*[15]. These observations, the uneven distribution of substance P in the central nervous system, and the finding that on the subcellular level substance P is concentrated in the "synaptosomal" fraction, all support the suggestion[17] that substance P may participate in chemical neurotransmission by sensory neuronal pathways.

Brain tissue extracts must be purified before they can be reliably bioassayed for substance P because bioassays are hampered by the presence of other pharmacologically active agents which affect gut contraction[18], blood pressure[19] and salivation[12]. The radioimmunoassay is 100 times more sensitive than the sialogic bioassay, and has permitted some interesting conclusions. It has enabled us to confirm using crude extracts, the distribution of substance P in nervous tissue, which was previously determined by bioassay grossly[20] and at the subcellular level[13]. It has given evidence for structural similarity between substance P in the bovine, in the rat and in humans, and has allowed us to set an upper limit of 2 ng/ml. for the concentration of substance P in the circulation

Table 1 Concentrations of Substance P in Human Brain

Human brain preparation	Immunoreactive substance P (ng/g)
Substantia nigra	500
Spinal cord	65
Hypothalamus	120
Medulla	40
Frontal cortex	10

in man. Because of the sensitivity, precision, specificity and ease of operation of the radioimmunoassay compared to bioassay, it should be useful in studies aimed at defining the physiological role of the peptide.

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Evidence for Transformation of Human B Lymphocytes by PHA

PHYTOHAEMAGGLUTININ (PHA) induced transformation of lymphocytes is generally considered specific for thymus derived (T) cells. Thus in neonatally thymectomized mice[1] and chickens[2] the PHA response is reduced or absent, and studies with chromosome markers[3] and anti-theta antibody[4,5] indicate that, in the mouse, the PHA responding cells are thymus derived. In Man, absence of the thymus (DiGeorge's syndrome) is associated with a diminished or absent PHA response[6,7] which would appear to be evidence for specific T cell transformation. Here we present evidence, however, suggesting that in Man, B cells do respond to PHA.

Peripheral lymphocytes were obtained from normal healthy adults. The blood was defibrinated with glass beads, and lymphocytes separated on a 'Triosill/Ficoll' gradient[8]. They were then washed three times with Eagle's minimum essential medium (MEM) before staining or culturing. Tonsils (collected in cold MEM immediately after removal) were teased in MEM to produce a cell suspension. Polymorphs were removed by incubation with carbonyl iron, and the remaining lymphocytes washed three times in MEM before staining or column fractionation. Bacterial contamination was further reduced before culturing by washing three times in MEM containing 10 per cent foetal calf serum.

Lymphocytes were cultured in MEM supplemented with 10 per cent foetal calf serum, or where stated with 10 per cent human AB serum (0.5×10^6 cells ml.$^{-1}$, 95% air, 5% CO_2). PHA (Difco) was used at a concentration of 1/100. Tonsil lymphocytes were cultured with added gentamicin (Roussel) and vancomycin (Lilly) (25 μg ml.$^{-1}$) to counteract bacterial contamination. ^{14}C-thymidine (0.25 μCi ml.$^{-1}$) was added to cultures 4 h before harvesting.

Lymphocyte populations enriched for non-Ig bearing (T) cells, or Ig bearing (B) cells were obtained by fractionation at 4° on immunoabsorbent columns[9,10]. 'Degalan' polymethyl meta acrylic beads (250μ, Degussa Wolfgang Ag. Germany) were equilibrated with 0.15 M phosphate buffer, *p*H 6.1, and incubated with purified rabbit antihuman light chain (ALC), or normal rabbit Ig at 45° for 2 h. Approximately 12 mg of protein was thus bound to 5 g of beads, giving a 10 cm × 1 cm column. The columns were equilibrated for 30 min at room temperature, and then 4° C with Hanks 2 per cent BSA–5 mM EDTA (HBE). Lymphocytes (10^7 in 1 ml. HBE) were layered on to the column and eluted in 5 ml. HBE (2 ml. min^{-1}). This procedure could be repeated for a maximum of 10 aliquots per ALC column, to give eluates containing less than 2% Ig bearing cells.

'Sephadex G-200' was brought to *p*H 10.5 with 0.2 M NaOH, activated with cyanogen bromide (2 mg ml.$^{-1}$ 7 min), and incubated with purified ALC, or normal rabbit Ig. Approximately 8 mg of protein bound to 1 g of Sephadex. Columns 8 cm × 2 cm, were prepared from 0.25 g of Sephadex, and equilibrated for 30 min with Hanks 5% foetal calf serum–5 mM EDTA (HFE) at 37° and then at 4° C. Lymphocytes (10^7 in 1 ml. HFE) were layered on to the column and eluted in 3 ml. HFE (4 ml. min^{-1}). A total of 10^8 cells were normally fractionated in this way. Ig bearing cells were recovered from the column by dextranase digestion of the Sephadex (1.0 mg "dextranase" International Enzymes 1 h at 37°). Lymphocytes to be stained for Ig after recovery were fractionated in the presence of 8 mM sodium azide to block capping.

Staining for surface immunoglobulin was carried out at 4° on suspensions of viable lymphocytes[11]. Aliquots of 5×10^6 cells were incubated for 30 min with 0.1 ml. ALC or normal rabbit serum, washed three times in cold MEM and incubated for 30 min with fluorescein conjugated goat anti-rabbit antiserum. After a further three washes in MEM they were examined as a suspension in glycerol PBS with a 'Leitz Ortholux' microscope under incident ultraviolet light. Blast cells were identified from their phase contrast appearance. Autoradiographs were prepared from cytosmears of lymphocyte cultures harvested at 72 h[5]. They were stained with Giemsa after development and examined under a light microscope. Where incorporation of ^{14}C-thymidine was to be measured, TCA precipitates of 5×10^6 cultured lymphocytes were prepared for liquid scintillation counting[12].

Approximately 30% of blasts from PHA cultures of peripheral blood lymphocytes stained with ALC. This percentage appeared to be independent of cell concentration in culture (Table 1). PHA blasts from tonsil lymphocytes (1 donor) were stained with 6 different ALC antisera (1 commercial, Hoechst Pharmaceuticals, 5 prepared in this laboratory) giving a mean percentage of 32 positive (s.d. 7.6). It would therefore seem unlikely that staining was due to antigen specificity other than light chains.

The following results suggest that ALC staining of blasts is not due to a T cell membrane component. 30–60% of tonsil lymphocytes were light chain positive, varying with the donor.