LEISHMANIA PROMASTIGOTES ARE RECOGNIZED BY THE MACROPHAGE RECEPTOR FOR ADVANCED GLYCOSYLATION ENDPRODUCTS

By DAVID M. MOSSER, HELEN VLASSARA, PAUL J. EDELSON, and ANTHONY CERAMI

From the Department of Pediatrics, Division of Infectious Diseases and Immunology, New York Hospital and Cornell University Medical College, New York 10021; and the Laboratory of Medical Biochemistry, The Rockefeller University, New York 10021

The various organisms responsible for visceral, cutaneous, and mucocutaneous leishmaniasis have evolved characteristically the strategy of evading the host immune system by invading reticuloendothelial cells. Their ability to find safety in phagocytic cells prompts many intriguing questions. Foremost among these are how they are taken up by these cells and how they avoid being killed. Recent work on the uptake question points to the complement receptor for C3bi, CR3, as a major mechanism for the uptake of leishmania promastigotes (1, 2). A monoclonal antibody (anti-Mac-1) that is specific to CR3 can block uptake by >50%. However, this inhibition cannot be increased by the addition of more antibody. The inability of this monoclonal antibody to further inhibit binding suggests that an additional mechanism for uptake may be operating.

A novel receptor has recently been noted in macrophages that binds selectively to proteins containing covalently attached advanced glycosylation endproducts (AGE)[1] (3, 4). These AGEs arise from the time-dependent nonenzymatic reaction of glucose with proteins (5). The precise chemical identity of only one of this family of AGEs has been established (6). In this case, two glucose molecules and two protein amino groups condense to form the fluorescent yellow pigment 2-furoyl-4(5)-(2-1-*H*-furanyl)-imidazole (FFI). The receptor on macrophages that binds the AGE moieties is distinct from other scavenger receptors (7) and may play a role in the removal of senescent proteins and cells. Reasoning that parasites of the macrophage may wish to gain entry via such a scavenger pathway, we have evaluated the role of the AGE-protein receptor in the binding and uptake of leishmania. In the present communication, we report that binding of promastigotes of *Leishmania major* is inhibited by 50% by the addition of the ligand AGE-albumin (AGE-BSA). This inhibition is additive with inhibition of the macrophage CR3 by anti-Mac-1, and in combination, anti-Mac-1 and AGE-BSA specifically inhibit leishmania uptake by 90%.

Address correspondence to D. M. Mosser.

[1] *Abbreviations used in this paper:* AGE, advanced glycosylated endproducts; FFI, 2-furoyl-4(5)-(2-1-*H* furanyl)-imidazole.

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Materials and Methods

Macrophages. Resident macrophages were obtained from the peritoneal cavity of BALB/c mice (Rockefeller University LARC) as described previously (8). The adherent cells were incubated in DMEM supplemented with 10% heat inactivated FCS for 1 h, washed, and incubated overnight at 37°C. Before the addition of parasites, cells were washed two times with phagocytosis buffer that contained 0.25% BSA and equal parts of DMEM and TC-199 buffered with 25 mM Hepes buffer, pH 7.2.

Parasites. The National Institutes of Health S-strain of *Leishmania major*, originally isolated from a patient with a cutaneous ulcer in West Africa, was kindly provided by Dr. David Wyler, Tufts University Medical Center, Boston, MA (9). Promastigotes were grown in Schneider's Complete Drosophila Medium as described previously (10).

Parasite Binding Assay. For the binding assay, promastigotes were radiolabeled with [^{3}H]uracil as previously described (10). Briefly, cultivated promastigotes, at a cell density of ~4 × 10^7 parasites/ml, were washed and resuspended in 5 ml Schneider's Complete Medium (10) containing 30 μCi[^{3}H]uracil (sp act 26.8 Ci/mmol; New England Nuclear, Boston, MA) for 4 h. Parasites were washed three times and resuspended to a concentration of 2 × 10^7 parasites/ml in phagocytosis buffer. 50 μl of labeled parasites were added to monolayers of macrophages that had been seeded on 13-mm coverslips in multiwell plates, in the presence of the designated inhibitors, and the overlay was brought to 0.4 ml with phagocytosis buffer. Parallel binding assays were also quantitated at the light level to allow calculation of binding on a per-cell basis, as described (10).

Inhibitors. Anti-Mac-1, a monoclonal antibody to the CR3, initially described by Springer et al. (11), was precipitated from culture supernatants by ammonium sulfate and purified on DEAE-cellulose. It was used at a final concentration of 7 μg/ml as described previously (1). AGE-BSA was made by incubating BSA in 50 mM glucose in 0.1 M PBS, pH 7.4 at 37°C for 3–4 wk in the presence of protease inhibitors and antibiotics, as described (3). FFI-BSA was prepared as described previously (3).

Cytochrome C Reduction Assay. Macrophage superoxide production was quantitated by measuring the reduction of extracellular cytochrome C as described by Johnston (12). Monolayers were incubated in HBSS containing 4 mg/ml glucose and 80 μM cytochrome C (Sigma Chemical Co., St. Louis, MO). Parasites in HBSS were added for 30 min at 37°C, at which time the cytochrome-containing overlay was removed and centrifuged to remove free parasites. The absorbance at 550 nm was determined against an appropriate cell-free blank. Cell-free wells containing parasites alone or inhibitors alone were run in parallel as controls. Monolayers were also exposed to parasites in the presence of 150 U superoxide dismutase (Sigma Chemical Co.).

Results and Discussion

Incubation of *L. major* promastigotes with macrophages in serum-free medium leads to parasite binding and uptake. After 45 min, at a parasite inoculum of 2.5 × 10^6 parasites/ml, ~55% of the 10^5 macrophages present on 13-mm-diam round coverslips have bound at least one parasite, with a total of 1.5 × 10^5 parasites being bound on average per coverslip. ~85% of the attached parasites are internalized during this period. Preincubation of macrophage cultures for 20 min with AGE-BSA decreases, in a concentration-dependent manner, the total number of parasites bound (Fig. 1) and the number of macrophages binding at least one parasite. Addition of underivatized BSA (1 mg/ml) to cultures did not interfere with binding or uptake. With this particular preparation of AGE-BSA, maximal inhibition occurred at 125 μg/ml, decreasing the total number of parasites taken up per monolayer by 60.4 ± 5.2%. The average number of parasites bound decreases in the presence of AGE-BSA from 1.65 parasites per macrophage to 0.79 (Table I), while the percentage of cells containing at least one parasite decreases from 55 to 36%. Other AGE-BSA preparations inhibited

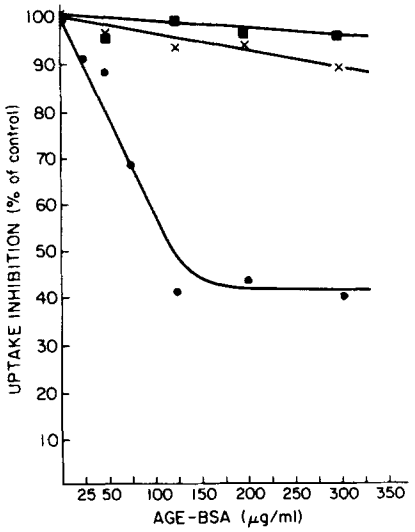

FIGURE 1. The inhibition of promastigote binding to macrophages by AGE-BSA. Increasing concentrations of AGE-BSA were added to macrophages for 20 min before, and during the addition of target particles. The percent inhibition is calculated by dividing the number of particles bound per macrophage in the presence of inhibitors by the number bound without inhibitors and subtracting that value from 1. Target particles included *L. major* promastigotes (*circles*), zymosan (×), or C3bi–RBCs (*squares*). Zymosan and C3bi-RBC binding were scored microscopically, while *L. major* binding was quantitated with the parasite radiobinding assay as described in Materials and Methods.

TABLE I

Effects of Glycosylated BSA Derivatives on Binding of L. major Promastigotes to Macrophages and their Stimulation of the Respiratory Burst

Stimulus	Treatment*	*n*	Particles/ macrophage‡	Reduced oxygen (nmol/mg)§
L. major	None	5	1.65	139.2 ± 6.0
	AGE-BSA	2	0.79	73.8 ± 14.4
	Anti-Mac-1	3	0.70	75.0 ± 9.0
	AGE-BSA + anti-Mac-1	3	0.19	24.1 ± 6.2
	FFI-BSA	2	1.77	144.0 ± 18.0
	BSA	2	1.56	128.5 ± 14.4
C3bi-RBC	None	2	(+)‖	10.5 ± 2.2
	AGE-BSA	2	(+)	17.5 ± 4.8
Zymosan	None	2	(+)¶	242.6 ± 19.5
	AGE-BSA	2	(+)	231.0 ± 22.4

* AGE-BSA, FFI-BSA, and native BSA were each used at a final concentration of 125 μg/ml. Anti-Mac-1 was used at a final concentration of 7 μg/ml.

‡ The number of particles/cell determined by the radiobinding assay for parasites and by light microscopy for C3bi-RBCs and zymosan. The variance in the radiobinding assay is ±15%.

§ The respiratory burst was quantitated by measuring the reduction of cytochrome C as described in Materials and Methods. It is expressed in nanomoles of reduced O_2 per milligram of protein.

‖ 74% of the macrophages formed rosettes with three or more red blood cells.

¶ 87% of the cells had bound three or more zymosan particles.

uptake to approximately the same extent but exerted a maximal effect with different amounts of material, ranging from 80 to 300 μg/ml. This presumably reflects the presence of differing amounts of particular AGEs in the AGE-BSA preparation. The inhibition of parasite binding was most dramatic when macrophages were first pretreated with AGE-BSA and the parasites were added in the continuous presence of the inhibitor. The data presented in Table I and Fig. 1 were obtained under this condition. Macrophage pretreatment for 20 min, with subsequent inhibitor, washout before the addition of parasites also resulted in inhibition of parasite binding. Pretreated monolayers bound from 64 to 72% of the amount of organisms bound to parallel untreated monolayers (two independent experiments done in triplicate). This is an inhibition of 28–36%, which is approximately half the inhibition (60%) seen when parasites are added to monolayers in the continuous presence of AGE-BSA. Fig. 1 shows a concentration-dependent inhibition by AGE-BSA ranging from 25 to 300 μg/ml. Incubation of monolayers with AGE-BSA at 300 μg/ml caused no evidence of cell toxicity. Monolayers retained the same number of cells (in the range of $1.0–1.2 \times 10^5$ cells/coverslip) with unchanged morphology. The protein content of untreated monolayers (20.6 μg) was comparable to BSA-treated monolayers (23.2 μg) and those treated with AGE-BSA (21.4 μg). Further, the inhibition of macrophage uptake of leishmania promastigotes by AGE-BSA was specific. The binding and uptake of either C3bi-coated red blood cells or zymosan by macrophages was not affected by preincubation with AGE-BSA (Fig. 1). After a 45-min incubation, 87% of the macrophages in a monolayer were positive for zymosan uptake, scored positive when a cell had internalized three or more particles, and 74% of the cells had formed rosettes with C3bi–red blood cells. Preincubation of parallel monolayers with AGE-BSA did not affect their recognition, with 83% of the cells in the monolayer continuing to efficiently take up zymosan, and 78% of the cells rosetting C3bi–red blood cells. The monoclonal antibody to the macrophage CR3, anti-Mac-1, inhibits the rosetting of the same preparation of C3bi-RBC by 75%, while AGE-BSA does not. In addition to demonstrating the specificity of AGE-BSA inhibition, these data emphasize the distinction between the C3bi receptor and the AGE-protein receptor. The inhibition of parasite binding by AGE-BSA works at the level of the macrophage, as promastigotes that were preincubated with AGE-BSA (100 μg/ml) for 30 min and then washed before incubation with macrophages bound to the same extent as control parasites.

Preincubation of macrophages with BSA linked to the specific AGE product, FFI, did not reduce uptake (Table I). This latter result is surprising, since FFI-BSA has been observed (4) to inhibit the binding and uptake of AGE-BSA by macrophages. Since the AGE products are a complex family of molecules, this result may reflect increased binding of a subset of AGE-products that are able to compete with leishmania, or it may reflect a subset of receptors for which AGE and leishmania can compete but FFI can not.

Preincubation of macrophages with AGE-BSA and anti-Mac-1, a monoclonal antibody to the CR3, inhibits the uptake of promastigotes by macrophages by 88% (Table I). The combined effect of these two agents is specific, as the uptake of latex beads or IgG-coated red blood cells proceeds uninhibited in their presence (data not shown).

Previous workers (13, 14) have demonstrated a macrophage respiratory burst triggered by the uptake of leishmania promastigotes. Addition of either AGE-BSA or anti-Mac-1 to the monolayer reduced the amount of parasite binding by ~50%, with a concomitant reduction in the macrophage respiratory burst. In combination, these two agents reduced the burst by 80% (Table I). Incubation of the monolayers with either unconjugated BSA or FFI-BSA did not affect the respiratory burst, arguing against a nonspecific quench. Further, the burst triggered by zymosan (242.6 nmol of O_2 per milligram protein) is not affected by the presence of 125 μg/ml AGE-BSA (231.0 nmol of O_2 per milligram protein). The presence of superoxide dismutase (150 U) decreases by ~90% the respiratory burst triggered by leishmania ($n = 3$), equivalent to an average of 10.2 nmol O_2 per milligram protein. Parasites alone cause only a very slight reduction of cytochrome C, (equivalent to 14.2 ± 2.7 nmol O_2 per milligram protein; $n = 5$), as did parasites added to glutaraldehyde-treated macrophages. Neither exceeded 15% of simultaneous values for stimulated cells. Thus, parasites entering via either the AGE receptor or the receptor for C3bi can trigger the respiratory burst.

Further work is needed to ascertain the role of both these uptake systems in different phagocytes and on the fate of various leishmania organisms. These future studies may provide insight into specific cellular tropisms characteristic of a given species, and to the pathological sequelae associated with leishmania infection.

Summary

In this paper we demonstrate the involvement of the macrophage receptor for advanced glycosylation endproducts (AGE) in the phagocytosis of *Leishmania major* promastigotes. Blocking of this receptor with the ligand, AGE-BSA, leads to a 50% decrease in phagocytosis relative to controls, and a comparable decrease in the respiratory burst. The inhibition of phagocytosis by AGE-BSA was specific to leishmania. The binding of zymosan or C3bi-RBC and the phagocytosis of IgG-RBC or latex beads was not affected by the presence of AGE-BSA. Blocking of both the AGE receptor and CR3 decreases leishmania binding by nearly 90%, and reduces the respiratory burst by 80%, indicating that the two receptors account for the bulk of *L. tropica* promastigote recognition and uptake by the macrophage.

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