Non-specific binding of advanced-glycosylation end-products to macrophages outweighs specific receptor-mediated interactions

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On binding to murine peritoneal macrophages, maleylated BSA exhibited saturable-binding kinetics, with about 24000 sites/cell. Prolonged incubation of BSA with > 20 mM glucose or 2 months incubation with ≥ 0.5 M glucose induced the modified protein to readily bind non-specifically to both cell and tube surfaces. Kinetic studies on the binding of advanced glycosylated end-products (AGEs) and other modified proteins to macrophages and hepatocytes showed no evidence for specific receptor binding, as neither binding saturation nor cross-competition (homologous or heterologous) was detected. Although there was evidence for uptake of

BSA which had been incubated with 0.5 M glucose for 2 months, there was no uptake or degradation of AGEs which had been produced at physiological concentrations of glucose. This has implications for the role of macrophages in the recognition of AGEs, and suggests that the non-specific binding may be important in adhesion of AGEs, particularly in poorly controlled diabetics, and might act as a 'damage limitation' mechanism in the potential development of diabetic complications, while low macrophage levels in the blood could seriously potentiate the long-term effects of non-enzymic post-translational protein modifications.

INTRODUCTION

It has been suggested that macrophages possess 100000–120000 receptors/cell, with a K_d of 5–20 nM, capable of recognizing aldehyde-modified proteins [advanced glycosylation end-products (AGEs)] from *in vitro* or *in vivo* preparations (Vlassara et al., 1987). Acetylated low-density lipoprotein (acetyl-LDL) appeared to bind to two populations of sites on macrophages: 19000 sites with a K_d of 9.6 nM and 2700 sites with a K_d of 39.4 pM (Haberland et al., 1989). A separate receptor for maleyl-BSA appeared to have a K_d of 5–15 M, though with about 10^6 receptors/per cell (Haberland et al., 1989; Takata et al., 1989). The characteristics necessary for the modified proteins to be recognized and removed remain unclear, but are believed 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 to be evidence that non-enzymic glycation has replaced cationic or neutral charges on the protein surface with anionic ones. On this basis the modified BSA that has been used as a model has been maleylated to generate a further 55–60 anionic charges on its surface. Limited research using native or *in vitro*-glycated BSA has claimed to have shown similar results, despite the degree of modification being a magnitude lower than by maleylation. A membrane protein has been purified and put forward 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 rôle as a scavenger receptor.

The existence of a scavenger receptor, which could recognize 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 was claimed

by Goldstein et al. (1979), capable of recognizing acetylated LDL and maleylated BSA. During the last 10 years such a receptor has been implicated in the apparent recognition of not only several glycated proteins, but possibly also those with other aldehyde-derived adducts.

LDL treated with malonaldehyde, maleic anhydride or acetic acid was recognized by macrophages. If the LDL is modified in a so-far-unelucidated manner (either by the epithelial cells or by free-radical oxidation in the plasma), it becomes epithelial cell-modified LDL (EC-LDL), which is no longer recognized by LDL receptors, but readily endocytosed by the macrophages. The cholesterol from within the complex accumulates in the macrophage. Atherosclerosis is characterized by the appearance of cholesterol-ester-rich macrophages or 'foam cells' where $\geq 50\%$ of total cellular cholesterol occurs in the esterified form (Fogelman et al., 1980). Acetylated LDL, which had been shown to compete with EC-LDL *in vitro* (Henrikson et al., 1981), has also been claimed to compete with maleylated-BSA *in vitro* for binding and uptake by different receptors (Goldstein et al., 1979). Purification of a receptor using either modified protein supports the hypothesis that they share the same receptor.

A receptor for modified LDL has been purified to near homogeneity, producing a 220 kDa protein, a trimer of 77 kDa subunits (Kodama, 1990). Post-translationally modified BSA and polyanionic compounds acted as competitive inhibitors.

Sequencing and cloning demonstrated the presence of similar receptors in bovine macrophages and a murine macrophage cell line, P388D1. Two types were found (Freeman et al., 1990), the type II having an attenuated, extracellular C-terminus. The structure of type I possesses a triphasic, extracellular structure: an α -helical coiled coil, a collagenous region, then a cysteine-rich terminal region. Though the type II receptor lacks nearly all the cysteine-rich region, cDNA probes of each type do map to the same locus on the murine chromosome 8, suggesting a single mRNA is responsible for both.

The hypothesis is that the collagenous region may be a broad-

Abbreviations used: AGEs, advanced-glycosylation end-products; LDL, low-density lipoprotein; EC-, epithelial-cell-modified; NBT, Nitroblue Tetrazolium; DMEM, Dulbecco's modified Eagle's medium (with 25 mM Hepes); FCS, foetal-calf serum; E.C.A.C.C., European Collection of Animal Cell Cultures; N.C.T.C., National Collection of Type Cultures.

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range receptor for a multitude of proteins, particularly those that have been modified in some way, or for compounds of a polyanionic nature. Specificity for modified proteins other than acetylated LDL has yet to be demonstrated, the apparent non-reciprocal competition between acetylated LDL and modified proteins being the most significant indication to date. It is therefore important to determine whether macrophages possess a receptor which can act as a specific receptor for AGEs to remove post-translationally modified proteins, particularly long-lived proteins, which have been implicated in the development of diabetic complications (Cerami and Crabbe, 1986; Brownlee et al., 1987).

Here we report studies on the binding of AGEs and other modified proteins to macrophages and hepatocytes. While we confirm the existence of a specific macrophage receptor for maleylated protein, there was no evidence for a specific AGE receptor; rather we found non-saturable binding of AGEs to macrophages and hepatocytes. This has implications for the role of macrophages in the recognition of AGEs, and suggests that the non-specific binding may be important in adsorbing quantities of AGEs, particularly in poorly controlled diabetics, and acting as a 'damage-limitation' mechanism in the potential development of diabetic complications.

MATERIALS AND METHODS

Materials

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

Methods

Incubation of Maillard products

(a) BSA and glucose. Several incubation series were used to generate Maillard products from the interaction of glucose and BSA, though a similar methodology was used in all. The concentration of BSA was kept constant at 50 mg/ml and the glucose varied depending on the aims of each incubation. Two methods were used.

(i) An adaptation of those of Pongor et al. (1984) and Chang et al. (1985). An incubation solution (350 ml) was created consisting of 0.15 M PBS, pH 7.4, 1.5 mM phenylmethanesulphonyl fluoride, 0.5 mM EDTA, 80 mg/ml gentamicin and 200 units/ml penicillin. Of this solution, 260 ml were used to dissolve 13 g of BSA to give a final concentration of 50 mM. Fractions (50 ml) of the BSA solution were used to dissolve 4.5, 9.0, 13.5, 18.0 and 22.5 g of glucose to give a concentration series of 0.5, 1.0, 1.5, 2.0 and 2.5 M glucose. Controls were the remaining 10 ml of the BSA solution, and 10 ml volumes of the incubation solution containing 0.9, 1.8, 2.7, 3.6 and 4.5 g of glucose (0.5, 1.5, 2.0 and 2.5 M concentrations). Labelling was by the addition of $[U-^{14}C]$ glucose to each of the 50 ml assay solutions at 24 kBq/mmol of glucose. Each solution was filter-sterilized and placed in 100 ml or 20 ml bottles before incubation at 37 °C in the dark. After 2 months incubation, 2 ml samples were taken from each ^{14}C -labelled incubation, precipitated with 25% (w/v) trichloroacetic acid and resuspended in 0.15 M PBS/5 mM EDTA. The precipitation and resuspension were repeated until the supernatant showed only background radio-

activity. The precipitated protein samples were suspended in 5 ml of 0.15 M PBS and the protein concentration determined using the Bradford assay. The protein recovery rate was > 95%, i.e., 100 mg of protein in 5 ml of buffer. The degree of glycation was estimated by measuring the ^{14}C radioactivity associated with known masses of the proteins and using the Nitroblue Tetrazolium (NBT) assay described below. This was correlated with the absorption spectra obtained from spectrophotometric scans of samples and controls (Shaw and Crabbe, 1994b).

(ii) An adaptation of the method of Vlassara et al. (1985). BSA at 2.5 g/50 ml of PBS buffer was incubated with 75 mM glucose for 1 month at 37 °C in the dark. The controls were again buffer only or buffer containing only BSA or 75 mM glucose. The degree of glycation was again confirmed using the ^{14}C tracer. The intention was of glycosylating BSA at only one site.

Incubation series of BSA with 2.5–2560 mM glucose

An exponential series of glucose concentrations was created to simulate likely physiological concentrations at the lower end of the scale and to compare with the carbohydrate concentrations in the incubations mentioned above. Near-physiological concentrations were 2.5 mM (chronic hypoglycaemia), 5 mM (normal serum glucose concentration) and 10 and 20 mM (chronic hyperglycaemia). The high glucose concentrations used were 320, 640, 1280 and 2560 mM. BSA was dissolved in 0.15 M PBS, pH 7.4 (13 g in 130 ml) to give a final concentration of 100 mg/ml and 27.648 g of glucose were dissolved in 30 ml of 0.15 M PBS, pH 7.4, to give a final concentration of 5120 mM. Aliquots of the BSA solution (10 ml) were placed in each of eleven Universal bottles. Glucose solution and 0.15 M PBS were added to each of these bottles to give a 20 ml solution with the required glucose concentration and 50 mg/ml BSA. Control incubations contained BSA only or 2560 mM glucose only.

Incubation was for 9 months at 37 °C in the dark. No $[^{14}C]$ glucose tracer was included, the development of Maillard products being monitored by absorption spectrophotometry (Shaw and Crabbe, 1994b). Samples were again purified by repeated precipitation with 25% trichloroacetic acid; ten cycles were considered sufficient from previous observation of preparations containing ^{14}C tracer. Analysis of glycation used the NBT assay, absorption spectrophotometry and scintillation counting.

BSA maleylation

The method used was adapted from that described by Takata et al. (1989). BSA was dissolved in 0.15 M pyrophosphate buffer, pH 8.5, to a concentration of 10 mg/ml. Maleic anhydride was dissolved in 1,4-dioxan to a concentration of 1.0 M. The incubation was done on ice with sufficient of the maleic anhydride solution being added to that of the protein to give a final concentration of 0.038 M. The final volume is unimportant; 50 ml were used in this preparation with 0.5 g BSA and 1.9 ml of maleic anhydride. The reaction was allowed to proceed for 5 min with 5 M NaOH solution being added *ad libitum* to maintain the pH at 8.5. The reaction was terminated by the addition of 1 vol. of 0.5 M PBS, pH 7.4. For a 50 ml incubation, the maleic acid remaining was removed by 48 hours dialysis in 4 litres of ice-cold buffer (0.15 M NaCl/20 mM sodium phosphate buffer, pH 7.4).

LDL acetylation

The method was adapted from that described Goldstein et al. 1979. LDL (5 mg) was dissolved in 1 ml of dialysis buffer

consisting of 0.15 M NaCl, 0.3 mM EDTA, pH 7.4 at 4 °C. To this was added 1 ml saturated sodium acetate solution at 0 °C, and the reaction mixture incubated in an ice-cold water bath with stirring. Acetic anhydride was added as 2 ml aliquots every 15 min for 1 h; the final mass needed to be 7.5 mg, 150 % that of the protein. The incubation was continued for a further 30 min. Dialysis was for 48 h, twice for 24 h in 5 litres of dialysis buffer, ice-cold with continuous stirring.

Assay for Maillard products

This was done using the Nitroblue tetrazolium method described by Ghiggeri et al. (1988).

Cell culture

(a) M5076 murine monocyte-macrophages. The cell line was generously donated by Dr. Ian Hart at the Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, U.K. The cells existed in the semi-adherent phase and required RPMI 1640 buffer with 10 % horse serum as the growth medium. Antibiotics were included in the medium: 50 µl/ml penicillin, neomycin and streptomycin, and 100 µl/ml Fungizone. Culturing was almost exclusively in 5 ml of medium in 25 cm² disposable flasks at 37 °C under an air/CO₂ (19:1) atmosphere. 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 (10⁵ cells/mouse) to maintain viability. The mice were maintained on standard feed for 3 weeks, then killed and the abdominal cavity washed with 5 units/ml heparin in Iscove's Dulbecco's modified Eagle's medium (with 25 mM Hepes) (DMEM). After centrifugation the cells were cultured in flasks as described above; non-adherent contaminating cells could be removed after incubation overnight at 37 °C under air/CO₂(19:1). Adherent contaminating cells would be rendered negligible by the passaging of the cells at least three times before any use in assays.

(b) J774.2 murine monocyte-macrophages. This was a standard cell line obtained from the European Collection of Animal Cell Cultures (E.C.A.C.C.). The cells existed in a semi-adherent phase and required Iscove's DMEM buffer with 10 % foetal-calf serum (FCS) as growth medium. Antibiotics were included as with the M5076 cell-line. Culturing was in both 25 and 75 cm² disposable flasks using 4 and 12 ml of medium respectively, at 37 °C under a normal atmosphere. Cells were obtained by gentle centrifugation of the removed medium; after washing and resuspension in fresh Iscove's DMEM they were stored at 4 °C and used within a few hours. Cells were stored in liquid nitrogen after freezing. Cells were resuspended after washing to (4–10) × 10⁵ cells/ml of freezing medium containing 90 % FCS/10 % glycerol (> 20 % FCS and 10 % glycerol in the normal medium is the recommended freezing medium for mammalian cells). Aliquots of 1 ml were placed in 1.5 ml Nunc Cryovials and left overnight in a –70 °C freezer in an insulated polystyrene box. Once frozen, the vials were transferred to liquid nitrogen storage.

(c) NCTC 1469 murine hepatocytes. This was a standard cell line obtained from the E.C.A.C.C. The cells existed in a semi-adherent phase and required DMEM buffer with 10 % horse serum as growth medium. Culturing was in both 25 and 75 cm² disposable flasks using 4 and 12 ml of medium respectively, at 37 °C under a normal atmosphere. Cells were obtained by gentle

centrifugation of the removed medium; after washing and resuspension in fresh DMEM they were stored at 4 °C and used within a few hours.

Storage of the cell-line in liquid nitrogen followed the same procedure as for the J774.2 cell line, except that horse serum was used in preference to FCS.

(d) L929 murine fibroblasts. These were obtained from the E.C.A.C.C. in an adherent phase, requiring DMEM buffer with 10 % FCS. Storage was as for the J774.2 cell line.

(e) Native murine peritoneal macrophages. These were extracted from the abdominal cavity of CO₂-gassed CD1 mice by the injection and removal of 5 ml of Iscove's DMEM/heparin medium. The cells were gently centrifuged and either plated out in Iscove's DMEM/10 % FCS [37 °C, air/CO₂ (19:1)] or used in a 0.15 M PBS suspension. Those plated out were allowed to mature for 3 days before use; those in suspension were stored at 4 °C and used within a few hours. All cell viability was assessed by using the Trypan Blue exclusion test.

Siliconization

Since it was found that the modified proteins had an increased affinity for binding to plastics, greatly interfering with assays where low concentrations of protein were used (Shaw and Crabbe, 1994a,c), some surface treatment of the polypropylene surface was necessary. Silicone oil was applied to microcentrifuge tubes with a small pipette dropper, two drops of oil being placed 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 tip held upright in a Rainin autoclavable box. The combination of coating and vaporization of the remaining oil during autoclaving ensured efficient preparation.

Iodination of modified proteins

This was derived from 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 microcentrifuge tubes and allowed to evaporate in a fume hood. The coated tubes could then be stored in 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 K¹²⁵I stock solution was diluted in 0.15 M PBS (3 µl in 300 µl of buffer) and 20 µl of the dilution were added to each tube. The tubes were left at room temperature for 20 min before the addition of 250–300 µl of 0.05 M PBS. The tubes were left for a further 10 min while the Iodogen process was terminated and then the solution was filtered through approx. 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 γ-radiation in an LKB 1261 Multi-Gamma γ-radiation counter using Pharmacia RIACalc software. The fractions containing protein were collected and stored at –20 °C in an Amersham ¹²⁵I storage box. The degree of labelling was estimated by placing aliquots in a counter and the ratio of bound to free iodine estimated using dotting on thin-layer chromatograms.

Binding of modified proteins

(a) Concentration-based binding. 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 unlabelled 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 μ l of 0.15 M PBS in pre-siliconized micro-centrifuge tubes suspended above ice at 4 °C. Approx. 5×10^5 cells were used in each tube, with at least 1 h in suspension prior to the assay. The cells were added in 100 μ l aliquots, the remaining 100 μ l being made up with buffer, labelled protein and excess unlabelled protein. Incubation was for 1 h (stated if different), terminated by centrifugation and repeated washing with 200 μ l of ice-cold PBS. Counting was done by transferring cells to fresh tubes and counting both these tubes and the originals in a counter.

(b) Competitive binding. Competition assays were carried out similarly to the binding assay described above, except that the 100 μ l buffer making up the incubation volume was partially replaced by co-reagents dissolved in 0.15 M PBS. The co-reagents could be unlabelled competing protein or one of dextran sulphate, fucoidan (0–125 μ g/ml) and polyinosinic acid (0–50 μ g/ml) as a polyanionic competitor for maleyl-BSA. Bars on the Figures indicate ± 1 S.D., and the results are from at least three separate experiments. Where bars are not shown, the S.D. lies within the plotted points.

Uptake and degradation of modified proteins

Similar incubations were used to those described for binding assays, and took place in a water bath at 37 °C. Tubes were removed at appropriate times and cell pellets prepared as for binding assays. Trichloroacetic acid [final concn. 5% (w/v)] was added to the samples of the incubation medium to separate labelled protein from free iodine or iodinated amino acids. Experiments were also performed using chloroquine (0–200 μ M) to inhibit lysosomal degradation.

RESULTS

Binding of modified proteins to the M5076 monocyte-macrophage cell-line

Binding of BSA incubated with 2.0 M glucose for 2 months (AGE-2.0M/2)

It was found that binding was completely non-saturable and non-specific; quenching of specific binding by the addition of large amounts of unlabelled protein produced no decrease in binding to the assay. Figure 1(a) shows that, where 0–20 nM AGE-2.0M/2 was used to assay binding with 7.5 μ M unlabelled protein in a parallel control assay, the assay produced a linear graph as expected, since the published K_d had been approx. 15–20 nM (Vlassara et al., 1987). When this binding was used to calculate the number of molecules present, and hence the number of binding sites/cell, it was found that there appeared to be > 2 million molecules bound/cell rather than the expected 120 000 at most. The control incubation shown in Figure 1(a) yielded similar results; there was no measurable decrease in the binding,

Figure 1 Binding of AGEs to M5076 monocyte-macrophages

(a) Binding of BSA incubated with 2 M glucose for 2 months (AGE-2.0M/2) to M5076 monocyte-macrophages; ○, assay; ●, control with 7.5 μ M unlabelled protein. The binding is linear, which was expected for 0–20 nM protein concentrations, but the amount of protein bound was high. With 500 000 cells in each incubation, 0.8 pmol of protein would be the equivalent of approx. 1.0×10^6 sites/cell. An excess of unlabelled protein should reduce any binding to the non-specific component; here there is no reduction, indicating all the binding is non-specific. (b) Binding of AGE-2.0M/2 to M5076 monocyte-macrophages up to 100 nM. The linearity of the binding observed in (a) continues well above the expected K_d of 20 nM; no saturation is observable, and the binding at 100 nM is approx. 1.5×10^6 sites/cell. (c) The percentage binding of AGE-2.0M/2 to M5076 monocyte-macrophages when increasing concentrations of unlabelled AGE-2.0M/2 protein are added to each incubation. ○, Assay with 500 000 cells; ●, assay with 100 000 cells. The binding appears to remain at 40–50% (4–5 fmol), despite the increased number of cells and protein, indicating non-specific binding.

indicative of the insignificance of specific binding. When the assay was carried out with the reagent concentration extended to 100 nM, sufficient to allow maximum binding (calculated to be approx. 3.75×10^6 sites/cell at 100 nM), there was no indication of reaching a horizontal asymptote. Expressed as percentage binding, the data consistently gave values of 30–35% incorporation of the protein in the assay, equivalent to 3.75×10^6 sites/cell.

Two possibilities arose that may have explained, in part, this apparently anomalous behaviour compared with previously published results. Firstly, it had been assumed that non-specific binding to the tubes was insignificant because the cell concentration in the assays was such that the plastic surface was out-competed by binding to cells, both specific and non-specific. The assay covering 0–10 nM also contained a parallel incubation that lacked cells, from which it was calculated that the binding to the tubes was approx. 10–15% of the cell binding observed in Figure 1(b). While it may be assumed that this percentage would have decreased in the assay itself due to the competition offered by the cells present, 15% is still markedly higher than the $\ll 5\%$ non-specific binding normally expected in such assays. Secondly, linear binding may have resulted from inadequate quenching of specific binding by high concentrations of unlabelled protein (7.5 μ M).

The assay covering 10 nM of unlabelled AGE-2.0M/2 was quenched by exponentially increasing the concentration of unlabelled protein from 19 nM to 100 μ M. Efficient quenching should reduce binding to the proportion of the labelled protein that binds non-specifically, normally less than 1% of the total label added. We found that the quenching had no effect upon the binding of the protein, indicative of negligible specific binding. The percentage of label bound was far in excess of the proportion that would have been expected for any binding assays (Figure 1c) and did not vary with the number of cells used. That this proportion appeared to increase slightly with the concentration of protein is probably not significant, but it remains a fact that the binding was unquenchable, i.e., non-specific. The possibility did exist that much of the non-specific binding occurred through protein being trapped by cell aggregation, a condition that would appear as unquenchable binding. Aggregates still invisible to the naked eye could be large enough to trap sufficient protein to produce high percentage non-specificity. The marked difference in the number of cells used in different parts of the assay argued against this; using 20% of the number of cells produced the same percentage binding.

Binding of maleyl-BSA

The binding consistently exhibited non-saturable activity, indicating the existence of at least 10×10^6 binding sites/cell. The expected saturable binding of a maximum 100–120 000 sites/cell was never observed. Differences were observed with changes in cell concentrations, but the amount of protein binding did not increase proportionally with the number of cells used.

Analysis of specific binding of a labelled protein, such as 125 I-maleyl-BSA, to a saturable receptor has to take into account non-specific binding as background activity. The latter was estimated by running a parallel incubation with large excess of unlabelled protein; since specific binding is limited, this is saturated by unlabelled protein, but the constant proportion of protein engaged in non-specific binding remains measurable. Since in the previous assays there was no indication of saturation by labelled protein nor competition by an excess of unlabelled protein, an assay was carried out in which the amount of

Figure 2 Binding of maleyl-BSA to M5076 monocyte macrophages

M5076 macrophage-monocytes (2.5×10^5 and 5.0×10^5) were incubated in 500 μ l PBS for 1 h at 4 °C with 10 nM 125 I-maleyl-BSA in the presence of 0–10 μ M unlabelled maleyl-BSA. ○, Assay with 5.0×10^5 cells; ●, assay with 2.5×10^5 cells. For an anticipated K_d of 5–20 nM maleyl-BSA, 10 nM should have exhibited specific binding that was completely blocked by the high concentrations of unlabelled maleyl-BSA. In fact, a constant percentage of the label was recovered, even in the presence of 10 μ M unlabelled maleyl-BSA, indicative of entirely non-specific binding.

unlabelled protein was increased to a 1000-fold the labelled protein's concentration. The amount of labelled binding was not found to decrease (Figure 2), and when the total amount of protein binding was calculated, a linear graph was obtained. Decreasing the number of cells from 500 000 to 250 000 resulted in a reduction of the binding by 60%.

It had been suggested that peritoneal macrophages contained approx. 120 000 receptors/cell; assuming the M5076 cell line had a similar number, 500 000 cells would permit 0.1 pmol of modified protein to bind. We found that an excess of unlabelled protein reduced the non-specific binding to a background of 0.04 pmol (2% of the labelled maleyl-BSA), which was the equivalent of 48 000 sites/cell. Even with efficient correction for non-specific binding, background activity of this level would make any estimation of receptor kinetics worthless.

Binding of BSA incubated with 20 mM (AGE-20/9) and 160 mM (AGE-160/9) glucose for 9 months

It was possible that a modified protein preparation, only lightly glycosylated in comparison with the above (AGE-2.5M/2), would be less prone to non-specific binding and might clearly exhibit specific binding to the putative receptor. Two preparations were used, namely AGE-20/9 and AGE-160/9, the former having been incubated at a glucose concentration more similar to that found *in vivo*. However, it was found that both exhibited the same non-specific activity as previously found with maleylated BSA, with quenching by excess unlabelled protein having no effect upon the binding detected, as shown in Figure 3 for AGE-20/9; AGE-160/9 produced identical linear results, indicating a predominance of non-specific binding.

Competition for binding to cell-line M5076

(a) Competition between BSA and AGE-20/9. The inclusion of up to 2 μ M unlabelled competing protein produced a small

Figure 3 Binding of glycated-BSA to M5076 monocyte-macrophages

The incubation of ^{125}I -AGE-20/9 ($\circ$) with 5.0×10^5 cells produced only linear binding. The inclusion of $15 \mu\text{M}$ unlabelled AGE-20/9 ($\bullet$) brought about no decrease in the linear binding, indicating that non-specific binding accounted for most or all of the binding observed.

Table 1 Competition between glycated BSA and the unmodified protein

For details, see the text.

[Competing protein] (nM)	^{125}I -BSA + AGE-20/9; BSA bound (pmol)	^{125}I -AGE-20/9 + BSA; AGE-20/9 bound (pmol)
0	0.1148 ± 0.0137	0.1366 ± 0.0357
500	0.1092 ± 0.0059	0.0954 ± 0.0121
1000	0.0716 ± 0.0228	0.0863 ± 0.0210
2000	0.0835 ± 0.0203	0.0946 ± 0.0019

decrease in the binding of the 50 nM labelled protein (Table 1), but not one that was statistically significant. If the putative receptor's function is to identify and remove modified proteins, then unmodified BSA should not provide competition. For non-specific adsorption to the cell surface, though, BSA should provide competition. Since our experiments suggest that the binding to the cell surface of the glycated protein is largely non-specific, it was expected that BSA should offer some competition. The fact that it did not implied that some form of site recognition had occurred, with AGE-20/9 binding preferentially to a non-specific binding site. Such a site population would need to have a large capacity for an unlabelled excess of the same modified protein not to provide competition. The adsorption of BSA appeared to have occurred at sites not recognized by the AGE-20/9.

(b) Competition between maleyl-BSA and acetyl-LDL. The competition between these two modified proteins appeared to be minimal, again complicated by the high degree of non-specific binding that occurred during the assays. The addition of up to $10 \mu\text{M}$ unlabelled acetyl-LDL to an incubation containing 100 nM maleyl-BSA (Figure 4a) produced no discernible decrease in binding to the cells (a M5076 monocyte-macrophage cell line).

Figure 4 Competitive binding to M5076 monocyte macrophages

(a) Incubation of 100 nM ^{125}I -maleyl-BSA with excess acetyl-LDL found that no competition was discernible between the two for the cell surface ($\circ$). Competition was found for the tube surface ($\bullet$), although it was siliconized to limit non-specific binding; acetyl-LDL appears to compete for 55–60% of the sites on the tube surface to which maleyl-BSA will bind. (b) Incubation of 1.5 nM acetyl-LDL with excess maleyl-BSA showed no discernible competition for binding to the cell surface ($\circ$). Competition was found with binding to the tube surface ($\bullet$) with 70–75% of the bound acetyl-LDL being replaced with maleyl-BSA.

Since an earlier assay quenching with high concentrations of unlabelled maleyl-BSA had brought about a decrease in the binding of maleyl-BSA to the same cell line (Figure 2), it was expected that any competition between maleyl-BSA and acetyl-LDL for a binding site, specific or non-specific, would exhibit similar behaviour. Since the excessive amount of acetyl-LDL present did not reduce the binding of maleyl-BSA it was concluded that there was no competition between the two proteins for binding to the cell surface. Some competition was observed with binding to the siliconized tube surface, where the excess unlabelled acetyl-LDL displaced 55–60% of the non-specifically bound maleyl-BSA.

The competition of maleyl-BSA with labelled acetyl-LDL produced similar results (Figure 4b) to those described above. No decrease in the binding of the acetyl-LDL to the cell surface was detected, but binding to the tube surface was decreased. This reinforced the conclusion that there was no discernible specific binding to the cell surface.

Figure 5 Binding of modified proteins to N.C.T.C.-1469 murine hepatocytes

(a) Binding of maleyl-BSA. Incubations with (○) and without (●) the presence of 30 μ M unlabelled maleyl-BSA. There was no statistical difference between assay and control binding, indicative of non-specific binding. (b) Binding of acetyl-LDL. The 125 I-acetyl-LDL (○) produced a linear plot in excess of the 100 fmol. The presence of 160 nM unlabelled lipoprotein (●) did not decrease binding activity, indicating it to be non-specific.

Binding of maleyl-BSA to the J774.2 monocyte-macrophage cell line gave results identical with those with the M5076 monocyte-macrophage cell line. The inclusion of 10 μ M unlabelled maleyl-BSA in the control incubation produced no decrease in the binding; similarly the inclusion of the polyanions fucoidan and dextran sulphate produced no effect on binding. The total binding to the cells (assay or control) did not exceed 0.02 pmol, equivalent to approx. 24000 sites/cell.

Binding of modified proteins to the N.C.T.C. (National Collection of Type Cultures) 1469 murine hepatocyte cell line

Binding of maleyl-BSA

The binding of maleyl-BSA to the hepatocyte cell line produced similar results to the binding to macrophage-derived cell lines (Figure 5a). A similar amount of labelled protein bound in both the absence and presence of a high concentration of unlabelled protein. If there was any specific binding of maleyl-BSA to a

Figure 6 Binding of maleyl-BSA to murine peritoneal macrophages

This showed some significant difference between assay (○) and control (●) activities. The calculated specific binding activity (—) indicated the presence of approx. 24000 sites/cell.

receptor, then it was a trace activity that was masked by the excess of non-specific binding that had occurred.

Binding of acetyl-LDL

As with the binding of acetyl-LDL to other cell types and as with the binding of maleyl-BSA to this cell line, no specific binding was discernible. The inclusion of 160 nM unlabelled acetyl-LDL produced no reduction of the linear binding observed over a range of lipoprotein concentrations (Figure 5b). The amount of binding was approx. 100 fmol.

Binding to murine peritoneal macrophages

Binding of maleyl-BSA

Some evidence for the specific binding of maleyl-BSA to peritoneal macrophages was found. A difference in the assay and control activity was apparent (Figure 6), and appropriate curve-fitting could be made to each (saturable and linear respectively). Though the binding was sufficiently low for background radioactivity to substantially increase the statistical error, leading to some apparently negative values in the data obtained after statistical analysis, the clear difference in total and non-specific binding indicated the presence of specific binding to the macrophages. The specific binding found was the equivalent of approx. 24000 sites/cell, similar to that suggested for the postulated scavenger receptor (Vlassara et al., 1987).

Dextran sulphate and fucoidan, both polyanions, were included in parallel incubations as potential inhibitors of the specific binding of maleyl-BSA to the macrophages. However, no decrease in total binding, which would be expected in the presence of competitive molecules, was observed.

Binding of BSA incubated with 75 mM glucose for 2 months (AGE-5/2) and 20 mM glucose for 9 months

A maximum non-saturable protein binding of 0.43 ± 0.23 pmol was calculated for both AGEs. Since a receptor population estimated at 100–200000 sites/cell would be expected to produce

Figure 7 Uptake of heavily glycosylated BSA

○, uptake of BSA incubated with 0.5 M glucose for 2 months (AGE-0.5/2) by M5076 monocyte-macrophages; ●, uptake as before, but with the addition of 400 nM unlabelled AGE-0.5/2. For details, see the text.

a maximum binding of 0.080–0.100 pmol, the variation in the binding found meant that it was difficult to make an estimate of the order of magnitude of the receptor population, as it was masked by the non-specific binding.

Uptake and degradation of modified proteins

BSA incubated with glucose (0.5 M) for 2 months gave non-saturable binding to M5076 monocyte-macrophages; binding was reduced by 60% on the addition of 400 nM unlabelled glycosylated BSA (BSA incubated with 0.5 M glucose for 2 months), as shown in Figure 7. This reduction would be expected if high concentrations of glycosylated substrate were needed to achieve significant uptake. Lower, more physiological, concentrations of glycosylated BSA (BSA incubated with 10 mM glucose) gave no evidence of such a reduction on the addition of unlabelled glycosylated BSA, suggesting that only high, non-physiological, concentrations of glycosylated BSA could be taken up by the macrophage cell line. Similar results were obtained with the J774.2 monocyte-macrophage cell line and with an L929 fibroblast cell line. With 500 000 cells and a protein concentration of 50 nM, 0.2–0.5 pmol, approx. 240–600 000 molecules/cell, of associated protein was consistently found in macrophages and fibroblasts. There was no reduction in the presence of unlabelled excess protein, and no detectable degradation of AGEs by macrophages. The addition of up to 200 μ M chloroquine in cell incubations with glycosylated BSA [incubated for up to 9 months in physiological concentrations of glucose (10 mM)] did not result in any significant difference of association with the radiolabelled protein. The effects of chloroquine were identical when J774.2 monocyte-macrophage cells and L929 fibroblast cells were used.

DISCUSSION

Prolonged incubation of BSA with > 20 mM glucose or 2 months incubation with $\geq$ 0.5 M glucose induced the modified protein to readily bind non-specifically to both cell and tube surfaces. Kinetic studies on the binding of AGEs and other modified proteins to macrophages and hepatocytes showed no evidence

for specific receptor binding, as neither binding saturation nor cross-competition (homologous or heterologous) was detected. At physiological concentrations of glucose, there was no evidence for uptake or degradation of AGEs. Though the AGE-75/2 exhibited quenchable binding and would have been expected to be recognized by a scavenger receptor for non-enzymically glycosylated proteins, the binding found was non-saturable over the ligand concentration range used. The quenchability of the binding by an excess concentration of unlabelled AGE-75/2 meant that saturation of the binding was possible, but required a much higher concentration than the 600 nM used. The maximum binding found was approx. 0.15 pmol over 5×10^5 macrophages, the equivalent of 180 000 molecules bound to each cell. Since the expected number of sites/cell was 100–120 000 (Vlassara et al., 1987), the presence of > 180 000 sites was within the anticipated order of population. However, the binding of the maleyl-BSA to the native murine peritoneal macrophages showed saturability over 200 nM concentration to a population of 24 000 sites/cell, an order of magnitude less than that possible for AGE-75/2. At this receptor concentration, assaying for specific binding was made difficult by the 125 I-label merging into the background activity at lower concentrations. Thus we find that while native murine peritoneal macrophages contain a receptor for maleylated proteins, such a receptor does not appear to be involved in binding AGEs. The non-saturability of maleylated BSA binding to cell lines may be because culture conditions might have modified any specific receptors; even in native murine peritoneal macrophages, specific maleylated protein binding was difficult to detect.

Competition with polyanions such as fucoidan and dextran sulphate was expected to block binding to a receptor that recognized modified proteins through the increase in anionic charges. Such competition was not found, suggesting that the recognition was not due to protein charge alterations. The binding of maleyl-BSA to the monocyte-macrophage cell line J774.2 also produced a value of 24 000 sites/cell, though it was unquenchable, and without apparent competition offered by either dextran sulphate or fucoidan. The binding of both non-enzymically glycosylated BSA to other cell lines, M5076 monocyte-macrophages and N.C.T.C. 1469 hepatocytes, produced no evidence of saturable or quenchable binding. Similar results were obtained for binding of lysine-glucose-derived Maillard products (Shaw and Crabbe, 1994c). Analysis of the non-specific binding to cell surfaces shown by all the modified proteins indicated that some modifications encouraged non-specific binding activity more than others. Maleylation (maleyl-BSA) and non-enzymic glycation with $\leq$ 20 mM glucose were less susceptible to non-specific binding than heavily non-enzymically glycosylated protein. Aging of the protein concerned also played a rôle, with proteins that had been incubated for longer generally being more adherent. This suggests the number of glycosyl adducts per protein to have been of more consequence than the formation of mature advanced glycosylation end-products.

Competition for binding between the modified proteins produced no blocking, or only partial blocking, of any apparent binding of a specific modified protein to the cell surfaces, whether the modifications were maleylation or non-enzymic glycation. This suggests that each modified protein could adhere to a range of non-specific sites on the cell surfaces, of which differently modified proteins could adhere to only a proportion. Such non-specific binding offered one possible explanation for how non-enzymically glycosylated proteins could adhere to one another *in vivo*, e.g., albumin becoming attached to the collagen of basement membranes. However, it raised the question of how a specific receptor could function if the substrate readily attached, non-

specifically, to the materials around it with high affinity and possibly irreversibly. If such a scavenger receptor existed then it would need to possess a very high affinity indeed for modified proteins. Certainly, the protein-binding activities described here were the product of more than a single binding site and possibly of many. One of these possible sites may be a scavenger receptor, but it is unlikely that one particular site could function as a specific receptor.

The uptake and degradation experiments showed that there was no evidence for uptake or degradation of AGEs produced at physiological concentrations of glucose. Rather, the association was concerned with non-specific adsorption to cell surfaces. The results with chloroquine would support this, whether that compound inhibits initial endocytosis or prevents fusion of the endosomes/lysosomes to the endoplasmic reticulum. Heavily glycosylated BSA exhibited some uptake, although the physiological significance of this appears remote, owing to the high concentration (0.5 M) of glucose used in the glycation. This might imply either that such heavy glycation allows the product to be taken up by another receptor, i.e. non-specifically, or that there is a receptor on the macrophage which only responds to non-physiologically glycosylated proteins. Under physiological conditions, while the macrophages adsorb the AGEs, we found no evidence for any significant uptake or degradation.

The putative ability of macrophage cells to identify and remove modified proteins could well be a result of the non-specific binding activities of the modified proteins acting upon a limited population of non-specific binding sites on the cell surface. This could have important implications for the 'control' of AGE concentrations and so the development of diabetic complications, particularly in poorly controlled diabetics. Low macrophage levels in the blood could seriously potentiate the long-term effects of non-enzymic post-translational protein modifications.

We thank the Agricultural and Food Research Council, and the British Diabetic Association, for support.

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