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Non-specific binding of lysine–glucose-derived Maillard products to macrophages outweighs specific receptor-mediated interactions

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Studies on the binding of lysine–glucose-derived Maillard products to siliconised assay tubes indicate that caution must be exercised when interpreting binding parameters, owing to the saturable binding kinetics observed (B_{max} = 490 pmol, K_d = 22·2/nM). When these are taken into account, kinetic studies on the binding of such late-stage Maillard products to macrophages show that any specific receptor binding is swamped by non-specific binding to both cell lines and native macrophages, as neither binding saturation nor cross-competition (homologous or heterologous) was detected.

This has implications for the role of macrophages in the recognition of Maillard products, and suggests that non-specific binding may be quantitatively more important than specific receptor binding in the cellular recognition of such food products.

INTRODUCTION

Free amino acids and reducing sugars play an important role in flavour formation and food storage and processing. The importance of the Maillard reaction in flavour formation during processing of foodstuffs and in the production of distilled beverages is well established (Hodge, 1953; Tress *et al.*, 1983; Ledl & Schleicher, 1990), but the cellular recognition of such food products is less well understood. Maillard products are absorbed into the body via the buccal cavity and gastro-intestinal system. Late stages of non-enzymatic browning, which occur when foods are stored, heat-treated or processed, decreases bioavailability (Finot, 1982).

Brown pigments with spectral and fluorescent properties similar to those of late-stage Maillard products have been observed in association with long-lived proteins (Cerami & Crabbe, 1986; Harding & Crabbe, 1992). These Maillard products have been related to the pathology of hyperglycaemia, vascular permeability and complement activation (Brownlee *et al.*, 1987). The molecular recognition of such products in the body has been shown to involve macrophages (Vlassara *et al.*, 1984).

The existence of a scavenger receptor that could recognise proteins solely by Maillard adducts, almost irrespective of the identity of the protein, was postulated after the identification of a mannose–fucose scavenger receptor on macrophages. The existence of a Maillard-adduct scavenger receptor capable of recognising acetylated-LDL and maleylated-BSA was claimed by Goldstein *et al.* (1979). During the last 10 years it has been implicated in the recognition of not only several glycosylated proteins but possibly also those with other aldehyde-derived adducts. The characteristics necessary for the modified proteins to be recognised and removed remain unclear but are suggested to be based on marked alterations in the surface charge of the proteins. Polyanions such as polyinositol and dextran sulphate are believed to compete with the modified proteins for binding to the receptor (Radoff *et al.*, 1991; Acton *et al.*, 1993). This competition is regarded as evidence that non-enzymatic glycosylation has replaced cationic or neutral charges on the protein surface with anionic ones. On this basis, the modified bovine serum albumin (BSA) that has been used as a model has been maleylated to generate a further 55–60 anionic charges on its surface. A membrane protein has been purified and proposed as a prime candidate for the scavenger receptor (Kodama *et al.*, 1990). Many, though not all, modified proteins will bind to the purified membrane protein but it remains unclear whether this binding is specific in nature or if the protein actually does play a role as a scavenger receptor.

The hypothesis is that the collagenous region of this protein is a broad range receptor for a multitude of proteins, particularly those that have been modified in some way, or for compounds of a polyanionic nature.

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It has also been suggested that it can act in such a way as to exhibit non-reciprocal competition between recognised proteins. Thus it could act as a receptor both for Maillard food products and for advanced glycosylation endproducts (AGEs).

This paper reports on studies on the Maillard products obtained from lysine and glucose, and the binding of such products to macrophages. It is shown that any specific receptor binding is swamped by non-specific binding to these cells. This has implications for the role of macrophages in the recognition of Maillard products, and suggests that this non-specific binding may be quantitatively more important than specific receptor binding in the recognition of such products.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma with the following exceptions. Acrylamide/bis-acrylamide premixed (19:1 ratio) solution was bought from Severn Biotech Ltd. Ampholytes, ammonium persulphate, glycine, sodium hydroxide and orthophosphoric acid were obtained from Bio-Rad. All cell-culture chemicals were from Gibco. $K^{125}I$ and ^{14}C-[UL]-glucose were obtained from Amersham and ^{14}C-[1]-glucose from Du Pont.

Methods

Lysine–glucose incubations
Incubations contained 22·5 g glucose (2·5 M) and either 2·5 g BSA or 0·396 g lysine in 50 ml 0·15 M PBS, pH 7·4. Controls were incubations comprising only buffer or buffer containing either lysine or BSA. Incubation was at 37°C in the dark.

Monitoring was by absorption spectrophotometry (using a Beckman DU 70 stable beam spectrophotometer) in the early stages of the incubation; free amino acids undergo non-enzymatic glycosylation more readily than proteins and it was expected that the incubation rate of the lysine and glucose would rapidly exceed that of BSA and glucose. After approximately 2 months of observation, the incubation was allowed to continue for approximately 2 years at 37°C. Analysis of the eventual product was by absorption spectrophotometry.

Temperature-dependent incubations of lysine with glucose
A number of lysine–glucose based incubations were carried out over a wide temperature range, the time varying inversely with the temperature used. The concentrations of lysine and glucose were maintained throughout at 45 mM lysine and 2·5 M glucose. Incubations of 1 ml were carried out in 1.5-ml polyethylene micro-centrifuge tubes in a Techne Dri-Block DB-2P heating block. Analysis was by monitoring changes in spectrophotometric absorption, assuming initially that the ratio of rate to temperature was similar to that expressed in the Q_{10}-rule.

Incubations of lysine with glucose developed Maillard products with absorption spectra similar to that found with BSA and glucose (Shaw & Crabbe, in press), but at a far slower rate. The rate was also noted to increase markedly as the incubation temperature was raised, far above that possible for BSA, which would denature at approximately 50°C, and to increase also as the concentrations of the reagents were increased. Therefore, incubations were carried out with high glucose concentrations at high temperatures to follow the development of the products by spectrophotometry in more detail. Lysine and glucose (45 mM and 2·5 M, respectively) were incubated in 0·15 M PBS, pH 7·4, in 1-ml aliquots in 1·5-ml polyethylene micro-centrifuge tubes in a heating block at 100°C. Incubation time was from 0 to 15 min, a tube for either each minute of the incubation or for five-minute intervals. The incubation for each tube was terminated by removal from the heating block and immersion in ice. Samples of 0·5 ml were then taken for spectrophotometric assay. There was no decrease in incubation volume caused by evaporation; the tubes remained sealed throughout the incubation and the samples were removed only after cooling. The controls used were solutions of glucose only and lysine only, plus the zero time incubation.

Assay for Maillard products
Maillard products were assayed using Nitro blue tetrazolium, following the method of Ghiggeri *et al.* (1988).

Cell culture
M5076 murine monocyte-macrophages. The cell-line was generously donated by Dr Ian Hart at the Imperial Cancer Research Fund, Lincoln Inn Fields, London. The cells existed in the semi-adherent phase and required RPMI 1640 buffer with 10% horse serum (HS) as the growth medium. Antibiotics were included in the medium: 50 μl/ml penicillin, neomycin, streptomycin (PNS) and 100 μl/ml Fungizone. Culturing was almost exclusively in 5 ml medium in 25 cm^2 disposable flasks at 37°C in 5% CO_2. Cells were obtained by gentle tapping of the flasks to dislodge the cells followed by centrifugation of the removed medium. After washing and resuspension in 0·15 M PBS they were stored at 4°C and used within a few hours in the assays described below.

The cell-line was regularly injected into the abdominal cavity of 6-month-old C57 mice (10^5 cells per mouse) to maintain viability. The mice were maintained on standard feed for 3 weeks, then killed and the abdominal cavity washed with 5 U/ml heparin in Iscove's DMEM medium. After centrifugation, the cells were cultured in flasks as above; non-adherent contaminating cells could be removed after incubation overnight at 37°C, 5% CO_2. Adherent contaminating cells would be rendered negligible by the passaging of the cells at least three times before any use in assays.

Native murine peritoneal macrophages. These were extracted from the abdominal cavity of CO_2-gassed

CD1 mice by the injection and removal of 5 ml Iscove's DMEM/heparin medium. The cells were gently centrifuged and either plated out in Iscove's DMEM/10% FCS (37°C, 5% CO_2) or used in a 0·15 M PBS suspension. Those plated out were allowed to mature for three days before use; those in suspension were stored at 4°C and used within a few hours.

All cell viability was assessed using trypan blue.

Siliconisation

The authors used siliconisation of polypropylene tubes, as it was found that post-translationally modified proteins had an increased affinity for binding to plastics compared with unmodified proteins, greatly interfering with binding assays where low concentrations of proteins were necessary (Shaw & Crabbe, 1994*a*). Two preparations were used:

(a) *Sigmacote* (a suspension of silicon oil in heptane). A 1·5-ml micro-centrifuge tube was filled with some of the solution, shaken gently to ensure the whole surface was covered, and the solution transferred to the next tube. The process was repeated, with further additions of the Sigmacote, until sufficient tubes had been coated. The open tubes were then allowed to dry for a few hours in a fume-hood and could be used immediately or stored indefinitely at room temperature.

(b) *Silicone oil* (low melting point bath oil). Silicone oil was applied to micro-centrifuge tubes with a small pipette dropper, two drops of oil in every tube. The tubes were then sealed, placed in a foil-covered beaker and autoclaved. During autoclaving the tubes were forced open by the internal air pressure and most of the excess silicone oil was able to drain to the bottom of the beaker. Before use, the remaining oil needed to be removed from the bottom of each tube either with a needle by tap-driven suction or by draining. The first method was faster but care had to be taken not to scratch the sides of the tubes, exposing uncoated polypropylene.

Coating of pipette tips was carried out by adding a drop of oil to each pipette held upright in a Rainin autoclavable box. The combination of coating and vaporisation of the remaining oil during autoclaving ensured efficient preparation.

The latter method (b) was generally employed to allow the pre-processing of a large number of tubes.

Iodination of lysine–glucose complex

The iodination followed the method described by Fraker and Speck (1978). A 1% solution was prepared of glycouril in methylene chloride. Volumes of 100 μl were placed in 1·5-ml micro-centrifuge tubes and allowed to evaporate in a fume-hood. The coated tubes could then be stored at 4°C indefinitely or used immediately.

A 20–100 μg sample of the protein to be labelled was dissolved in 50 μl of 0·25 M PBS and placed in a coated tube. An aliquot of the $K^{125}I$ stock solution was diluted in 0·15 M PBS (3 μl in 300 μl buffer) and 20 ml of the dilution were added to each tube. The tubes were allowed to stand at room temperature for 20 min before the addition of 250–300 μl 0·05 M PBS. The tubes stood for a further 10 min while the Iodogen process was terminated and then the solution was filtered through approximately 2 ml of Sephadex G-25. (The filtering columns were prepared from 2-ml syringes with the plungers removed and filter paper placed over the eluting hole.) Fractions obtained from this separation were assayed for activity in a LKB 1261 Multi-Gamma counter using Pharmacia RIACalc software. The fractions containing protein were collected and stored at –20°C in an Amersham ^{125}I storage box. The degree of labelling was estimated by placing aliquots in a counter and the ratio of bound : free iodine estimated using dotting on thin-layer chromatography.

Binding of lysine–glucose complex to cells

Assays were done by incubation of the test proteins at specific concentrations with cells at 4°C, followed by counting of the protein's radioactive label to estimate the amount bound to each cell. Control assays used a large amount of cold protein in addition to the assay amount. The technique assumed that the receptor binding was limited and would be completely masked by the constant percentage binding of non-specific activity; the binding activity found in control assays was therefore an accurate indication of non-specific activity at assay concentrations.

All assays were carried out in 200 μl of 0·15 M PBS in pre-siliconised micro-centrifuge tubes suspended above ice at 4°C. Approximately 5×10^5 cells were used in each tube with at least an hour in suspension prior to the assay. The cells were added in 100 μl aliquots, the remaining 100 μl being made up with buffer, labelled protein and excess unlabelled protein. Incubation was for one hour (stated if different), terminated by centrifugation and repeated washing with 200 μl ice-cold PBS. Counting was by transferring cells to fresh tubes and counting both these tubes and the originals in a counter.

Bar lines indicate ± 1 SD, and the result of at least three separate experiments. Where bar lines are not shown, the standard deviations lie within the plotted points.

RESULTS

Comparison of the browning of BSA with lysine

Parallel incubations of BSA and lysine with 2·5 M glucose ran for 2 years with absorption spectroscopy used for analysis of the Maillard products that formed. While the browning was more rapid in the lysine–glucose mixture initially (Fig. 1), after 2 years both sets of incubations exhibited similar absorption spectra. Minimal browning was observed in each of the controls containing lysine or BSA without glucose. A number of

peaks appear, initially at 238 and 316 nm; after 2 years' incubation, six peaks become apparent across the spectrum at 226, 320, 370, 480, 556 and 712 nm. The absorption eventually exceeded three units (<0·1% transmission, calculated after appropriate dilution) across most of the spectrum.

Non-specific adsorption of the lysine–glucose complex to surfaces

It was found that the siliconisation was effective in reducing the adsorption that occurred on the tube surface (Table 1). At low protein concentrations this reduction still left sufficient adsorption to interfere with a receptor assay; attempts to measure a K_d of <50 nM would be unreliable.

The kinetics of the adsorption of modified proteins to a siliconised surface

Since the proportion of complex binding to the tube surface decreased as the protein concentration increased, it appeared that this adsorption exhibited saturation. This meant that a binding assay carried out on the tube surface rather than on a cell population should produce kinetic data similar to a specific receptor. Such an assay was carried out using the lysine–glucose complex (incubated for 24 months), and showed that saturable binding was obtained, identical to that which would result from a specific receptor. Figure 2 shows that there was a high percentage binding at low substrate concentrations, declining to background binding above 2000 nM. With the background activity removed, curve-fitting indicated a B_{max} of 490 pmol and an apparent K_d of 22·2 nM.

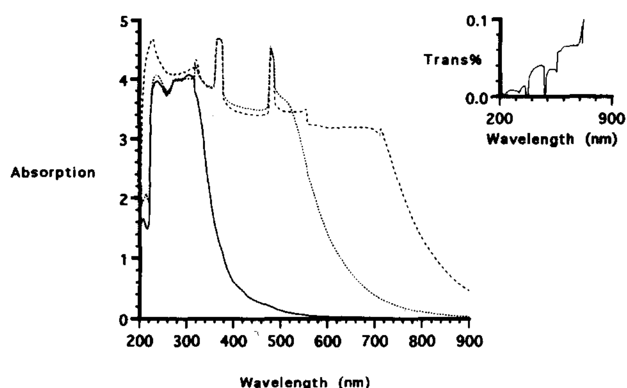

Fig. 1. Development of the absorption spectrum when lysine (45 mM) and glucose (2·5 M) are incubated at 37°C. A number of peaks appear, initially at 238 and 316 nm; after 2 years of incubation, six peaks become apparent across the spectrum at 226, 320, 370, 480, 556 and 712 nm. (—) After 14 days of incubation; (·········) after 208 days of incubation; (- - - -) after 24 months of incubation. The absorption eventually exceeds three units (<0·1% transmission) across most of the spectrum. The insert shows the per cent transmission spectrum when lysine (45 mM) and glucose (2·5 mM) were incubated at 37°C for 24 months.

Binding of the lysine–glucose complex incubated for 24 months (KG-2·5M/24) to the M5076 monocyte–macrophage cell line

The complex readily underwent non-specific binding and did not exhibit any activity that could be identified as specific binding. The binding was the same magnitude as that of the expected specific binding but linear in nature and unsaturable by an excess of unlabelled complex (Fig. 3). Where large proportions of excess unlabelled complex were used to quench binding activity (Fig. 4), it was found that the binding was partially reducible but required a 4000-fold excess of unlabelled complex. In binding assays the usual range of excess unlabelled substrate for quenching is approximately 50-fold that of the binding maximum. The high concentration of unlabelled complex required for quenching was therefore indicative of a high capacity on the cell surfaces for the adsorption of the complex. This was in agreement with the unsaturable binding observed with modified BSA, both by glycosylation and maleylation.

When two concentrations (0·5 and 0·1 $\mu g/\mu l$) of dextran sulphate — to provide a polyanion that would compete with the lysine–glucose complex for binding to the putative receptor, since that had been claimed to function by recognising polyanionic molecules — were run against the lysine–glucose complex, no decrease in

Table 1. The decrease in the percentage binding of the lysine–glucose complex (KG-2·5M/24) to polypropylene micro-centrifuge tubes after pre-treatment with silicone oil. The non-specific adsorption would cause a significant error if concentrations <100 mM were used

Lysine–glucose (nM)	Untreated	Silicone oil
20	11·9±3·74	4·65±1·46
100	6·25±0·85	0·33±0·10
500	4·07±0·26	0·15±0·06

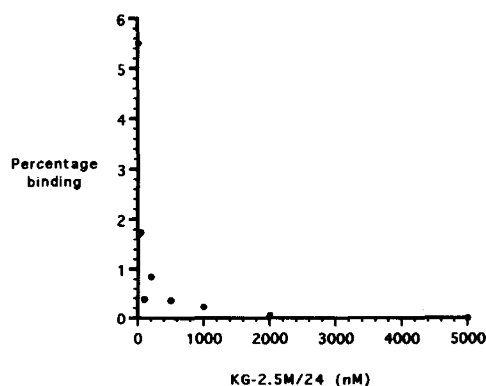

Fig. 2. The percentage binding of the lysine–glucose complex, KG-2·5M/24, (lysine incubated with 2·5 M glucose for 24 months) to siliconised polypropylene micro-centrifuge tubes. At low concentrations (≤100 nM) the percentage rises markedly, comparable to the binding recorded in Table 1. This graph has been corrected for the background binding observable at high concentrations (≤ 2000 nM); the background binding was of the order of 0·175% (1·75 pmol).

binding was observed, the binding remaining constant at 0·012 pmol of complex bound. This was in agreement with the conclusion that the lysine–glucose complex was not recognised by a putative receptor, binding to the cell only non-specifically.

Binding of the lysine–glucose complex to murine peritoneal macrophages

With murine peritoneal macrophages, a similar result of non-specific binding was obtained even with the concentration range extended to 2000 nM (Fig. 5). It was concluded from this that the complex had the ability to adsorb to the cell surface in large quantities in addition to the amounts attached to the micro-centrifuge tubes. We found no evidence for a putative scavenger receptor capable of recognising the complex; the putative maximum binding for the number of cells in this assay was 100 fmol, the maximum observed here being 250 fmol in the assay and 100-fold that in the control.

DISCUSSION

The *in-vitro* incubation of lysine and glucose for up to 24 months produced late-stage Maillard products with absorption maxima in the UV and visible regions of the spectrum similar to those observed with glycated proteins (Shaw & Crabbe, 1994*a*). These products were used to investigate binding of Maillard products to macrophages. The elucidation of specific saturable cell-surface receptors requires minimal non-specific binding, both to cells and surfaces used in assay procedures. While binding of albumins to rat hepatocytes had suggested the presence of a saturable high-affinity receptor on the cell surface (Reed & Burrington, 1989), later work showed that at low protein concentrations, non-specific binding to material surfaces used in binding activity assays demonstrated that a specific receptor was spurious (Reed, 1990).

The results presented here are in agreement with the suggestion that Maillard products exhibit marked non-specific binding effects, as have been found with modified proteins (Shaw & Crabbe, 1994*a,b*). Although the authors have been at pains to limit non-specific adsorption to cell and tube surfaces, it is shown here that, despite such techniques, it is possible to demonstrate spurious receptor kinetics which may colour interpretation if cells contain low numbers of specific receptors.

The putative ability of macrophage cells to identify and remove Maillard products could well be a result of the non-specific binding activities of the products acting upon a limited population of non-specific binding sites upon the cell surface. Any specific receptors on macrophages must be swamped out by the non-specific binding of these late-stage Maillard products. These findings are in accord with those using maleylated- and acetylated-bovine serum albumin and advanced glycosylated Endproducts (AGEs), where binding to macrophages and hepatocytes shows that the low

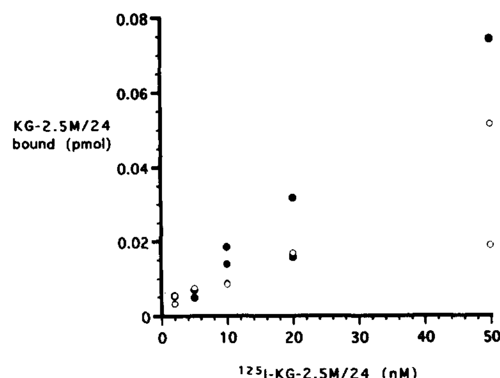

Fig. 3. Incubation of ^{125}I-labelled lysine–glucose complex with M5076 monocyte macrophages: (○) without and (●) with 200 μM of unlabelled complex. Inclusion of excess unlabelled complex (●) indicated that binding was linear and non-specific.

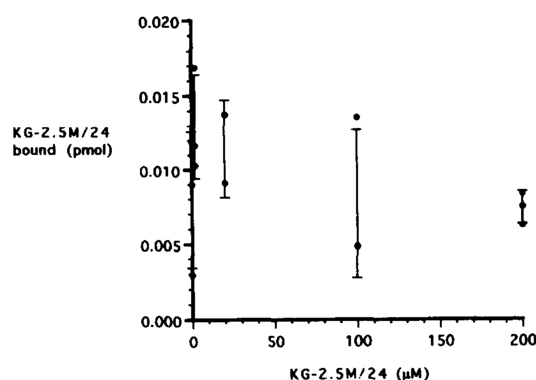

Fig. 4. A 50 nM preparation of ^{125}I-labelled lysine–glucose complex was incubated with M5076 monocyte macrophages, with increasing concentrations of unlabelled complex to determine the concentration required to satisfactorily quench the binding of the labelled complex to the cell-line. A 4000-fold excess of unlabelled complex reduced the binding less than 50%.

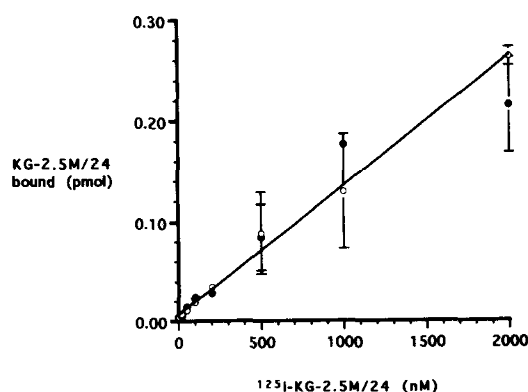

Fig. 5. The binding of the lysine–glucose complex (KG-2·5M/24) (lysine incubated with 2·5 M glucose for 24 months) to murine peritoneal macrophages (○) was found to be linear into the micromolar range. The control assay incorporating 200 mM excess unlabelled complex (●) did not reduce this linear binding, demonstrating the binding to be non-specific in nature.

amount of specific receptor binding is swamped by non-specific binding to both cell lines and native macrophages (Shaw & Crabbe, 1994*b*). Food-derived Maillard products may compromise the effectiveness of macrophages in the 'removal' of AGEs in ageing, and in conditions such as diabetes.

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