Advanced Glycosylation Endproduct-specific Receptors on Human and Rat T-Lymphocytes Mediate Synthesis of Interferon γ: Role in Tissue Remodeling

By Farhad Imani,* Yasuhiro Horii,* Manikkam Suthanthiran,‡ Edward Y. Skolnik,* Zenji Makita,* Vijay Sharma,‡ Prabodh Sehajpal,‡ and Helen Vlassara*

*From *The Picower Institute for Medical Research, Manhasset, New York 11030 and ‡Rogosin Institute, Immunogenetics and Transplantation Center, New York 10021*

Summary

During normal aging and in chronic diabetes the excessive accumulation of reactive glucose-protein or glucose-lipid adducts known as advanced glycosylation endproducts (AGEs) has been shown to induce tissue dysfunction, in part through interaction with AGE-specific receptors on monocyte/macrophages and other cells. Recognizing that circulating lymphocytes trafficking through tissues interact with tissue AGEs, we searched for the expression of AGE-binding sites on peripheral blood T lymphocytes. Resting rat and human T cells bound ^{125}I-AGE-albumin with an affinity of 7.8×10^7 M^{-1}, whereas, after stimulation with phytohemagglutinin (PHA) for 48 h, binding affinity increased to 5.8×10^8 M^{-1}. Flow cytometric analysis of resting rat T cells using polyclonal antibodies raised against rat liver AGE-binding proteins (p60 and p90) revealed the constitutive expression of both immunoreactivities. The number of resting $CD4^+$ and $CD8^+$ T cells positive for anti-p60 antibody binding (34.2 and 58.5%, respectively) increased to 92 and 90% of cells after 48-h stimulation with PHA. Exposure of PHA-activated T lymphocytes to AGE-albumin enhanced expression of interferon γ (IFN-γ) mRNA 10-fold and induced greater elaboration of the mature protein than did exposure to unmodified protein or PHA treatment alone. These data indicate that T cells contain an inducible system of surface receptors for AGE-modified proteins, and that receptor occupancy is linked to lymphokine production. This T cell AGE-receptor system might serve to target lymphocytes to AGE-rich tissues and involve them in the regulation of tissue homeostasis either by assisting in macrophage-dependent clearance of AGE-proteins, or by exerting direct antiproliferative action on mesenchymal cells. Under conditions of excessive AGE-protein and AGE lipid accumulation (e.g., aging and diabetes), enhanced production of AGE-induced IFN-γ may accelerate immune responses that contribute to tissue injury.

Reducing sugars such as glucose can react nonenzymatically with protein amino groups in a time- and concentration-dependent manner to form a family of complex structures which are referred to as advanced glycosylation endproducts (AGE)[1] (1–3). The excessive deposition of these covalent adducts on various body proteins has been shown to contribute to the development of the complications of aging and diabetes by both direct chemical (covalent crosslink formation), and cell surface receptor–mediated pathways (3).

Cells of the monocyte/macrophage lineage were the first found to display a surface receptor system mediating the binding and internalization of AGE-modified macromolecules. These receptors are structurally and functionally distinct from other known macrophage scavenger receptors (4, 5). AGE-specific affinity labeling of cell surface proteins in the murine macrophage-like cell line RAW 264.7 revealed a single AGE-associated band at ~90 kD (6, 7). Subsequently, two AGE-binding proteins, one of 60 and the other of 90 kD were isolated from rat liver member preparations and partially sequenced (8).

Rat liver p60 and p90 were used to raise Abs, which recognized surface determinants on rat monocytes and macrophages (8). The same Abs inhibit AGE binding and neutralize

[1] *Abbreviations used in this paper:* AGE, advanced glycosylation endproduct; FBS, fetal bovine serum; f-BSA, formaldehyde-modified BSA; FFI, 2-furoyl-4-(5)-2-furanyl)-1-*H*-imidazole; HSA, human serum albumin; $NaBH_4$, sodium borohydride; TPA, O-Tetradecanoylphorbol 13-acetate.

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AGE-dependent responses on human monocytes/macrophages (9) and on murine mesangial cells (10), suggesting that the AGE-receptor system involves proteins with highly conserved domains. In addition to exhibiting significant transendothelial migratory activity toward AGE-modified matrix in vitro and in vivo (11), AGE-stimulated monocyte/macrophages are induced to secrete several cytokines and growth factors, such as TNF-α, IL-1β, platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF)-IA (9, 11, 12). These coordinate activities suggest that the macrophage AGE-receptor, mediating the selective removal of AGEs, may also be involved in growth regulatory activities within tissues.

Other cell types, e.g., human endothelial cells (13–15), mesangial cells (10, 16), and fibroblasts (17), have been shown to contain AGE-specific binding proteins that are linked to diverse functions. For instance, the exposure of vascular endothelial cells *in vitro* to increasing levels of AGE-protein results in disruption of normal barrier function and enhancement of cell-associated procoagulant activity (13), whereas the interaction of AGE ligands with the renal mesangial cell AGE-receptors is associated with increased matrix protein synthesis (10, 16). Based on the different binding specificities and functional characteristics of the cell type–specific AGE-receptors identified thus far, it remains unclear whether these molecules are structurally related to the macrophage AGE-receptor proteins. Nevertheless, the identification of an AGE-receptor system has introduced a new mechanism by which macrophages can participate in both the maintenance of normal extracellular matrix, and in certain pathological processes, such diabetic vascular disease and atherosclerosis (18).

AGE-receptor–mediated macrophage activation may be one mechanism by which immune cell activation may promote atherogenesis (19). In fact, several lines of evidence have suggested that other immune cells such as lymphocytes may play an important role in this progressive disorder which has been recently attributed to a chronic inflammatory process. T lymphocytes, which are ubiquitously present in inflammatory states, are also abundant in the vessel wall during the early stages of atherogenesis (19, 20). Cells of both the $CD4^+$, and $CD8^+$ phenotype are present and they express surface antigens that suggest an activated state (21). The stimulus for the recruitment and activation of such T cells remains unknown, as is their specific function in this slowly evolving process.

Given the spontaneous and progressive accumulation of AGEs within the tissues of diabetics, and in particular, the vascular matrix, we postulated that lymphocytes may express AGE-specific receptors which allow them to interact with tissue AGEs and contribute to the immune activation and effector activities within the vessel wall. We describe here the presence of two such AGE-binding proteins on rat and human T cells. These appear antigenically related to the p60 and p90 AGE-receptor polypeptides present on monocyte/macrophages and can be upregulated upon stimulation with mitogens. AGE-receptor–ligand interaction on the surface of activated lymphocytes results in the synthesis and secretion of IFN-γ.

Materials and Methods

Chemicals and Reagents. Human serum albumin (HSA), BSA (Fraction V, low endotoxin), and Triton X-100 were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bovine RNase and glucose 6-phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium ^{125}I (sp act 10 mCi/100 μl) was obtained from New England Nuclear (Boston, MA). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). The chemically synthesized model AGE, 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole-hexanoic acid (FFI-HA) was provided by Dr. Peter Ulrich (Picower Institute). PHA, *O*-tetradecanoylphorbol 13-acetate (TPA) (Sigma Chemical Co.), and ionomycin, were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Avian anti–rat polyclonal Abs raised against rat liver AGE-binding proteins p60 and p90 were prepared as described (8). Monoclonal anti–rat-$CD5^+$, -$CD4^+$, -$CD8^+$ were purchased from Bioproducts for Science, Inc. (Indianapolis, IN).

Preparation and Radiolabeling of Ligands. AGE-bovine or HSA and AGE-RNase were made by incubating each protein with 0.5 M glucose at 37°C for 6 wk in 10 mM PBS, pH 7.4, in the presence of protease inhibitors and antibiotics as previously described (4, 8). Unincorporated glucose was removed by dialysis against PBS. AGE and unmodified proteins were purified over heparin–Sepharose CL-6B (Pharmacia LKB, Piscataway, NJ) to remove contaminants (10). Endotoxin content in all samples was measured by Limulus amoebocyte lysate assay (E-toxate, Sigma Chemical Co.) and found to be <0.2 ng/ml. AGE content was assessed by an AGE-specific ELISA (22). AGE-BSA contained ~7.0 AGE U/mg; AGE-HSA, 8.3 U/mg; and AGE-RNase, 5.7 AGE U/mg. Unmodified BSA and HSA contained 0.02 AGE U/mg each.

To examine the effect of reducing early glycosylation product on ligand binding, AGE-BSA was incubated with 200-molar excess $NaBH_4$ (Sigma Chemical Co.) for 10 min at 4°C and for 1 h at room temperature (16). The reduced AGE-BSA was then dialyzed against PBS. The chemically defined model AGE, FFI-HA, was synthesized and linked to BSA with 100 mM carbodiimide as described previously (23). Formaldehyde-modified BSA (f-BSA) was prepared as described (5). In brief, BSA was incubated in 0.1 M sodium carbonate buffer (pH 10) with 0.33 M formaldehyde at 37°C for 5 h, followed by extensive dialysis against PBS.

AGE-ligands were iodinated with carrier-free ^{125}I by the Iodogen method (Bio-Rad Laboratories, Cambridge, MA) (24). Samples were dialyzed against PBS until >95% of radioactivity was TCA precipitable.

Cell Preparation. Heparinized blood was drawn from male Sprague-Dawley rats (200–300 g) by cardiac puncture. Heparinized human blood was drawn from healthy volunteers. 3 ml of dextran T-500 (Pharmacia Fine Chemical, Piscataway, NJ) were added to 10 ml of blood and the mixtures were allowed to sediment for 45 min. The PBMC-containing fraction was separated on a Ficoll-Paque gradient (25) and plated with medium containing 10% FBS for 30 min at 37°C to allow monocytes to adhere. Nonadherent cells were collected and passed through a nylon wool column (Polyscience, Inc., Warrington, PA) (26). Collected cells were >95% $CD3^+$ T cells, as determined by flow cytometry, using PE-conjugated Leu-4 (anti-CD3) (Becton Dickinson & Co., Mountain View, CA). Highly purified human T cells were isolated from normal PBMC with a sequential four-step procedure as described (26).

Membrane Preparation. Purified rat and human peripheral blood T cells were washed in PBS. After centrifugation, cells were disrupted with a tight Dounce homogenizer, in a solution of PBS

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containing 3% EDTA and protease inhibitors (2 mM PMSF, 10 μg/ml aprotinin, 5 ng/ml pepstatin, 1 mM benzamidine). The nuclear and organelle-enriched fractions were removed by centrifugation at 1,000 *g* for 10 min. Membranes then were isolated from the supernatant by centrifugation at 10,000 *g* for 20 min at 4°C. The resulting enriched membrane fraction was solubilized in PBS containing 1% Triton X-100, and protease inhibitors as stated above. The protein concentration was determined by the method of Bradford (27). This material then was used for ligand and Western blot studies.

Binding Studies. Radioligand binding studies were performed on rat or human T cells in 6-well plates (10^6 cells/well). The studies were performed in 0.1 ml of binding medium (RPMI 1640, 25 mM Hepes, pH 7.2, 3 mg/ml BSA, 0.02% NaN_3) in the presence of ^{125}I-AGE-BSA (sp act 7 × 10^7 cpm/ng) for 3 h at 4°C. At the end of the incubation, cells were layered on 0.3 ml fetal bovine serum (FBS) in a 0.4-ml polypropylene tube and centrifuged at 9,000 rpm for 90 s. Each tube was cut just above the cell pellet, and radioactivity was measured by Gamma Trac 1193 (Tm Analytic, Elk Grove Village, IL). Nonspecific binding of ^{125}I-AGE-BSA was determined in parallel incubations by adding 100-fold excess of unlabeled AGE-BSA. Protein concentration was determined by the method of Bradford (27). Specific binding was defined as the difference between total binding (cells incubated with radioligand alone) and nonspecific binding (cells incubated with radiolabeled ligand plus 100-fold excess unlabeled ligand). Scatchard analysis of the data was performed to determine the binding affinity constant and the receptor number as described previously (16, 28).

Uptake and Degradation. T cell uptake and degradation of AGE-BSA was performed according to previously described procedures (4, 16) with minor modifications. Briefly, cells were plated in 6-well plates in RPMI containing 20% FBS and insulin. Intracellular accumulation of radioactive ligand was assessed by incubating cells with various concentrations of ^{125}I-AGE-BSA, in the presence and absence of 100-fold excess of unlabeled AGE-BSA, for 6 h at 37°C. After suspending the cells on FBS and centrifuging for 90 s, the amount of cell-associated radioactivity was determined as described above. Specific uptake was defined by the same criteria as used for the binding studies. Degradation was determined by measuring TCA-soluble radioactivity in the aspirated medium.

Ligand Blotting. Human PBLs or highly purified T cells were collected either unstimulated or after stimulation with PHA (2 μg/ml) plus TPA (1 ng/ml) or ionomycin (1 μM) plus TPA (1 ng/ml) for 48 h. T cells (10^7) cell membrane preparations were electrophoresed on a nonreducing SDS-PAGE (12%), and then electroblotted onto a nitrocellulose filter, as previously described (8). After blocking for 1 h in a solution of PBS containing 1.5% BSA and 0.1% Triton X-100, the nitrocellulose filter was probed with ^{125}I-AGE-BSA in the presence of 100-fold excess of either BSA or AGE-BSA. The blot was washed three times with PBS and exposed to Kodak XAR-5 film at −80°C.

Flow Cytometric Analysis. Double color cell staining was performed by incubating one million cells with biotinylated anti-60 kD, anti-90 kD, or preimmune antisera and FITC-conjugated anti-CD4, anti-CD5, and anti-CD8 Abs at saturating concentrations, as primary Abs for 30 min at 4°C. For secondary staining, cells were washed in staining buffer (PBS, 3% FBS, 0.1% NaN_3) and then incubated with avidin-PE (Becton Dickinson & Co.). Background fluorescence was determined by staining the cells with a relevant isotypic control Ab, e.g., biotinylated chicken IgG. Cells were analyzed using a FACScan® (Becton Dickinson & Co.). The data were analyzed by Paint-A-Gate software (Hewlett-Packard, Arondale, CA).

RNA Preparation and PCR Assay for Human IFN-γ mRNA. Purified human T cells (2.0 × 10^6/well) were cultured in RPMI 1640 supplemented with 10% FBS, 5 × 10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin for 48 h, in the presence or absence of PHA (1–10 μg/ml). Cells then were transferred to 24-well plates coated with AGE-HSA or HSA (at 5 μg/cm^2), prepared as described (10). After 6 h, T cells were washed by PBS and total RNA was extracted as described (9) by using RNAzol (Cinna/Biotex, Friendswood, TX), and resuspended in 20 μl of diethylpyrocarbonate (DEPC)-treated water. 1 μg of total RNA was incubated with 10 μl of 5× RT buffer (250 mM Tris-HCL (pH 8.3), 375 mM KCl, 15 mM MgC_{12}), 200 U of murine mammary leukemia virus (M-MLV) reverse transcriptase (GIBCO BRL, Gaithersburg, MD), 1 mM dNTPs (Perkin-Elmer Corp., Norwalk, CT), 1 μg/ml BSA (GIBCO BRL), and oligo $(dT)_{12-18}$ (Pharmacia LKB), for 1 h at 37°C. 5 μl of cDNA was amplified in 0.5 ml GeneAmp reaction tubes (Perkin-Elmer Corp.) in the presence of 200 nM final concentration of 5′ (5′-ATG-AAATATACAAGTTATATCTTGGCTTT-3′) and 3′ (5′-CATGCT-CTTCGACCTCGAAACAGCAT-3′) primers, 200 μM dNTPs, 0.5 U Taq polymerase (Perkin-Elmer Corp.), and PCR buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgC_{12}, and 0.001% gelatin in a final volume of 80 μl. PCR was performed in a DNA thermal cycler (Perkin-Elmer Corp.) for 35 cycles: 1 min denaturation at 92°C, 1 min annealing at 55°C, and 1 min extension at 72°C. 15 μl of reaction mixture was electrophoresed in 2% agarose containing 0.5 μg/ml ethidium bromide. A 123-bp DNA ladder was used as a molecular weight marker (GIBCO BRL).

Assay for IFN-γ Secretion. After a 48-h prestimulation with PHA (1–10 μg/ml), purified human T cells (6 × 10^6) were plated on AGE-HSA or HSA-coated wells (5 μg/cm^2) with RPMI containing FBS (10%) for 24 h. The supernatants were collected and IFN-γ concentration was determined using the InterTest-γ ELISA kit (Genzyme, Cambridge, MA).

Results

AGE-binding on Rat and Human T Cells. Freshly isolated rat PBL lymphocytes were tested for AGE-specific binding as described above (4, 16). With increased concentrations of ^{125}I-AGE-BSA, specific binding increased in a saturable fashion (Fig. 1 *A*). Scatchard analysis of binding data revealed a single linear regression line, indicating a single set of binding sites on resting T cells (12.2 × 10^5 sites/cell) (Fig. 1 *A*, *inset*) and a binding affinity of 7.78 × 10^7 M^{-1}. After a 48-h incubation with PHA alone (10 μg/ml), the specific binding for AGE-modified albumin was markedly enhanced, and the binding affinity (K_a) increased to 5.8 × 10^8 M^{-1}, whereas the receptor number increased to 84.6 × 10^5 sites/cell (Fig. 1 *B*, and its *inset*).

The specificity of AGE binding to T cells was tested in competition experiments using either an AGE-modified protein distinct from albumin (AGE-RNase), or BSA modified either by the synthetic AGE model compound FFI (FFI-BSA), or by formaldehyde (f-BSA). Although AGE-RNase competed effectively for binding against ^{125}I-AGE-BSA (Fig. 1 *C*, *a*), neither FFI-BSA nor f-BSA were inhibitory (Fig. 1 *C*, *c*, and *d*). Also, unmodified HSA (Fig. 1 *C*, *f*) and mannan, a polysaccharide recognized by a distinct class of mannose-specific lymphocyte receptors (29) failed to compete for AGE-binding when added in concentrations up to 300 μg/ml (Fig.

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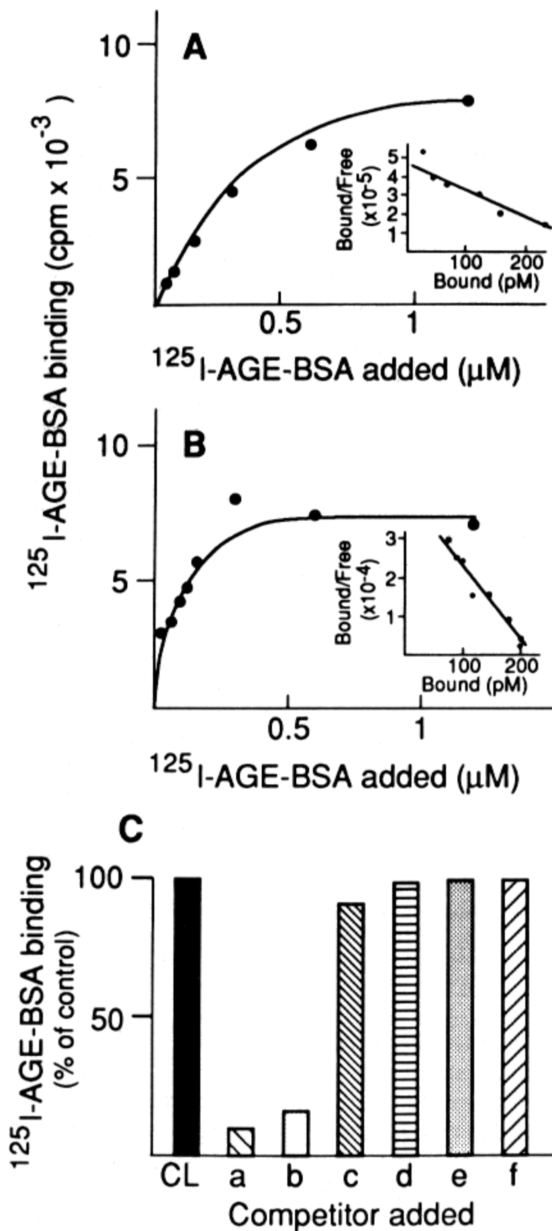

Figure 1. Binding of ^{125}I-AGE-BSA to resting (*A*) and PHA-stimulated rat peripheral blood T cells (*B*). Purified T cells were incubated with various concentrations of iodinated AGE-BSA for 2 h at 4°C, in the presence or absence of 100-fold excess unlabeled AGE-BSA. Specific binding was obtained by subtracting nonspecific binding from total binding. (*Insets*) Scatchard plots for the specific binding data (*Bound*, pM, *Free*, nM). (*C*) Competitive inhibition of ^{125}I-AGE-BSA (25 µg/ml) binding to rat T cells by different ligands added at a 100-fold excess: (*a*) AGE-ribonuclease, (*b*) $NaBH_4$-reduced AGE-BSA, (*c*) FFI-BSA, (*d*) f-BSA, (*e*) mannan (300 µg/ml, (*f*) unmodified HSA. Data are representative of five experiments, each performed in triplicate.

1 *C, e*). $NaBH_4$-reduced AGE-BSA competed equally well with unreduced AGE-BSA for binding by T cells, ruling out the possibility that the observed binding activity was due to early glycosylation products (4, 5) (Fig. 1 *C, b*).

In additional studies, the ability of T cells to internalize radiolabeled AGE-BSA was tested by extending incubations with the ligand for up to 6 h at 37°C. No significant changes were noted in either cell-associated or in TCA-soluble radioactivity of the media (data not shown).

To determine whether the T cell AGE-binding sites contained epitopes related to those found on monocyte/macrophages, ^{125}I-AGE-BSA binding inhibition experiments were carried out using Abs raised against two AGE-binding proteins, p60 and p90, isolated from rat liver (8). These Abs have been shown to inhibit binding of AGE-ligands on rat monocyte/macrophages (8). Each Ab was used separately as well as in combination (Fig. 2). In the presence of a constant amount of ^{125}I-AGE-BSA as ligand and with increasing concentrations of either anti-p60 or anti-p90 Ab as competitor, significant inhibition of AGE-ligand binding was observed (up to 90%), and the combination of both Abs at a concentration of 2 µg/ml yielded a >95% inhibition of AGE-ligand binding. In contrast, no inhibition of binding was observed when a preimmune chicken IgG was used as a control (Fig. 2).

Flow Cytometry of Rat T Lymphocytes. Two major subgroups of T lymphocytes, $CD4^+$ and $CD8^+$ were examined for surface expression of epitopes crossreactive with existing antimacrophage AGE-receptor Abs, using flow cytometry. One million cells were stained with biotinylated anti-p60 or anti-p90 and avidin-PE, FITC-conjugated monoclonal anti-rat CD5, CD4, and CD8 Abs. As shown in Fig. 3, using resting cells, 36% of $CD5^+$ cells stained positively with anti-p60 Ab, whereas 35% of $cD4^+$ and 58% of $CD8^+$ cells stained positively with the same Ab. No immunoreactivity was revealed when nonspecific IgG was used as a control (data not shown). After stimulation with PHA/TPA for 48 h, the expression of the p60 molecule was upregulated significantly (Fig. 3). Similarly, 18, 24, and 25% of $CD5^+$, $CD4^+$, and $CD8^+$ T cells, respectively, stained with anti-p90 Abs. After

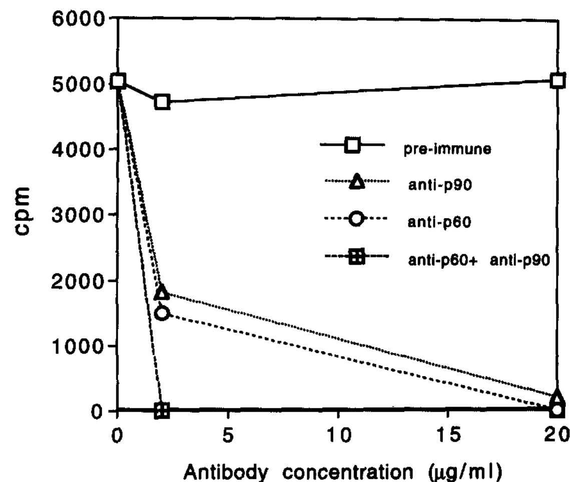

Figure 2. Inhibition of ^{125}I-AGE-BSA binding on rat peripheral T cell surface by chicken anti-p60 and anti-p90 Abs. Cells were incubated with the radioligand (25 µg/ml) in the presence of each Ab added alone or in combination, or in preimmune serum. Data are expressed as cpm of specifically bound AGE-BSA in the presence of the respective Ab and represent the mean of duplicate experiments, each performed in triplicate.

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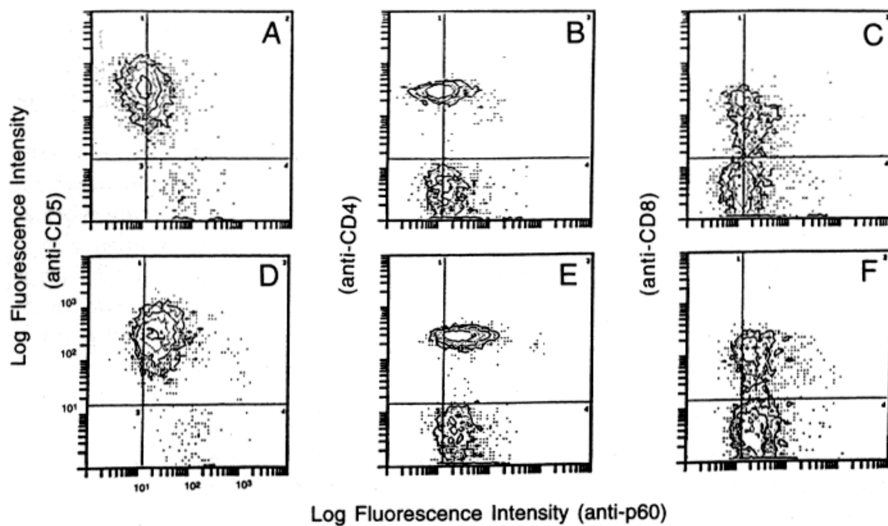

Figure 3. Demonstration by flow cytometry of expression of cross-reactivity with anti-AGE-proteins on purified rat peripheral T cells. Representative experiment using anti-p60 AB. (*A–C*) Resting T cells. (*D–F*) T cells prestimulated with PHA (2 μg/ml) + TPA (1 μg/ml) for 48 h. Double color cell staining was performed using biotinylated chicken anti-p60 (5 μg/ml), or FITC-conjugated anti-CD4, anti-CD5, anti-CD8 as primary Abs, followed by avidin-PE.

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activation, no enhancement in the expression of p90 was noted on these cells despite stimulation (data not shown).

Ligand Blot from Human T Cells. The presence of AGE-binding proteins on the surface of human T cells was further explored using either unfractionated PBLs or highly purified T cell membranes, before or after a 48-h activation by PHA/TPA or TPA/ionomycin. Membrane components were separated by nonreducing SDS-PAGE (12%), then transferred onto nitrocellulose membranes and blotted with the ligand ^{125}I-AGE-BSA. As shown in Fig. 4, an AGE-binding protein with an apparent molecular mass of ~55–60 kD was readily visible in membranes of activated PBLs (Fig. 4, lane *B*), but not of resting PBLs (Fig. 4, lane *A*). The presence of 100-fold excess unlabeled AGE-BSA effectively inhibited the binding of radiolabeled AGE-BSA to the ~55-kD protein band, indicating binding specificity (Fig. 4, lane *C*). Similarly, an AGE-binding protein with identical mobility was identified in membranes prepared from activated (Fig. 4, lanes *E* and *F*), but not from resting, purified T cells (Fig. 4, lane *D*). The method of T cell activation did not appear to influence the level of induction of the 55-kD AGE-binding protein, as both combinations of either PHA/TPA (Fig. 4, lane *E*) or TPA/ionomycin (Fig. 4, lane *F*) yielded identical results. The addition of excess AGE-BSA completely abrogated the appearance of the 55-kD protein (Fig. 4, lane *G*).

IFN-γ mRNA Expression Is Induced by AGE-BSA in PHA-stimulated Human T Cells. cDNA amplification experiments showed that purified human T cells can be induced by AGE-modified HSA to express mRNA for IFN-γ. As shown in Fig. 5, lane *C*, IFN-γ transcripts were readily detected in lymphocytes pretreated with PHA for 48 h and then exposed to AGE-HSA coated dishes for 6 h. Similar treatment of PHA-

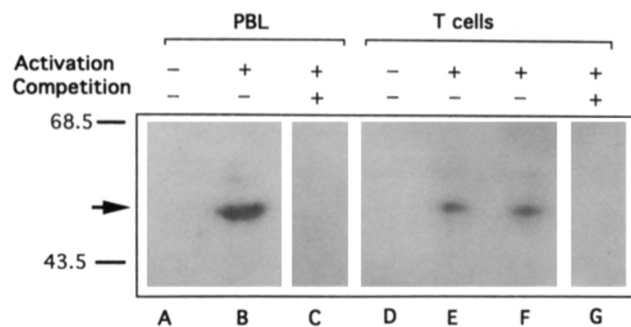

Figure 4. Ligand blot analysis of resting (*A* and *D*) and activated (*B*, *C*, *E*, *F*, and *G*) human PBL or highly purified T cells. Membrane proteins from 10^7 cells were used either untreated (resting) or after activation with PHA or ionomycin plus TPA, were electrophoresed on a nonreducing SDS-PAGE (12%), and electroblotted on nitrocellulose (NC) filter, and probed with ^{125}I-AGE-BSA, in the presence (*C* and *G*) or absence (*A*, *B*, *D*, *E*, and *F*) of 100-fold excess AGE/BSA. Data are representative of three identical experiments.

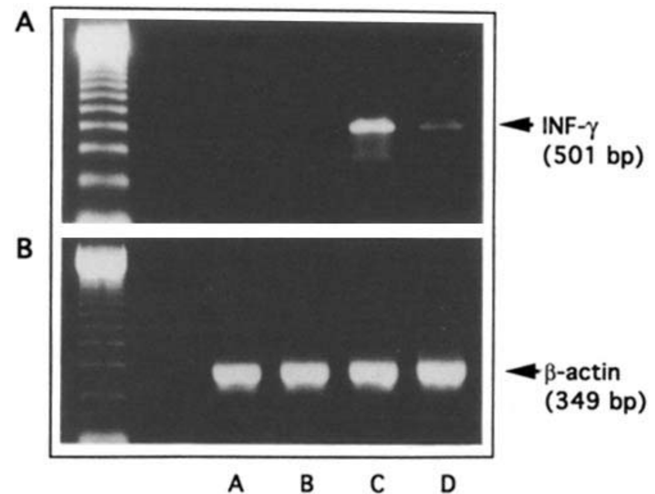

Figure 5. Detection of IFN-γ mRNA in PHA-activated human peripheral blood T cells by cDNA amplification. After prestimulation with PHA, cells were incubated on AGE-HSA or HSA coated wells for 6 h. Data are representative of three identical experiments. Lane *A*, PHA alone; lane *B*, AGE-HSA alone; lane *C*, PHA plus AGE-HSA; and lane *D*, PHA plus HSA.

stimulated T cells with unmodified HSA failed to upregulate IFN-γ mRNA, (Fig. 5, lane *D*), indicating that elevated levels were a specific response to AGE-modified protein. AGE-HSA alone did not elicit detectable levels of IFNγ mRNA (Fig. 5, lane *B*), nor did PHA alone under the conditions used (Fig. 5, lane *A*). To ensure that the PCR product generated using IFN-γ primers originated from IFN-γ mRNA, the isolated fragment was digested with the restriction enzymes BamHI and DraI. After amplification, isolation, and digestion of the full-length fragment with the respective restriction enzymes, individual fragment sizes were assessed by gel electrophoresis, which yielded fragments of the expected sizes (data not shown).

IFN-γ Secretion Is Induced by AGE-BSA in PHA-stimulated T Cells. Since induction of mRNA is not always followed by the secretion of the corresponding protein, the secretion of IFN-γ protein in PHA-activated human T cells in response to AGE-HSA was determined. As shown in Fig. 6, a significant dose-dependent release of IFN-γ into the medium occurred when cells stimulated with PHA for 48 h were exposed to an AGE-HSA-coated surface for 6 h. PHA stimulation alone or PHA followed by the addition of unmodified HSA failed to elicit this response.

Discussion

The results presented in this report indicate that human and rat peripheral blood T lymphocytes express surface binding sites specific for molecules modified by AGEs. The evidence is severalfold. Resting T lymphocytes from both species bind the in vitro–generated ligand AGE-albumin in a saturable fashion, with a binding affinity (K_a) of $7.7 \times 10^7\ M^{-1}$ at resting state, which rises to $5.8 \times 10^8\ M^{-1}$ after stimulation with PHA. binding of AGEs by intact T cells is specific for the AGE moiety, since an AGE-modified protein distinct from albumin, AGE-RNase, competed for binding whereas unmodified albumin or albumin modified by other agents (e.g., formaldehyde) did not. Radiolabeled AGE-ligand binding was inhibited by polyclonal Abs, raised against two AGE-receptor peptides isolated from rat liver, p60, and p90 kD (8), and FACS® analysis confirmed the presence of two corresponding cell surface epitopes, distributed primarily among cD4$^+$ and CD8$^+$ cells. Ligand blotting studies on membrane proteins from resting and stimulated T cells confirmed the presence of at least one major AGE-Binding membrane protein with an estimated molecular mass of 55–60 kD.

Although by ligand blotting no AGE-binding band corresponding to 90 kD was observed, the presence of a second binding protein on the cell surface could be inferred from the ligand-binding inhibition experiments using the anti-p90 Ab, in conjunction with the flow cytometry data which indicated a lack of responsiveness to PHA-stimulation compared with that obtained with the anti-p60 Ab. A similar discrepancy in binding activity between intact cells and detergent-processed, nitrocellulose-immobilized membranes was observed previously by the corresponding 90-kD AGE-binding protein isolated from rat liver membranes (8). This protein, although active on intact cells, consistently lost AGE-binding activity once transferred to nitrocellulose (8). From the available evidence, it is tempting to speculate that, in a manner analogous to the monocytes/macrophage system (8), only the 55-kD protein is capable of binding AGEs, whereas an additional one or more membrane structures may be important for the stabilization of the ligand–55 kD interaction on the cell surface. This is supported by the ability of the anti-p90 Ab to inhibit this interaction.

Binding studies showed that prestimulation of resting T cells with PHA significantly upregulated both binding affinity and the number of AGE binding sites per cell. This was further evidenced by FACS® analysis and radioligand blotting using different lymphocyte activators. The enhanced AGE-receptor activity, when evaluated flow cytometrically appeared attributable mostly to the increased expression of the AGE-binding protein that was recognized by the anti-p60 Ab, since the putative membrane protein recognized by the anti-p90 Ab remained unchanged upon stimulation. The actual composition and the dynamic nature of the cooperation of the AGE-receptors on T cells remains conjectural until approaches are used that allow the determination of the size of native receptors without the denaturing conditions employed here.

Binding of AGEs to the T cell surface is not followed by internalization or degradation of the bound ligand to a discernible degree. However, once activated by an independent stimulant, binding markedly enhances synthesis and release of IFN-γ, a response not achieved even when using maximal doses of PHA alone. In this regard, the function of this TCR system is distinct from that of the AGE-receptors present on

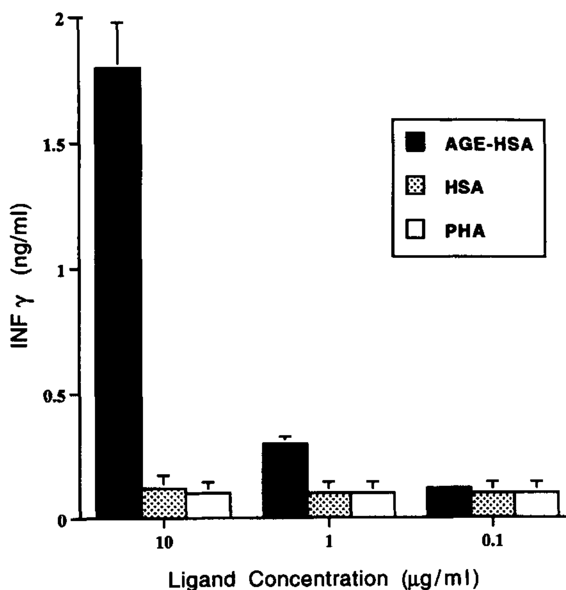

Figure 6. Secretion of IFN-γ into medium by human peripheral PHA-activated T cells. After prestimulation with PHA, cells were incubated on AGE-HSA (*solid bars*) or HSA-coated wells (*dotted bars*) for 24 h, as described. IFN-γ concentration was determined in the supernatants by ELISA. (*Open bars*) Cells exposed to PHA alone. Data are expressed as ng/ml of IFN-γ and represent the mean ± SEM of triplicate determinations.

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monocyte/macrophages (8), an important function of which is the endocytosis and destruction of AGE-modified molecules and cells (4, 5, 30). Previous studies have demonstrated that AGEs can induce macrophage activation, followed by cytokine (TNF-α, IL-1β) (12) and growth factor (PDGF, IGF-IA) release (9, 11) possibly contributing to the growth-promoting pattern involved in the turnover of extracellular matrix. In contrast, AGEs alone could not prompt T cell activation and lymphokine production, suggesting that, not unlike other TCRs, including the two subunits of the IL-2 receptor (31), AGE-receptors are constitutively expressed, but are not functional until the T cells are activated. In order for AGE-ligands to induce an increase in the message for, as well as the secretion of IFN-γ T cells required a 48-h prestimulation. The inability of AGE-HSA to prompt lymphokine release in the absence of a primary stimulus suggests an inducible system subject to regulation by other lymphocyte-specific antigens or activators. The possibility that the release of IFN-γ is due to a specific subset of cells, e.g., NK cells cannot be ruled out from these studies, although this is unlikely given the magnitude of the response obtained from preparations containing >95% T cells.

The observation that T cells, like macrophages have receptors for AGE-modified proteins suggests a mechanism for communication and cooperation between these two cell systems during tissue homeostasis and repair. The precise steps toward this interaction remain largely obscure. Extracellular matrix components are known to form spontaneously AGEs at levels increasing with age and with diabetes (1–3). In both these conditions, the incidence of atherosclerosis is highly prevalent (1–3). T lymphocytes, which are frequently observed at sites of tissue inflammation and repair, are also found within chronic inflammatory/degenerative lesions such as in atherosclerotic tissues, where they are reported to be activated (19–21, 33), expressing IL-2 receptors and IFN-γ (32, 33). Although the mechanism of this activation is still unclear, an interaction with local macrophages has been suggested.

The observations presented raise the possibility that lymphocytic AGE-specific receptors may serve as anchors of T cells to extracellular matrix components. The interaction of these cells and macrophages with AGE-modified structures could elicit a complex cytokine response by both cell types which could influence mesenchymal cell functions such as cell proliferation, and clearance or resynthesis of modified structural proteins. In this regard, through lymphokine secretion, which can regulate gene expression of such systems as endothelial, and smooth muscle cells (34, 35), T cells are shown to be capable of controlling important vessel wall functions.

In summary, the identification of an inducible AGE-specific receptor system on human T cells introduces a novel mechanism for the cooperative interaction between tissue macrophages and lymphocytes during tissue homeostasis or repair. Under conditions of excessive AGE-protein/lipid accumulation (e.g., in aging or chronic diabetes), this orderly system may be disturbed so that inappropriate lymphokine activity, in synergy with macrophage-derived cytokine activity, could lead to tissue injury. The nature of these complex interactions warrants further investigation.

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Address correspondence to Dr. Helen Vlassara, The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030.

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