

Expression of a Mannosyl-Fucosyl Receptor for Endocytosis on Cultured Primary Macrophages and Their Hybrids

PHILIP STAHL and SIAMON GORDON

Department of Physiology and Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110; and Sir William Dunn School of Pathology, University of Oxford, Oxford, England

ABSTRACT The presence of a pinocytosis receptor, specific for mannose-fucose terminated glycoproteins, has been established on murine resident peritoneal macrophages, thioglycollate-elicited peritoneal macrophages, and macrophages derived from bone-marrow in culture. Macrophagelike cell lines (J-774 and P338.D1), a myelomonocytic cell line (427E), lymphocytes, polymorphonuclear leukocytes, and fibroblasts were negative. Binding and uptake of ^{125}I -mannose-BSA and ^{125}I - β -glucuronidase, respectively, into thioglycollate-induced peritoneal macrophages is saturable ($K_{d_{37^\circ\text{C}}} = 5.4 \times 10^{-9} \text{ M}$; $K_{\text{uptake}_{37^\circ\text{C}}} = 7 \times 10^{-7} \text{ M}$) and sugar specific. Macrophage-macrophage (rat \times mouse) hybrids prepared by fusing rat alveolar macrophages with J-774-B10 (HAT-sensitive macrophagelike cell line) express the mannose-fucose receptor. Karyotypes of the hybrids confirmed a 1:1 fusion of rat and mouse cells. The rat/mouse hybrids express a variety of rat and mouse antigens including Fc receptors. Fibroblast-macrophage hybrids and melanoma-macrophage hybrids were negative for mannose-fucose receptor activity. The expression of the mannose-fucose receptor by macrophages appears to be regulated independently of other macrophage markers.

Glycoproteins terminating in D-mannose or L-fucose, including various lysosomal enzymes, are recognized by alveolar macrophages (1, 2) and sugar-specific binding activity has been demonstrated in pulmonary macrophages from a number of mammalian species including human, rat, mouse and rabbit (3). A similar, if not identical, receptor, present on liver Kupffer cells (4–6), mediates rapid plasma clearance of injected glycoconjugates terminating in mannose or L-fucose and lysosomal glycosidases. The putative liver receptor has been isolated (7, 8). The macrophage receptor appears to have as a primary function the efficient uptake of mannose/L-fucose glycoconjugates. The rate of uptake is time and temperature dependent and saturates with increasing ligand concentration. Evidence to date suggests that the receptor is reused after internalization probably by being recycled back to the plasma membrane (3).

The cells of the mononuclear phagocytic system have a common origin in bone marrow but vary in their expression of functional traits. In this paper, the presence of mannose-fucose receptor activity is demonstrated on a variety of primary macrophages including macrophages derived in culture of mouse bone marrow and peritoneal macrophages. Several macrophage cell lines were found to be negative for binding and

uptake activity. Hybrids, prepared by fusing rat alveolar macrophages with a HAT-sensitive mouse macrophagelike cell line, J774-B10, were found to express the mannose-fucose receptor.

MATERIALS AND METHODS

Materials

Mannose-bovine serum albumin (BSA), L-fucose-BSA, and D-galactose-BSA were prepared and kindly donated by Y. C. Lee (9) (Johns Hopkins University, Baltimore, MD). The conjugates contained 33–37 moles of sugar per mole of protein. β -Glucuronidase was purified from rat preputial glands (10). Mannan, HEPES, and PIPES were from Sigma Chemical Co., St. Louis, MO. Na ^{125}I was purchased from Amersham Ltd. Dulbecco's modification of Eagles' minimal essential medium (DME) and fetal bovine serum (FBS) were obtained from Gibco-Biocult, Paisley, Scotland. Serum was heat-inactivated ($56^\circ\text{C}/30 \text{ min}$) before use. Kanamycin, 100 $\mu\text{g}/\text{ml}$, streptomycin, 50 $\mu\text{g}/\text{ml}$ and penicillin, 50 $\mu\text{g}/\text{ml}$, were added to all media. Phosphate buffered saline (PBS) (Dulbecco's) was obtained from Oxoid Ltd., Basingstoke, England. Pathology-Oxford (PO) mice were bred and housed at the Dunn School of Pathology.

Cells

Thioglycollate-elicited mouse peritoneal macrophages were obtained from mice 4–5 d after intraperitoneal injection of thioglycollate broth. Cells were

plated in DME plus 10% FBS. After 90 min at 37°C, the cultures were washed to remove unattached cells. Bone marrow-derived macrophages were cultured on gelatin-coated flasks in the presence of L-cell conditioned medium as described by Lin and Gordon (11). After 5–7 d in culture, the flasks were washed with PBS, trypsinized, and the cells were reseeded into Linbro multi-well plates (2 cm² surface area per well) in DME + 10% FBS supplemented with 10% L-cell conditioned medium. Rat bone marrow-derived macrophages were prepared in the same way except with 20% L-cell conditioned medium.

J-774-B10 was grown in DME plus 10% FBS. Cells were passaged by shaking nonadherent or poorly adherent cells from the flask and sub-culturing. J-774-B10 is HAT-sensitive and was isolated by thioguanine selection (10 µg/ml) without mutagenesis. (S. Gordon, unpublished observation). Rat alveolar macrophages were obtained by pulmonary lavage as described by Stahl et al. (1). The other cell lines used were obtained from frozen stocks at the Sir William Dunn School of Pathology (12).

Hybrids

Rat alveolar macrophage × J-774-B10 hybrids were prepared by plating 2 × 10⁵ J-774-B10 cells in Linbro multi-well plates. After overnight incubation, 2 × 10⁵ rat alveolar macrophages were added in a total volume of 0.5 ml/well. After 60 min at 37°C, the cell layer was washed twice with PBS and UV-inactivated Sendai virus (15,000 HA U/ml) was added at 125 (A), 250 (B), 500 (C), and 1,000 (D) U/well in a total volume of 0.2 ml Iscove's medium without serum. Heterokaryons were observed with all concentrations of virus. After 45-min incubation at 37°C, the virus was removed and 1 ml DME plus 10% FBS was added per well. After overnight incubation, half the media was gently removed and 0.5 ml DME plus 20% FBS containing double concentrated hypoxanthine, aminopterin, thymidine (HAT) medium was added. After several weeks in culture, cells were removed by lidocaine treatment and expanded in T-25 flasks. Hybrids were obtained from all wells and were cloned in 0.33% agar. Mouse bone marrow × J-774-B10 hybrids were prepared with 7-d cultured bone marrow cells. The latter were removed by trypsinization. The cells were washed once in glucose-free Hank's buffered salt solution (HBSS) and held on ice. J-774-B10 cells, shaken from the plastic flask in which they were growing, were resuspended in glucose-free HBSS. 2.3 × 10⁶ bone marrow-derived macrophages and the same number of J-774-B10 cells were mixed, centrifuged, and resuspended in 0.5 ml glucose-free HBSS to which was added 0.5 ml HBSS containing 5,000 HA units of UV inactivated Sendai virus. After a 30-min incubation at 4°C, the mixture was warmed to 37°C for 30 min. The reaction was quenched by the addition of 15 ml DME plus 20% FBS plus HAT. Cells were then plated in plastic wells or T-flasks in the same medium with or without 10% L-cell conditioned medium. After same period in culture, the cells were removed by Lidocaine treatment and studied as clones or as mass cultures. Both RAM × J-774-B10 and bone marrow × J-774-B10 were subsequently cultured on gelatin coated flasks as described by Lin and Gordon (11). Other hybrids, D2.6 and B82 × B10, were prepared by similar methods (S. Gordon, unpublished observation). Hybrids growing on gelatin-coated flasks were trypsinized and passaged or plated into multi-well plates (without gelatin) for binding, uptake, and degradation experiments after overnight culture in DMEM plus 10% FBS.

Binding, Uptake, and Degradation Assay

Mannose-BSA and β-glucuronidase were iodinated by the chloramine T method as described by Stahl et al. (1, 3). Binding studies were performed in DMEM plus 10% FBS buffered with 20 mM PIPES and 20 mM HEPES adjusted to pH 7.0. Cell layers (5 × 10⁵ – 2 × 10⁶ cells/well) were first washed with PBS, cooled to 4°C, and incubated for 90 min with ligand in a total volume of 0.5 ml in the absence or presence of yeast mannan (2 mg/ml). After the 4°C incubation, the medium was removed and the cells were washed four times with Ca⁺⁺/Mg⁺⁺ containing PBS. The cells were then taken up into 1.0 ml of Emulphogene (0.25%) for counting and for protein determination using a dye binding assay (Bio-Rad Laboratories, Richmond, CA). Uptake was performed in 0.4 ml DME + 10% FBS or Iscove's medium plus 10% FBS. Uptake was terminated and cells were extracted exactly as for binding experiments. For degradation, ¹²⁵I-mannose-BSA was added directly to the culture medium and samples were taken at the indicated times. Acid soluble-radioactivity was determined by 10% trichloroacetic acid precipitation.

Antigen Assays

Mouse and rat macrophage plasma membrane antigens were detected in trace indirect binding assays (IBA) by the method of Austyn and Gordon (12) with live adherent cells as targets. Saturating amounts of monoclonal rat or mouse antimacrophage antibodies were used with ¹²⁵I-rabbit Fab₂ or anti-mouse Fab as second antibody. The mouse anti-rat monoclonal antibodies OX-1 (leukocyte common antigen [13]), 29–18 (rat transplantation antigen [A. F. Williams, unpublished observations]), W3/13 and W3/25 (14) were obtained as culture

supernatants from Drs. N. Barclay and A. F. Williams Medical Research Council Immunology Unit, Oxford, England. The rat monoclonal antibody F4/80 which is specific for mature mouse macrophages was prepared by Dr. J. Austyn in our laboratory (12).

Fc receptor activity was assayed with sheep erythrocytes (E) coated with hyperimmune rabbit antibody (E1gG) (12). Mouse Fc receptor antigen was detected by IBA using the IgG or Fab fragment of the rat monoclonal antibody 2.4G2, a gift of Dr. J. C. Unkeless, Rockefeller University, New York, who also supplied a rabbit antiserum against mouse FcR.

RESULTS

Expression of the Mannose-Fucose Receptor on Primary Macrophages and Macrophagelike Cell Lines

PRIMARY CELLS: The presence of the mannose-fucose receptor (MFR) on primary macrophages and macrophagelike cell lines was tested using two ligands. ¹²⁵I-Mannose-BSA was used for binding and degradation studies whereas ¹²⁵I-β-glucuronidase was used to follow uptake. Binding was determined at 4°C where uptake is essentially blocked; uptake was measured at 37°C. Previous studies have shown that ¹²⁵I-mannose-BSA is subject to rapid degradation after uptake into lysosomes (3). In the present study, measurement of acid-soluble fragments which appear in the media after uptake and digestion of ¹²⁵I-mannose-BSA provided a very sensitive assay for a functional mannose glycoprotein pinocytosis receptor. β-Glucuronidase, a ligand that is more stable to lysosomal digestion and which, therefore, has an extended intracellular lifetime, was the ligand of choice for uptake studies. In all the assays used in this study, the nonspecific component was estimated by adding yeast mannan, a competitive inhibitor of ligand uptake, to companion cultures. Uptake in the presence of yeast mannan is nonspecific, linear, with time and nonsaturable. The binding, uptake, and degradation of ¹²⁵I-mannose-BSA, ¹²⁵I-β-glucuronidase and ¹²⁵I-mannose-BSA, respectively, by thioglycollate-elicited peritoneal macrophages is shown in Fig. 1. Cells were harvested by standard procedures and plated in Linbro multi-well plates at 2 × 10⁶ cells/well (50–70% of total cells were macrophages) in DME plus 10% heat-inactivated FBS. Incubation of ligand at 37°C in the absence of cells with medium or with macrophage conditioned medium resulted in no change in the precipitability of ¹²⁵I-mannose-BSA. Moreover, the addition of mannan to the culture media nearly completely suppressed the cellular uptake and degradation of ¹²⁵I-mannose-BSA. Mannan alone had no effect on cell viability even after 48 h in culture. The degradation of ¹²⁵I-mannose-BSA proceeds very rapidly and, consequently, served as a convenient, nondestructive assay of mannose-fucose receptor activity. The presence of FCS had no effect on ligand uptake and degradation whereas horse serum was slightly inhibitory.

Binding and degradation of ¹²⁵I-mannose-BSA were examined in bone marrow derived macrophages prepared using L-cell conditioned medium as a source of colony stimulating factor. 5- to 8-day cultures (11) growing on gelatin were trypsinized and plated into Linbro wells. After overnight incubation in the presence of L-cell conditioned medium, ¹²⁵I-mannose-BSA binding was measured exactly as described for peritoneal macrophages. The results were qualitatively and quantitatively similar to binding by the peritoneal cells. ¹²⁵I-Mannose-BSA was likewise rapidly digested by the bone marrow-derived macrophages. Polymorphonuclear leukocytes and lymphocytes were negative for mannose-BSA degradation.

SUGAR SPECIFICITY OF LIGAND UPTAKE INTO PRIMARY CELLS: The rat mannose-fucose receptor recog-

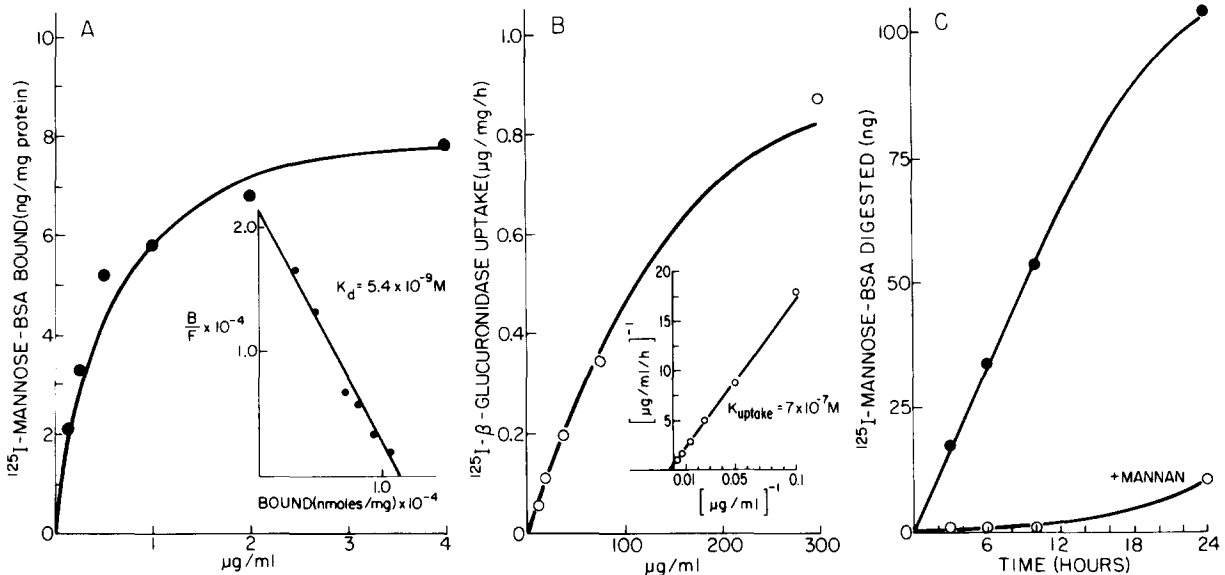


FIGURE 1 Binding, uptake, and degradation of mannose-terminated glycoproteins by thioglycollate-elicited peritoneal macrophages. Cells were harvested as described in Materials and Methods and plated in 2-cm² Linbro multi-well plates at $1.5\text{--}2.0 \times 10^6$ cells/well. Binding (A) was determined on ice (4°C) in 0.5 ml DME containing 0.02 M HEPES and 0.02 M PIPES, pH 7, and supplemented with 10% heat-inactivated FBS. ¹²⁵I-mannose-BSA ($1\text{--}5 \times 10^6$ cpm/ μ g) in medium was added to initiate the binding reaction. At 90 min, the medium was removed and the cell layer was washed 3–5 times with Dulbecco's phosphate saline. Cells were then taken up into 1.0 ml 0.25% Emulphogene. Protein was measured by a dye binding assay (Bio-Rad). Nonspecific binding (i.e., binding in the presence of mannan, 2 mg/ml) was always <20% of total binding and was subtracted from the total. A Scatchard plot (inset) was constructed from the specific binding data ($K_d = 5.4 \times 10^{-9}$ M). Uptake (B) of ¹²⁵I- β -glucuronidase (1×10^6 cpm/ μ g) was measured the same as for binding except in a total volume of 0.4 ml. Iscove's medium containing 10% heat-inactivated FBS. Uptake was measured after 60 min at 37°C. K_{uptake} was estimated at 7×10^{-7} M. Degradation of ¹²⁵I-mannose-BSA (C) was determined by incubating cells in 1.0 ml DME plus 10% heat-activated FBS containing ¹²⁵I-mannose-BSA (0.25 μ g/ml) in the presence or absence of mannan (1.25 mg/ml). Samples (100 μ l) of medium were withdrawn at the indicated times. 200 μ l of BSA (20 mg/ml) was added and protein was precipitated with 1.5 ml cold 10% trichloroacetic acid.

nizes glycoproteins terminating in mannose and fucose but not in galactose (2). To determine the specificity of the mouse uptake system, thioglycollate-induced peritoneal macrophages were prepared as described in Fig. 1 and incubated with 2.5 μ g/ml ¹²⁵I- β -glucuronidase (Fig. 2) in the absence or presence of increasing concentrations of mannose-BSA, L-fucose-BSA, or galactose-BSA. Mannose-BSA and L-fucose-BSA were found to be potent inhibitors of ¹²⁵I- β -glucuronidase uptake whereas galactose-BSA was inactive. These results demonstrate the specificity of the mouse MFR and indicate that the mouse and rat MFR have very similar sugar specificities. As shown previously (1) mannan, a mannose-rich glycoprotein, also blocks the uptake of β -glucuronidase by rat macrophages.

MACROPHAGELIKE CELL LINES AND NONMACROPHAGE CELL LINES: Several established cell lines are available which express various macrophage traits (e.g., FcR, lysozyme secretion; 12). J774-2, J774-B10 (HAT-sensitive), P388D1, and 427E (a myelo-monocytic line) were tested for uptake or degradation of ¹²⁵I-mannose-BSA. All were negative. J774-B10 was further tested for binding, uptake, and degradation—all results were negative. Attempts to induce MFR activity in J774-B10 by treatment with PMA, L-cell conditioned media, lymphokine from mouse spleen stimulated with Con A and by phagocytosis of zymosan were all unsuccessful. Non-macrophage cell lines which were negative for MFR activity were L-cells, PG19 (melanoma), and F4-80 (hybridoma).

Expression of the Mannose-Fucose Receptor by Macrophage-Macrophage Hybrids

Since the expression of MFR appeared to be restricted to primary macrophages, an attempt was made to capture MFR

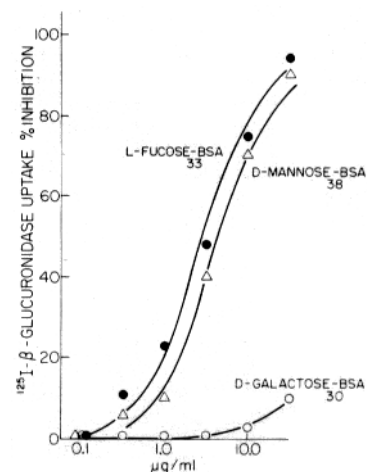


FIGURE 2 Inhibition of ¹²⁵I- β -glucuronidase uptake by neoglycoproteins. Thioglycollate-induced peritoneal macrophages were prepared as in Fig. 1. Cells were washed 3 times with PBS and incubated at 37°C with ¹²⁵I- β -glucuronidase (2.5 μ g/ml; 10^6 cpm/ μ g) for 60 min in the absence or presence of increasing concentrations of L-fucose-BSA or D-mannose-BSA. Cells were washed 3 times with PBS and solubilized in 1.0 ml 0.25% Emulphogene for counting. Results are expressed as the percent of inhibition of specific uptake by added neoglycoproteins. Specific uptake was determined by comparing uptake in the presence and absence of mannan (1.6 mg/ml).

expression in a macrophage hybrid by fusing a MFR⁺ primary cell with a MFR⁻ macrophagelike cell line. Rat alveolar macrophages, collected by pulmonary lavage (1) and J774-B10 cells, shaken from stationary cultures, were mixed in equal proportions. The mixed cells were fused with UV-inactivated

Sendai virus. The fused cells were expanded by growth in HAT-containing DME plus 10% FCS and the mixed hybrids were tested for degradation of ^{125}I -mannose-BSA. Degradation activity was found in all the wells. The cells were then cloned by dilution into 96 well plates. Colonies were picked and expanded in culture. The results in Fig. 3 show the degradation of ^{125}I -mannose-BSA by several clones. The level of expression of MFR, as reflected in the ability to digest ^{125}I -mannose-BSA varied considerably among the clones. For comparison, one parent, J774-B10, did not digest mannose-BSA at all whereas the other parent, the rat alveolar macrophage, was very active in the degradation assay. Two clones, D6-1000-B3 and D6-1000-B10 were studied in more detail. Fig. 4 shows photomicrographs of the parent rat alveolar macrophage and J774-B10 and the hybrid. The rat alveolar macrophages shown in Fig. 4 were allowed to attach briefly to glass and were photographed under phase contrast microscopy. These cells are rounded and show active plasma membrane ruffling. The cytoplasm contains numerous phase-dense and phase-lucent vesicles and mitochondria, except at the marginal zone. Some cells contain prominent refractile lipid droplets, perhaps resulting from the uptake of surfactant in the lung. Whereas the J774 parent attaches more loosely to glass or tissue culture plastic surfaces and has a rounded appearance with few endocytic vesicles, the hybrid cells are larger, often well-spread with prominent endocytic vesicles and mitochondria. The karyotype for D6-1000-B3 (Fig. 5) confirms the presence of rat and mouse chromosomes with a modal chromosome number of 100–101 for 50 cells examined (range 95–105); compatible with the fusion of $1 \times \text{J774-B10}$ (60) and $1 \times \text{RAM}$ (42).

Binding and Uptake Experiments with Macrophage Hybrids

A binding study was undertaken with the two parent cells and the hybrid (Fig. 6). The results show that RAM binds ^{125}I -

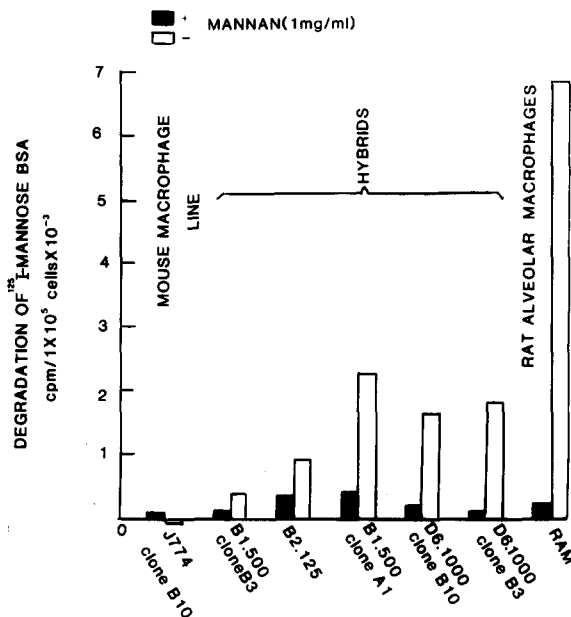


FIGURE 3 Degradation of ^{125}I -Mannose-BSA by macrophage hybrids. Rat alveolar macrophages, J-774-B10 and rat alveolar \times J-774-B10 hybrids were seeded at 1×10^6 /well in 24-well Linbro plates. Hybrids were grown in HAT-medium as described in Materials and Methods. ^{125}I -Mannose-BSA ($0.25 \mu\text{g}/\text{ml}$) was added as described in Fig. 1 and the media were sampled for determination of acid-soluble radioactivity after 24 h in culture in the presence or absence of mannan ($1 \text{ mg}/\text{ml}$).

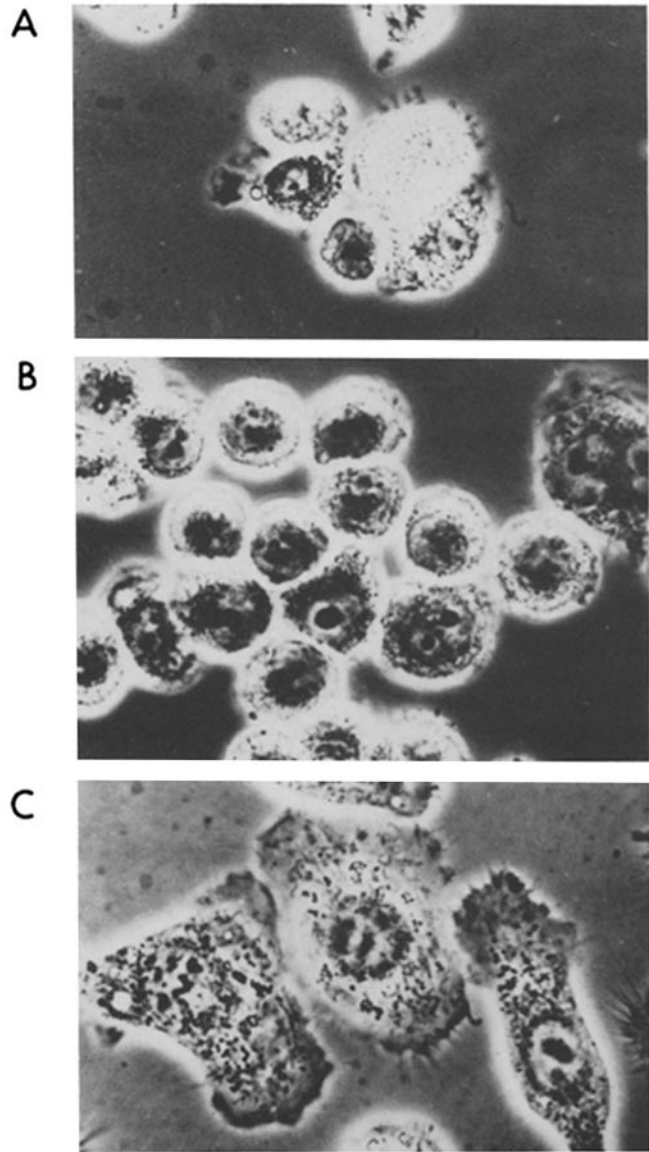


FIGURE 4 Phase-contrast photomicrographs. Photomicrographs of rat alveolar macrophages (A), J-774-B10 (B), and J-774-B10 \times rat alveolar macrophage hybrids (C).

mannose-BSA in a concentration dependent fashion as does D6-1000-B3. The RAM bound about four times more ligand on a cell protein basis than D6-1000-B3. The J774-B10 was negative for binding. Uptake of mannose-terminal glycoconjugates into D6-1000-B10 was studied with ^{125}I - β -glucuronidase. The results (Fig. 7) indicate that the receptor-mediated uptake mechanism is intact and that, on the basis of the K_{uptake} , is similar to the uptake mechanism in the mouse peritoneal macrophages or rat alveolar macrophages. ^{125}I - β -Glucuronidase uptake into the hybrid was fully inhibited by yeast mannan and L-fucose-BSA but not galactose-BSA.

Expression of Rat and Mouse Markers by Macrophage/Macrophage Hybrids

Rat alveolar macrophage \times mouse J774.B10 hybrids (D6-1000-B3) expressed several rat- and mouse-specific surface antigens when tested for binding of various monoclonal antibodies (Fig. 8). OX-1, a rat leukocyte common antigen (LC) (13) and 29-18, a determinant on the rat major transplantation

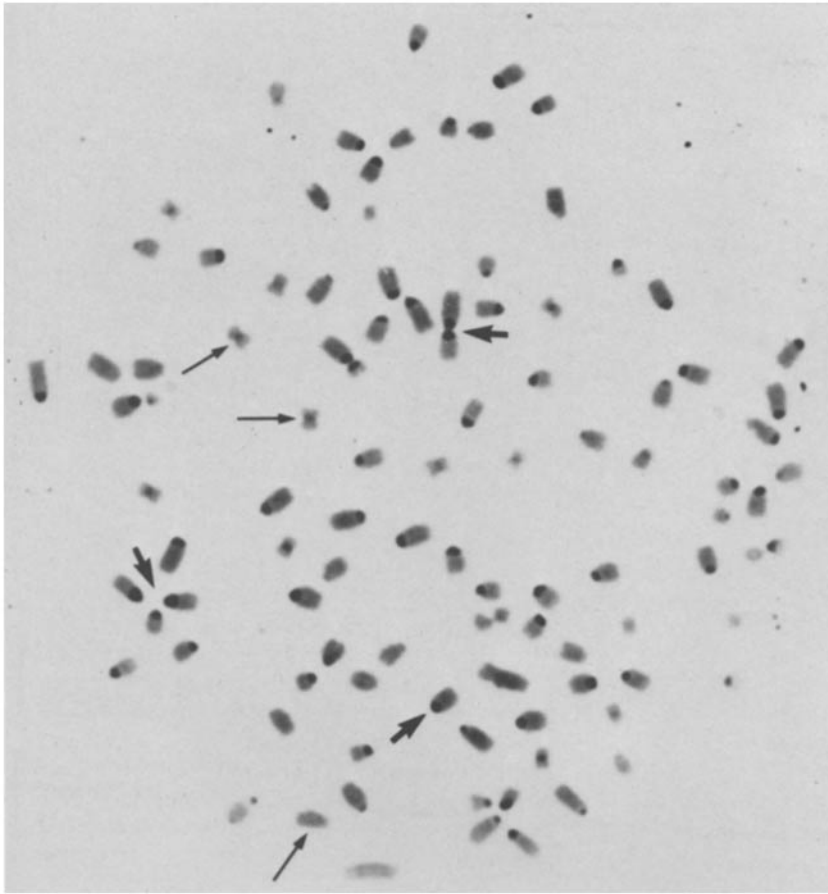


FIGURE 5 Karyotypic analysis of J-774-B10 x rat alveolar macrophage hybrid (D6-1000-B3). We are indebted to M. D. Burtenshaw and Dr. E. P. Evans for this analysis using the C-banding method (16). The thick arrows indicate typical examples of prominently C-banded mouse acrocentric chromosomes and the single-characteristic large metacentric chromosome from J774-B10, the thin arrows show examples of faintly C-banded rat chromosomes. The spread illustrated has 101 chromosomes with at least 55 mouse chromosomes; in a sample of 25 cells, 47-55 chromosomes per cell were recognized confidently as mouse chromosomes (minimal estimate).

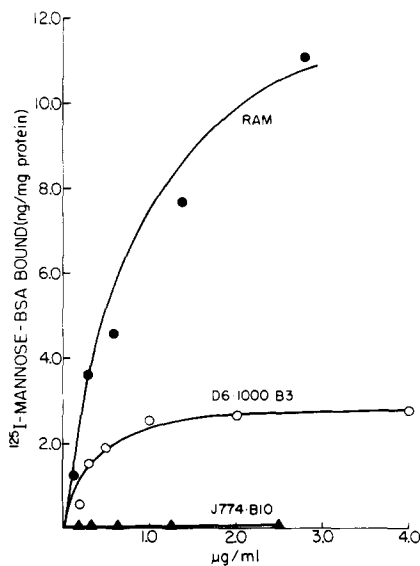


FIGURE 6 Binding of ^{125}I -Mannose-BSA to rat alveolar macrophages, D6-1000-B3 (J-774-B10 x RAM Hybrid), and J-774-B10. Binding of ^{125}I -mannose-BSA to D6-1000-B3 was carried out exactly as described in Fig. 1. ^{125}I -Mannose-BSA binding to rat alveolar macrophages and J-774-B10 cells was determined on suspended cells followed by centrifugation through silicon oil (3).

antigen RT-1 (A. F. Williams, unpublished observation) are expressed strongly on RAM and on the hybrid. The low binding of these mouse monoclonal antibodies to J774-B10 is probably via Fc receptors. W3/13 and W3/25 are rat antigens that were originally defined on lymphocytes (14). W3/13 is

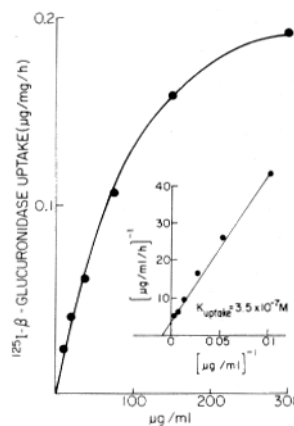


FIGURE 7 Uptake of ^{125}I - β -glucuronidase by D6-1000-B10 macrophage hybrid. Cells were seeded in Linbro wells at 5×10^5 cells/well. After 1 d in culture, uptake of ^{125}I - β -glucuronidase was determined as described in Fig. 1. Double-reciprocal plot was constructed from the specific uptake data.

expressed to a much greater extent by the macrophage hybrid than by either parent whereas low levels of W3/25 can be detected on the RAM and hybrid, but not J774-B10.

The mouse macrophage-specific antigen F4/80 (12) is expressed on J774.B10 and the hybrids, but not on RAM. The mouse Fc receptor antigen 2.4G2 (15) was detected on hybrids and on the mouse parent. Because the Fab fragment of 2.4G2 was used, binding via the Fc portion of the antibody can be excluded. Studies on Fc receptor function showed that the hybrids bind and ingest IgG-coated erythrocytes avidly and indicated that the hybrid cells express both rat and mouse Fc receptors. The rat antibody against mouse Fc receptors (2.4G2) and a rabbit anti-mouse Fc receptor antiserum blocked immune phagocytosis by the hybrid cells incompletely compared with complete inhibition or none with the mouse and rat parent cells, respectively.

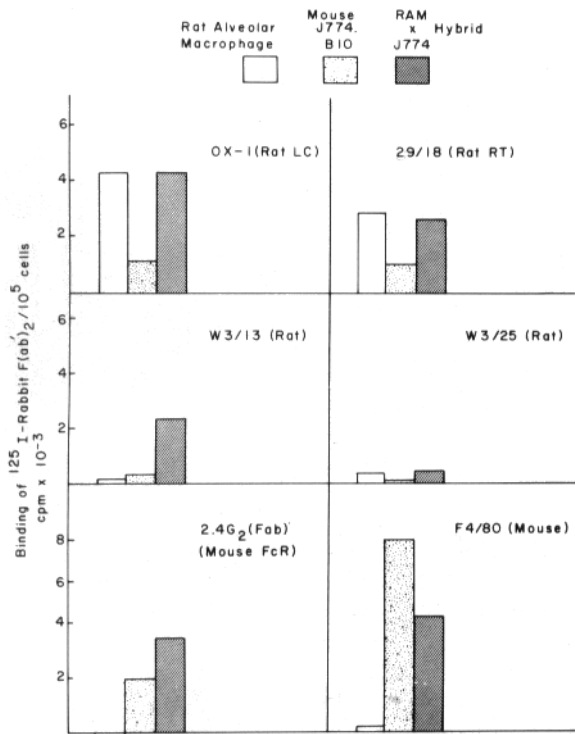


FIGURE 8 Expression of rat and mouse antigens by parental cells and rat alveolar macrophage \times J774-B10 hybrids. RAM obtained by lavage were plated in 96-well trays at 1.9×10^5 per well. J774-B10 detached by shaking and D6-1000-B3 hybrid cells by trypsinization were plated at 1×10^5 /well. Cells were cultured for 1 d before assay as described in Materials and Methods. Cells were cultured with rat or mouse monoclonal antibodies, 30 μ l/well and bound antibody was detected by 125 I-rabbit anti-rat or 125 I-rabbit antimouse antibodies, 2×10^6 cpm/well.

These studies confirmed that all the antigenic markers contributed by the parent cells were present in the hybrids and that other antigens such as W3/13 could also be induced by hybridization.

Subclones of D6-1000-B3

After several months in continuous culture, the uptake activity of D6-1000-B3 appeared to be falling off and the line was recloned in agar. A number of colonies were picked and, after expansion in culture, they were tested for specific degradation of 125 I-mannose-BSA. The results in Fig. 9 indicate the presence of widely varying activity amongst the subclones. Two (A6 and C4) having high and low activity were subjected to karyotypic analysis which indicated a shift in chromosome number from the parent hybrid. The A6 subclone had a mode of 91 chromosomes (range 85–97) and C4 subclone had a modal chromosome number of 89 (range 82–98). Subclones C4 and A6 were examined for their ability to take up 125 I- β -glucuronidase. C4 actively took up the ligand, whereas A6 was essentially negative. A6 was also negative for binding of 125 I-mannose BSA. The presence of the receptor in C3 and its near absence in A6 was further confirmed by fluorescence microscopy using a rabbit anti-rat receptor serum and a second antibody (FITC-Goat Anti-rabbit IgG) (Levy, Boshans, and Stahl, unpublished observations). This antisera was prepared by immunizing rabbits with mannose-binding protein (8) purified by affinity chromatography from rat liver. The loss of MFR activity by clone A6 was selective because both A6 and C3 continue to

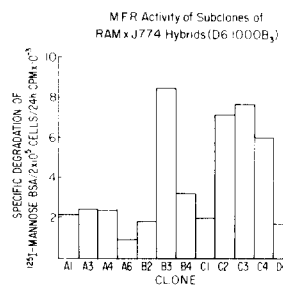


FIGURE 9 Degradation of 125 I-mannose-BSA by subclones of D6-1000-B3. D6-1000-B3 was recloned in agar as described in Materials and Methods. Colonies picked from the cloning experiment were plated in Linbro multiwell plates at 2×10^5 /well in standard media. After overnight incubation, 0.25 μ g 125 I-mannose-BSA was added to duplicate wells.

Mannan (2 mg/ml) was present along with the ligand in a second set of wells. After 24-h incubation, the acid-soluble radioactivity was estimated as described in Fig. 3.

express identical levels of the other rat antigens LC and RT 1 (not shown).

Control Hybrids

A variety of other mouse macrophage hybrids were tested for mannose receptor activity (Table I). Bone marrow-derived macrophage J774-B10 hybrids were active for uptake activity but the level of activity expressed was much less than RAM \times J774-B10 and was not increased by the inducers mentioned above (i.e., L-cell conditioned media and phorbol myristic acid). D 2.6 (peritoneal macrophage \times melanoma) was negative for uptake. B82 \times J774-B10, a control hybrid prepared by fusing a mouse fibroblast (TK⁻) line and the parent macrophage cell line, was negative for ligand uptake and degradation. All these hybrids, however, were positive for expression of Fc receptor activity and lysozyme secretion.

DISCUSSION

Binding and uptake of mannose-terminated glycoconjugates were studied in mouse thioglycollate-elicited peritoneal macrophages, bone marrow derived macrophages and several mouse macrophagelike cell lines. The results with thioglycollate-elicited peritoneal macrophages are presented in detail (Fig. 1). Binding (4°C) of 125 I-mannose-BSA was specific and saturable. A Scatchard plot of the binding data indicated the presence of a single class of binding sites which bind the ligand with high affinity ($K_d = 10^{-9}$ M). Uptake (37°C) of 125 I- β -glucuronidase was, likewise, saturable, specific and time dependent ($K_{\text{uptake}} = 7 \times 10^{-7}$ M). The sugar-specificity of the murine recognition system appears similar, if not identical, to that found in rat macrophages. Uptake (Fig. 2) is strongly inhibited by fucose-BSA and mannose-BSA but not galactose-BSA. Results from experiments with mouse bone marrow-derived macrophages and rat bone marrow-derived macrophages were essentially identical to those observed with thioglycollate-elicited peritoneal macrophages. Moreover, resident mouse peritoneal macrophages recognize and internalize mannose-BSA and β -glucuronidase (17). In sum, the results establish the presence of the MFR in a variety of mouse macrophages. Murine blood monocytes were not studied, however, human blood monocytes were observed to be negative for MFR activity. Culture of human monocytes for 4–6 days in the presence of autologous heat-inactivated human serum promoted spreading, formation of vacuoles and the appearance of MFR activity. In contrast, lymphocytes, polymorphonuclear leukocytes, fibroblasts, and several macrophage cell lines (J774-2, J774-B10 and P388-D1) were all negative for MFR activity. Although the expression of MFR is widespread among primary macrophages, the marker does appear to be regulated (17). In

TABLE I
Summary of Mannose-Receptor, F_c Receptor, and Lysozyme Secretion by Macrophages and Macrophage Hybrids

Cell	Species	Nature	MFR	FcR	Lysozyme
J774-B10 Line	Mouse	Macrophage	—	+	+
RAM macrophage	Rat	Primary	++++	+	+
J774-B10 × RAM	Mouse × rat	Hybrid	++	+	+
J774-B10 × MBM DM*	Mouse × mouse	Hybrid	+	+	+
D 2.6 Peritoneal macrophage × PG19 melanoma	Mouse × mouse	Hybrid	—	+	+
B82 (fibroblast; TK ⁻) × J774-B10	Mouse × mouse	Hybrid	—	+	+

* Mouse bone marrow derived macrophage.

peritoneal macrophages recruited by live BCG infection, for example, various antigens and receptors are differentially expressed. Ia antigen is enhanced while MFR, FcR, and antigen F4/80 are substantially reduced. Despite the fact that these markers are regulated, they served as good markers for primary macrophages since almost all cells in a population still express these receptors and antigens (17).

Expression of MFR has been captured in a macrophage/macrophage hybrid by fusion of rat alveolar macrophages with J774-B10. The rat alveolar macrophage parent was highly positive for receptor activity whereas J774-B10 was negative although it does express other macrophage markers (FcR, F4/80, and lysozyme secretion). J774-B10 had been rendered HAT-sensitive and was thereby selected against after fusion by growth in HAT medium whereas rat alveolar macrophages do not divide under these conditions. All the rat-mouse macrophage hybrids initially isolated showed MFR activity, although the level differed markedly. The presence of both rat and mouse chromosomes in an original clone of a strongly positive hybrid was established by karyotypic analysis and the total number of chromosomes indicate a 1:1 contribution by the two parents. Rat and mouse antigens and Fc receptors were also found on these hybrid cells. The rat liver mannosyl-fucosyl binding protein has been isolated and antisera raised against the purified protein was used to confirm that the hybrids express the rat MFR and not a latent mouse receptor derepressed by hybridization.

The binding and uptake of mannose glycoconjugates by receptor-positive hybrids were remarkably similar to that expressed by the primary cells. Binding and uptake were specific, saturable, and fully inhibited by mannose or fucose terminated glycoproteins. An original clone (D6-1000-B3) was recloned after some few months in continuous culture. The subclones varied substantially in activity. The most likely explanation for this is that the original rat × mouse hybrids are unstable and lose chromosomes during continuous culture. This was confirmed by karyotypic analysis of two such subclones. The loss of MFR activity by one subclone (A6) was due to loss of surface binding and was independent of other rat antigens present on the hybrids (LC and RT [rat major histocompatibility]). With more independent segregants, it may be possible to identify a chromosome required for MFR expression and to establish whether other genes involved in receptor-mediated endocytosis are lost coordinately with the MFR surface receptor.

These results indicate that factors produced by the mouse parent genome do not interfere with the ability of the hybrid to synthesize and deploy rat receptor molecules. A mouse bone marrow macrophage × J774-B10 fusion was undertaken to generate mouse × mouse hybrids. These cells were positive for mannose degradation but much less so than the RAM and J774-B10 hybrid. On the other hand, hybrids prepared without

a double macrophage input (macrophage × mouse melanoma and B82 [TK⁻ fibroblast] × J774-B10) were negative for MFR activity. Two questions arise from these results. (a) Why do J774-B10 and other lines not express MFR and (b) why, in contrast to macrophage × nonmacrophage hybrids, is there no extinction of MFR expression in the macrophage/macrophage hybrids. The lack of MFR expression by J774-B10 could be due to gene loss but this is unlikely because several other macrophage lines are also negative. These macrophage lines are relatively stable lines which express other macrophage markers such as FcR, F4/80, and secretion of lysozyme. Moreover, even in the mouse bone marrow × J774-B10 hybrids mentioned above, many (>10) isolates have very low MFR activity; it is unlikely that all have lost genes through instability. Alternatively, as macrophages are essentially nondividing cells, it could be that growth in and of itself or some growth factor causes extinction of MFR gene expression. The fact that primary bone marrow derived macrophages and J774-B10/RAM hybrids both grow and express MFR argues against this proposal. The most likely conclusion is that MFR expression is closely regulated independently of other markers on macrophages and macrophage cell lines.

Moreover, it seems likely that there may be other factors, introduced by the nonmacrophage input into macrophage × nonmacrophage hybrids which produce extinction of MFR activity. This could take the form of a critical dosage of macrophage input which is required for expression or the production of some factor by the nonmacrophage genome which suppresses MFR expression. The availability of other segregants may help to elucidate the mechanism(s) of MFR extinction in these hybrid cells.

We gratefully acknowledge the technical support by Maxine Bampton, Laurence Turley, and Chris Jenkins.

This work was supported in part by grants from the U. S. Department of Health, Education, and Welfare; the North Atlantic Treaty Organization; a Fogarty Foundation Fellowship (P. Stahl); United Kingdom Medical Research Council; and the Leukemia Society of America, Inc.

Received for publication 18 May 1981, and in revised form 9 November 1981.

REFERENCES

1. Stahl, P., J. Rodman, J. Miller, and P. Schlesinger. 1978. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates and lysosomal glycosidases by alveolar macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 75:1399-1403.
2. Shepherd, V. L., Y. C. Lee, P. H. Schlesinger, and P. D. Stahl. 1981. L-Fucose-terminated glycoconjugates are recognized by pinocytosis receptors on macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 78:1019-1022.
3. Stahl, P., P. H. Schlesinger, E. Sigardson, J. S. Rodman, and Y. C. Lee. 1980. Receptor-mediated pinocytosis of mannose-glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell*. 19:207-215.
4. Schlesinger, P. H., T. Doebber, B. Mandell, Ch. DeSchryver, J. Miller, J. Rodman, and P. Stahl. 1978. Plasma clearance of glycoproteins with terminal mannose and N-acetylglu-

- cosamine by liver non-parenchymal cells. *Biochem. J.* 176:103-111.
5. Achord, D. T., F. E. Brot, C. E. Bell, and W. S. Sly. 1978. Human β -glucuronidase: in vivo clearance and in vitro uptake by a glycoprotein recognition system on reticuloendothelial cells. *Cell* 15:269-278.
 6. Brown, T. L., L. A. Henderson, S. R. Thorpe, and J. W. Baynes. 1978. The effect of α -mannose-terminal oligosaccharides on the survival of glycoproteins in the circulation. *Arch. Biochem. Biophys.* 188:418-428.
 7. Kawasaki, T., R. Etoh, and I. Yamashita. 1978. Isolation and characterization of a mannan-binding protein from rabbit liver. *Biochem. Biophys. Res. Commun.* 81:1018-1024.
 8. Townsend, R. and P. Stahl. 1981. Isolation and characterization of a mannose/*N*-acetylglucosamine/fucose binding protein from rat liver. *Biochem. J.* 194:209-214.
 9. Lee, Y. C., C. Stowell, and M. J. Krantz. 1976. 2-imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins. *Biochemistry.* 15:3956-3962.
 10. Himeno, M., H. Ohara, Y. Arakawa, and K. Kato. 1978. β -Glucuronidase of rat preputial gland: crystallization, properties, carbohydrate composition and subunits. *J. Biochem. (Tokyo).* 77:427-438.
 11. Lin, H.-S. and S. Gordon. 1979. Secretion of plasminogen activator by bone marrow derived mononuclear phagocytes and its enhancement by colony stimulating factor. *J. Exp. Med.* 150:231-245.
 12. Austyn, J. M., and S. Gordon. 1981. Monoclonal antibodies directed specifically against the mouse macrophage. I. Production and characterization of antibody F4/80. Submitted for publication.
 13. Sunderland, C. A., W. R. McMaster, and A. F. Williams. 1979. Purification with monoclonal antibody of a predominant leukocyte common antigen and glycoprotein from rat thymocytes. *Eur. J. Immunol.* 9:155-159.
 14. Williams, A. F., G. Galfré, and C. Milstein. 1977. Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell.* 12:663-673.
 15. Unkeless, J. C. 1979. Characterization of monoclonal antibody directed against mouse macrophages and lymphocyte Fc receptors. *J. Exp. Med.* 150:580-596.
 16. Evans, E. P., C. V. Beechley, and M. D. Burtenshaw. 1978. Meiosis and fertility in XYY mice cytogenet. *Cell Genet.* 20:249-263.
 17. Ezekowitz, R. A. B., J. Austyn, P. D. Stahl, and S. Gordon. 1980. Surface properties of BCG-activated mouse macrophages. Reduced expression of mannose-specific endocytosis, Fc receptors and antigen F4/80 accompanies induction of Ia. *J. Exp. Med.* 154:60-76.