SURFACE PROPERTIES OF BACILLUS CALMETTE-GUÉRIN-ACTIVATED MOUSE MACROPHAGES

Reduced Expression of Mannose-specific Endocytosis, Fc Receptors, and Antigen F4/80 Accompanies Induction of Ia*

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After infection with bacillus Calmette-Guérin (BCG)[1] the host may acquire immunity to specific secondary challenge, protection against unrelated virulent organisms such as *Listeria monocytogenes*, and increased resistance to transplantable tumors (1–3). Macrophages from such animals spread rapidly in culture, secrete high levels of H_2O_2 (4) and plasminogen activator (PA) (5) when stimulated further, and display an enhanced capacity to kill microorganisms and tumor cells (6, 7). Although macrophages obtained from mice after intraperitoneal injection of thioglycollate broth also show increased spreading and secretion of PA compared with resident cells from untreated animals, the cells elicited by this inflammatory stimulus do not exhibit microbicidal or tumoricidal activity and do not release substantial levels of H_2O_2 (6).

Very little is known about the role of surface molecules of the BCG-activated macrophage in recognition of organisms and other cells and in control of the secretory activity of the macrophage. Recently, several specific receptors and antigens have been defined on macrophages including a lectin-like receptor that mediates endocytosis of mannose- or fucose-terminal glycoproteins (M,FR) (8), an Fc receptor (9) which binds and internalizes certain classes of immunoglobulin (FcR), and F4/80, an antigenic marker for the mature mouse macrophage.[2] In addition, macrophages can be induced to express Ia antigens by various infectious and other stimuli (10, 11). Specific ligands and monoclonal antibodies are therefore available to study the effects of cell activation on expression of these plasma membrane determinants.

We report here that intraperitoneal infection with live BCG organisms yields a population of macrophages, activated by conventional criteria, which differs strikingly in its surface properties when compared with thioglycollate-elicited and resident cells. Expression of the endocytic receptors and of F4/80 is markedly reduced in BCG-

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[1] *Abbreviations used in this paper:* BCG, bacillus Calmette-Guérin; BCG-PM, BCG-activated peritoneal macrophages; BSA, bovine serum albumin; DMEM, Dulbecco's modification of Eagle's minimal essential medium; FBS, fetal bovine serum; FcR, Fc receptor; M,FR mannose, fucosyl receptor; O_2^-, superoxide anion; PA, plasminogen activator; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; PO, Swiss Pathology Oxford; RAR, rabbit $F(ab')_2$ anti-rat Fab; RPM, resident peritoneal macrophages; TPM, thioglycollate-elicited peritoneal macrophages.

[2] Austyn, J. M., and S. Gordon. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Manuscript submitted for publication.

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activated macrophages at the same time as Ia antigen is enhanced. Cell activation, therefore, results in an apparent reversal in surface phenotype of the cultivated mouse peritoneal macrophage.

Materials and Methods

Animals. Mice were bred at the Sir William Dunn School of Pathology, University of Oxford, Oxford, and both sexes used at weights of 22–30 g. Swiss Pathology Oxford (PO) mice, CBA T_6T_6 (H-2^k), or BALB/c (H-2^d) mice were used for experiments as noted.

Media and Reagents. Dulbecco's modification of Eagle's minimal essential medium (DMEM) was obtained from Gibco-Biocult Ltd. (Paisley, Scotland). Fetal bovine serum (FBS) from the same source was routinely heat inactivated (56°C for 30 min) before use. 100 μg/ml Kanamycin, 50 μg/ml streptomycin, and 50 μg/ml penicillin were added to all media. Phosphate-buffered saline (PBS) was obtained from Oxoid Ltd. (Basingstoke, England) and routinely used without calcium or magnesium except for assays of mannose-specific endocytosis when calcium (1.2 mmol) and magnesium (1.2 mmol) were added.

Ligands. A glycoconjugate of mannose-bovine serum albumin (mannose-BSA) with 33–37 mol of sugar/1 mol protein was prepared by the method of Stahl et al. (8), trace-labeled with ^{125}I (12), and used at a sp act of 3.5×10^6 cpm/μg. β-Glucuronidase, a glycoprotein with terminal mannose, was purified from rat preputial glands, labeled, and used at a specific activity of 1×10^6 cpm/μg (13). Mannan from bakers' yeast was bought from Sigma Chemical Co., St. Louis, Mo. (catalogue M-7504).

Antibodies. OX-4, a monoclonal mouse anti $Ia^{k,s}$ antibody was a generous gift of Dr. A. F. Williams, University of Oxford (14). F4/80, a rat monoclonal antibody specific for mouse macrophages, was used as a concentrated supernate. The rat anti-mouse Fc receptor antibody, 2.4G2, was kindly provided as a purified protein by Dr. J. C. Unkeless, The Rockefeller University, New York (15). An affinity-purified $F(ab')_2$ fragment of rabbit anti-rat Fab (RAR) was prepared by S. Hirsch in our laboratory at the Sir William Dunn School of Pathology by the method of Jensenius and Williams (16), and trace-labeled for indirect binding assays as described elsewhere.[2] This reagent cross-reacts with mouse Ig.

Inhibitors. Superoxide dismutase (catalogue S.8254), catalase (catalogue C-100), and indomethacin were obtained from the Sigma Chemical Co. All other reagents used were highest grade available from standard commercial suppliers.

Peritoneal Cells. Resident peritoneal macrophages (RPM) were obtained from untreated animals and thioglycollate-elicited macrophages (TPM) 4–5 d after intraperitoneal injection of thioglycollate broth. BCG-activated peritoneal macrophages (BCG-PM) were obtained 10–21 d after intraperitoneal infection with 1×10^7 live organisms, Pasteur strain 1011, obtained as a generous gift from Dr. R. North, Trudeau Institute, Saranac Lake, N. Y. The organisms were stored at −70°C, thawed, and sonicated briefly before use. Peritoneal cells were washed, resuspended in medium (DMEM + 5% FBS), and plated in 24- or 96-well tissue culture trays (Linbro Chemical Co., Flow, Irvine, England) at 5×10^5 or 2×10^5 macrophages per well, respectively. The number of macrophages in peritoneal washouts was determined in a hemocytometer after staining with Turk's solution. BCG-infected animals yielded ~5×10^6 cells with 45–55% macrophages, 10–15% polymorphonuclear leukocytes (PMN), and 30–40% lymphocytes.

Adherent cell monolayers were prepared after a 4-h incubation at 37°C in the presence of 5% CO_2 by washing twice with PBS. Differential counts of glutaraldehyde-fixed adherent cells were made by phase-contrast microscopy using standard morphologic criteria and showed >90% macrophages in RPM and TPM preparations. BCG-activated adherent cells consisted of ~85% macrophages, 7% lymphocytes, 6% PMN, and 2% dendritic cells. No attempt was made to recover nonadherent macrophages in peritoneal populations. Adherent cell monolayers will be referred to as macrophages and were either used in various assays at this stage or after further cultivation in DMEM + 10% FBS for 60 h.

Cell viability was estimated by trypan blue dye exclusion, phase-contrast microscopy, and by lysozyme production (17). Adherent cells from all preparations showed >95% viability by these criteria. The recovery of adherent cells from different sources was estimated after lysis in

emulphogene (0.25%) and binding of a Bio-Rad dye reagent for cell protein (Bio-Rad Laboratories, Munich, Federal Republic of Germany). This method gave results similar to those obtained with the Lowry method (18), using an egg lysozyme standard. In all cases, the cell protein recovered was directly proportional to the number of macrophages plated.

Assays: Mannose-specific Endocytosis. The binding and uptake of ligands were assayed at saturating concentrations of ligand using trace-labeled mannose-BSA and β-glucuronidase in the presence or absence of yeast mannan, a potent competitor of mannose-specific endocytosis (19).[3] Adherent macrophages from different sources were assayed except for some binding assays that were also performed with total peritoneal cell populations freshly harvested from the animal.

Binding was assayed at 4°C. The reaction mixture contained Ca^{++} and Mg^{++}, which are required for binding, and FBS, which contains little inhibitor of mannose-specific binding, unlike horse serum.

Routinely, 4-h-adherent macrophages were washed three times in PBS and incubated in a reaction vol of 300 μl with DMEM + 10% FBS, with 10 mm Hepes buffer, pH 7.0, and 0.1–0.5 μg/well, ^{125}I-mannose BSA, with or without 1.25–2.5 mg mannan. Cells were incubated in duplicate for 60 min at 4°C and washed three times in ice-cold PBS with 10 mm sodium azide. 200 μl/well of 0.25% Emulphogene was added to dissolve the cells and the radioactivity bound measured in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Cell protein was assayed by dye binding. Results were expressed as nanograms of mannose-BSA specifically bound per 5×10^{-5} macrophages plated. Nonspecific binding was always <5% of specific binding.

Assays with ^{125}I-β-glucuronidase were done similarly except that a higher concentration of ligand (300 μg/ml) was required to saturate specific receptors. Cells in suspension were assayed in microfuge tubes over a layer of oil, and the amount of ligand bound estimated after centrifugation, as described elsewhere (8).

Uptake. Macrophages were incubated in 300 μl of DMEM + 10% FBS with 14 μg ^{125}I-mannose BSA, with or without 1.25–2.5 mg mannan. After 10–30 min at 37°C, the cells were washed five times, lysed, and analyzed as above. Control studies showed that specific uptake reached saturation after 10 min at the concentration of ligand used and that nonspecific uptake was <5%. Degradation of ^{125}I-mannose-BSA, as measured by appearance of trichloroacetic acid-soluble labeled material in the medium, was detectable after longer periods of incubation, but was less rapid with ^{125}I-β glucuronidase.

Saturation Indirect-binding Assays for Macrophage Antigens. Binding of monoclonal antibodies to live macrophages was detected by a second incubation with ^{125}I-labeled RAR. Both first- and second-stage antibodies were used at saturating concentrations so that the number of RAR molecules bound was proportional to the number of first-stage antibody molecules and thus represented a measure of antigen expression.[2] Adherent cells were incubated with monoclonal antibody at 4°C in the presence of sodium azide for 60 min, washed, and incubated further with 50 μl unlabeled RAR, (25 μg/ml), plus trace amounts of ^{125}I-RAR (2×10^5 cpm per well). Control preparations without first antibody showed <5% of binding. The number of molecules of RAR bound specifically was calculated using a value of 6×10^9 $F(ab')_2$ molecules/ng protein. Results were expressed as number of RAR molecules bound per macrophage plated.

Single-Cell Analysis: Uptake of ^{125}I-Mannose BSA. Cover slip preparations of 4-h-adherent macrophages were incubated with 16 μg/ml ^{125}I-mannose BSA with or without 1.25 mg/ml mannan, for 10 min at 37°C. The cells were then washed well, fixed in methanol, and processed for radioautography by standard procedures. Preparations were stained with Giemsa, and at least 300 cells examined in duplicate preparations. Cells with >10 grains were scored as labeled, and mannan-treated controls showed no labeling.

Antigen Analysis. Cover slip preparations were incubated with saturating concentrations of monoclonal antibody and with trace amounts of ^{125}I-RAR (6×10^5 cpm/coverslip). Radioautographs were prepared and scored as cells with >50, >20, or without grains.

Assays of Macrophage Secretory Products. Fibrinolysis by intact macrophages was assayed on ^{125}I-fibrin plates as described (20). Different concentrations of macrophages (6×10^4–5×10^5)

[3] Stahl, P., and S. Gordon. Expression of a mannosyl-fucosyl receptor for endocytosis on cultured primary macrophages and their hybrids. Manuscript submitted for publication.

were assayed after a 4-h adherence, using acid-treated dog serum as source of plasminogen. No plasminogen-independent fibrinolysis was detected.

Hydrogen peroxide release was assayed by the methods of Nathan and Root (4) using phorbol myristate acetate (0.02 μg/ml) as stimulus. Superoxide anion was assayed by the method of Johnston et al. (21).

Lysozyme was assayed by the lysoplate method with chick egg lysozyme as standard (22).

Uptake of BCG Organisms by RPM. RPM were plated for 4 h in DMEM + 5% FBS, washed, and then pulsed with 1×10^7 live organisms/5×10^5 macrophages for 100 min, washed twice in PBS, and then incubated at 37°C for 18 h in DMEM + 5% FBS (23). Binding of antibodies F4/80, 2.4G2, and OX-4, and uptake of ^{125}I-mannose-BSA were measured, and cell-associated organisms counted in Ziehl Neelson-stained preparations.

Results

General Considerations. Intraperitoneal infection by live BCG organisms provides a useful method to obtain activated macrophages in the mouse. These cells can be readily purified by 2–4 h adherence to tissue culture vessels in the presence of serum, and display rapid spreading and active membrane ruffling on glass cover slips. On examination by phase-contrast microscopy (Fig. 1 A), it was noted that BCG-PM differed morphologically from TPM and RPM. Although some cells were well spread and actively endocytic, other cells were smaller and had relatively few endocytic organelles in their cytoplasm, especially when compared with TPM. Morphologic features of intense plasma membrane activity combined with minimal endocytosis persisted for several days after further cultivation (Fig. 1 B). It was therefore of interest

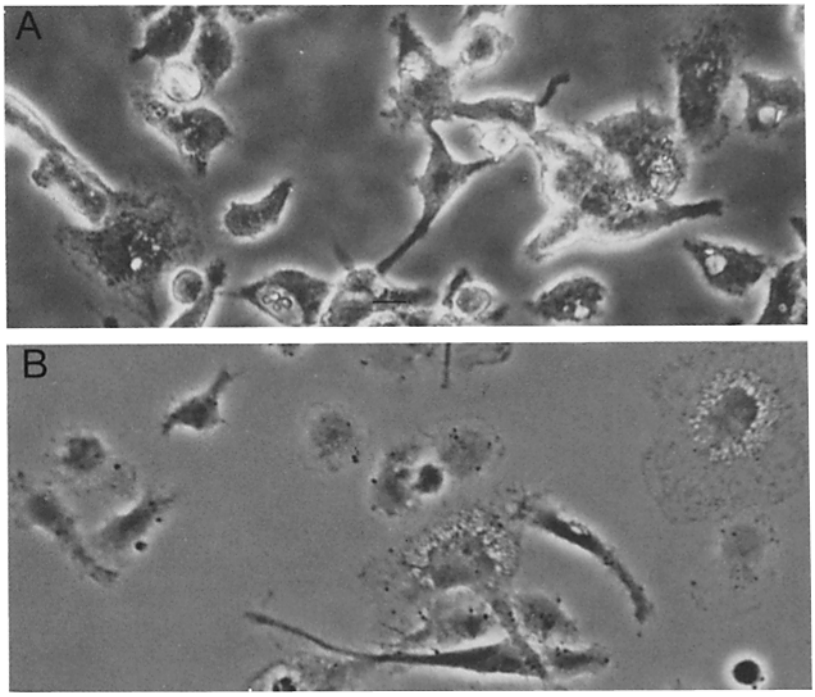

FIG. 1. Phase-contrast micrographs of BCG-PM (A) 4 h and (B) 60 h after cultivation in DMEM + 10% FBS. × 450.

to compare the ability of different peritoneal macrophages to endocytose ligands via specific surface receptors.

Mannose-specific Endocytosis. Rat alveolar macrophages bind and internalize a variety of glycoproteins and glycoconjugates via a specific plasma membrane receptor (19). A similar receptor has recently been detected on TPM,[3] but does not occur on other cells like PMN, lymphocytes, or fibroblasts. Binding and uptake of radiolabeled mannose-BSA or β-glucuronidase by macrophages can be specifically prevented by mannose-rich yeast mannan. Fig. 2 shows mannose-specific binding and uptake of ^{125}I-mannose-BSA by BCG-PM and TPM. Whereas TPM showed a high level of receptor activity, approaching saturation under conditions comparable with those reported earlier for rat alveolar macrophages, both binding and uptake of ligand by BCG-PM were markedly depressed: 6 and 25% of TPM activity, respectively. The low level of activity by BCG-PM was still saturable and completely inhibited by mannan, and was independent of the concentration of ligand or the duration of incubation. Mannose-specific degradation by BCG-PM was reduced to 20% of that by TPM. Similar results were obtained with a different ligand, ^{125}I-β-glucuronidase and diminished binding of ^{125}I-mannose-BSA was also observed with total BCG-peritoneal cells assayed in suspension, without fractionation by adherence. These results were highly reproducible and were confirmed in five independent experiments.

Radioautographic studies (Fig. 3) showed that all thioglycollate-elicited cells with macrophage morphology were labeled by ^{125}I-mannose-BSA (>10 grains/cell) and that BCG-PM were less heavily labeled. PMN and lymphocytes in both cell populations were unlabeled, and uptake by macrophages was inhibited by mannan. We concluded that mannose-specific uptake was confined to macrophages and that BCG-PM displayed defective endocytosis via this receptor.

Control experiments established that the decrease in receptor function was not a result of nonspecific cellular injury. Phase-contrast microscopy confirmed that cells were viable and >95% excluded trypan blue. The defect in mannose-specific endocytosis by BCG-PM persisted after 60 h of cultivation and still represented 25% of uptake by TPM and RPM, which displayed similar activity (Table I). In contrast, all three populations of macrophages secreted a comparable amount of lysozyme, an index of constitutive secretory activity (17). It was noted that these cultures contained few remaining lymphocytes and that the continued depression of endocytosis by BCG-PM was apparently stable and autonomous.

Role of H_2O_2 and PA Secretion. It is known from previous studies that BCG-PM obtained after intraperitoneal infection release H_2O_2 (24) and superoxide anion (O_2^-) in response to phorbol myristate acetate (PMA) and that peritoneal macrophages obtained after BCG infection by the intravenous route secrete high levels of PA, especially after further stimulation by purified protein derivative of tuberculin-induced lymphokines. Although TPM also produce O_2^- and PA, H_2O_2 seems to be consumed by an ingredient of thioglycollate broth, and since because TPM do not display enhanced microbicidal or cytocidal activity, they are therefore considered not to be activated. It was therefore important to examine the secretory activity of BCG-PM in our studies and to establish that they were indeed activated according to these criteria. Fig. 4 shows that BCG-PM with reduced specific uptake of ^{125}I-mannose-BSA produced similar levels of PA compared with TPM, and responded to PMA by releasing H_2O_2 at levels comparable with those reported by others. TPM did not

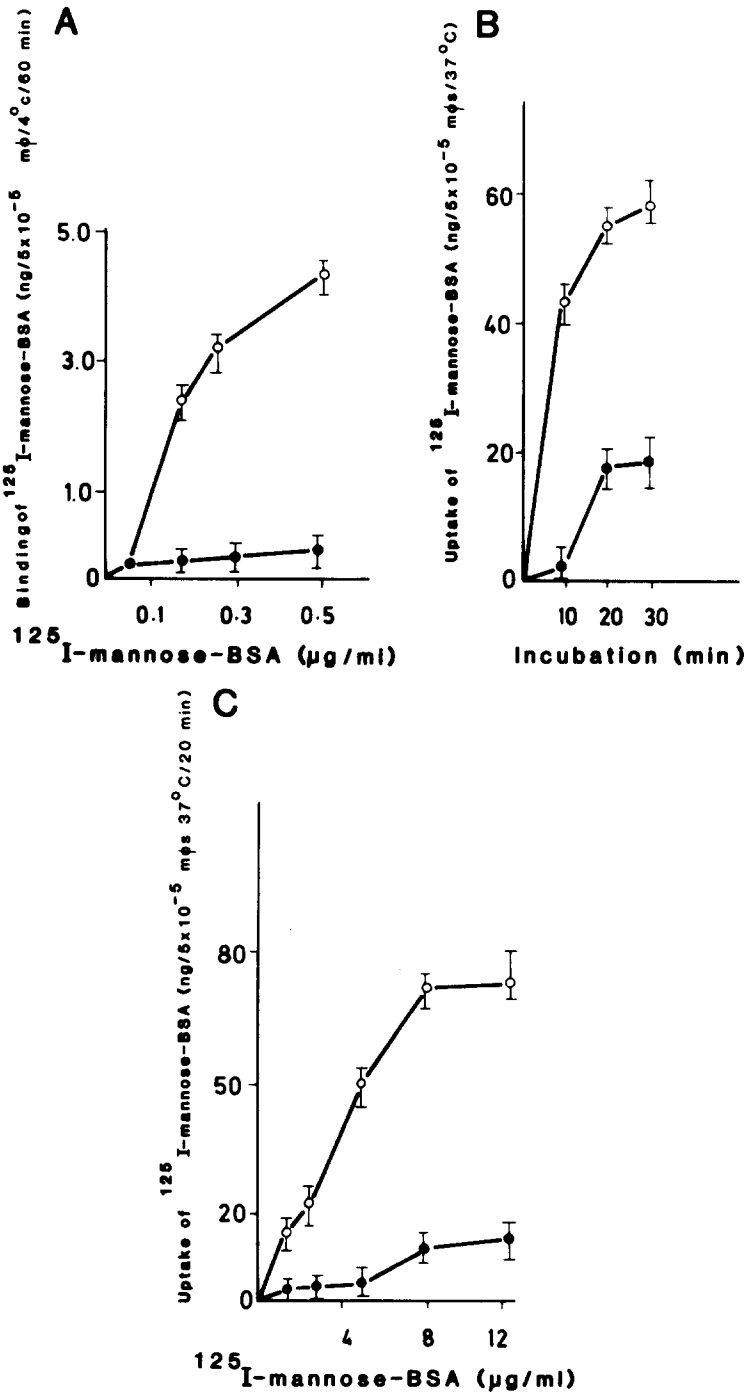

FIG. 2. Specific binding and uptake of ^{125}I-mannose-BSA by BCG-PM and TPM. 5×10^5 macrophages (Mϕ) were cultivated for 4 h before assay. ^{125}I-mannose-BSA (3×10^6 cpm/μg) was added with or without 1.25 mg mannan/well. Results show average ± SD of pooled results of five independent experiments. (○) TPM; (●) BCG-PM.

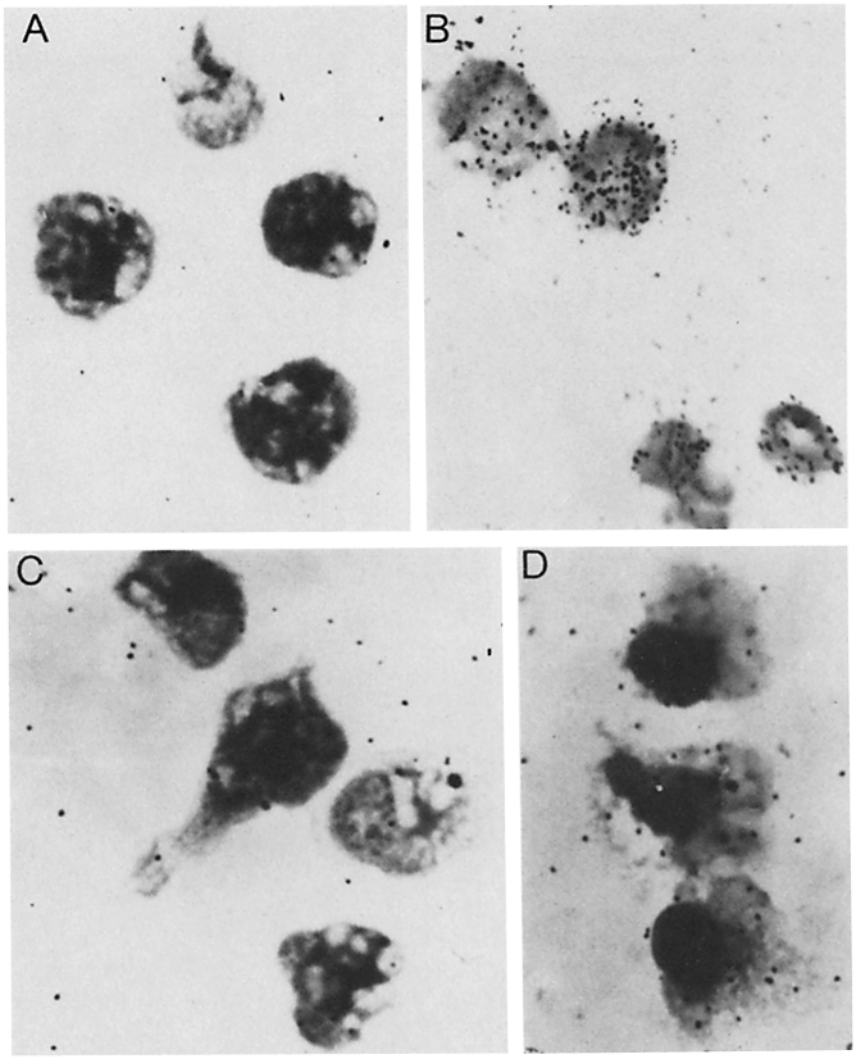

FIG. 3. Radioautographic analysis of uptake of ^{125}I-mannose-BSA by BCG-PM and TPM. 4-h adherent macrophages were exposed to ^{125}I-mannose (32 μg, 1×10^6 cpm/μg), with or without 1.25 mg mannan/ml. for 10 min at 37°C. TPM with (A) and without mannan (B), BCG-PM with (C) and without mannan (D). × 450.

produce H_2O_2 under these conditions, but both populations released high levels of O_2^- after PMA treatment. PMA treatment also enhanced PA secretion by both cell types threefold, but had no significant effect on mannose-specific endocytosis.

These studies confirmed that the BCG-PM were activated and lent further weight to the argument that the defect in endocytosis was not generalized. Because oxygen metabolites, especially H_2O_2, are known to have deleterious effects on cell membranes, and because other macrophage products such as proteinases and prostaglandins could also contribute to altered activity, experiments were performed to evaluate the role of secretion products in mannose-receptor function.

BCG-activated peritoneal cells were harvested and adherent cell macrophages

TABLE I

Mannose-specific Endocytosis and Secretion of Lysozyme by Peritoneal Macrophages after Cell Culture

Peritoneal Mϕ*	Specific uptake of ^{125}I mannose-BSA		Secretion of lysozyme‡
	Per 5×10^{-5} Mϕ	Per μg cell protein	
	ng/20 min at 37°C		*μg/5 × 10^5 Mϕ*
RPM	57 ± 4.1	2.0 ± .13	7.5 ± 1.3
TPM	72 ± 11	2.1 ± .20	6.9 ± 2.1
BCG-PM	20 ± 4.2	0.63 ± .10	7.2 ± 1.9

* 5×10^5 macrophages (Mϕ) were cultivated in DMEM + 10% heat-inactivated FBS for 2 h, washed, and then cultivated in the same medium for 60 h before assay.

‡ Conditioned medium collected over 2–60 h of culture. Pooled results of two independent experiments done in duplicate. Mean ± SD.

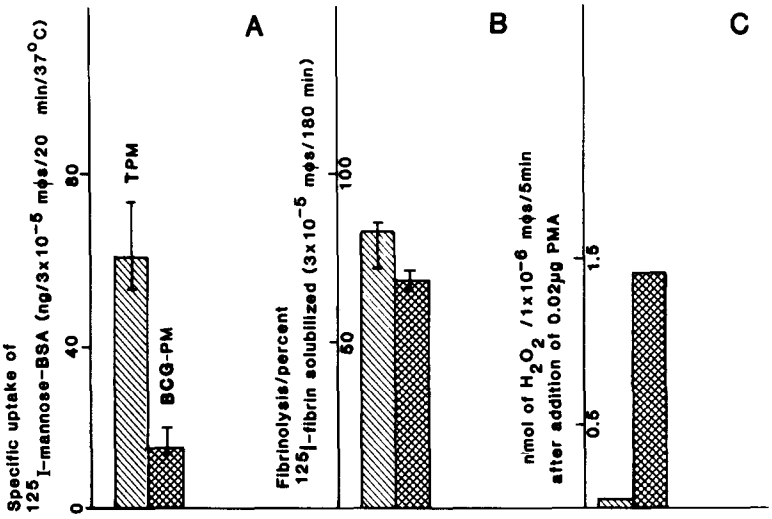

FIG. 4. Receptor-mediated endocytosis (A) and secretion of plasminogen activator (B) and H_2O_2 (C) by BCG-PM and TPM. (A) and (B) are matched experiments; (C) is independent. 0.2 nmol H_2O_2 produced by BCG cells before PMA treatment. 2,000 IU catalase + 25 μg/ml superoxide dismutase inhibited all H_2O_2 production. BCG-PM produced 301, and TPM 175 nmol superoxide/90 min per mg cell protein after treatment with PMA, respectively. Mϕ, macrophages.

prepared and assayed in the continuous presence of a cocktail of inhibitors. Table II shows that catalase and superoxide dismutase, at concentrations which inhibited H_2O_2 and O_2^- effectively in independent assays, did not restore M,FR activity, nor did the further addition of indomethacin. Minor inhibition of receptor activity was noted with both BCG-PM and TPM, but the cells remained viable. In other experiments (data not shown), sublethal concentrations of 0.1 mM reagent H_2O_2 added for 30 min, had no effect on uptake of ^{125}I-mannose-BSA by TPM or BCG-PM.

It is known that M,FR activity in rat alveolar macrophages is destroyed by treatment of intact cells with trypsin, but that receptors can be rapidly reexpressed on the cell surface from an intracellular pool (8). It seemed unlikely in the present

TABLE II

Inhibition of Oxygen Metabolites and of Prostaglandins Does Not Alter the Reduced Uptake of ^{125}I-Mannose BSA by BCG-PM

Inhibitors*	Specific uptake of ^{125}I-mannose-BSA	
	TPM	BCG-PM
	ng/5 × 10^5 Mϕ per 20 min at 37°C	
None	78 ± 11	17 ± 1.9
Catalase (2,000 U/ml) + superoxide dismutase (300 U/ml)	67 ± 6.2	14 ± 2.6
Catalase + superoxide dismutase + Indomethacin (10 μg/ml)	63 ± 5.2	14 ± 1.6

* Cells were maintained in the continuous presence of each inhibitor from time of harvest, including during all washes.

experiments that proteolysis at the surface of the cells could account for the stable loss in mannose-specific receptor activity because all macrophages were maintained in fetal bovine serum, which is strongly inhibitory for extracellular proteolysis, and because TPM, which secrete high levels of PA (25) and several other neutral proteinase activities showed no defect in receptor activity. Nevertheless, the role of macrophage proteinase secretion was examined by cultivating TPM in serum-free media and by cocultivating TPM with BCG-PM.

Adherent TPM were prepared in DMEM + 5% FBS that had been acid treated to reduce protease inhibitory activity (20). The monolayers were washed thoroughly and then cultivated in Dulbecco's medium with 0.1% lactalbumin hydrolysate for 24 h before assay. There was no reduction in ^{125}I-mannose-BSA uptake, but, rather, a small enhancement, probably a result of the absence of serum glycoproteins able to compete for binding of the ligand.

Equal numbers of TPM and BCG-PM were also mixed and cocultivated with or without serum for 24 h before assay. The presence of BCG-PM had no effect on TPM receptor activity compared with unmixed controls.

We concluded that diffusible secretory products could not account for reduced receptor expression on BCG-PM. These studies did not rule out an intracellular site of action for these products, unique enzymes produced only by BCG-PM, or pathways not inhibited by this limited range of inhibitors.

Quantitative Assays of Other Surface Markers. We next asked whether BCG-activated macrophages also expressed reduced levels of other receptors for endocytosis and of other macrophage surface antigens. We examined expression of FcR receptors, which bind and internalize immune complexes, and of macrophage-specific antigen F4/80, using rat monoclonal antibodies 2.4G2 and F4/80 in a quantitative indirect-binding assay. Antibody bound to macrophages was detected by ^{125}I-RAR, with both first- and second-stage antibodies at saturation. Similar assays were performed to quantitate expression of Ia antigens, which have been reported on BCG-PM (26). The mouse monoclonal antibody used, OX-4, reacts with polymorphic Ia determinants 17 or 18 (14), which are present in CBA (H-2^k), but not in BALB/c (H-2^d) mice. All antigens were therefore examined in matched groups of animals of both strains. Preliminary studies confirmed that peritoneal macrophages obtained after BCG infection of these

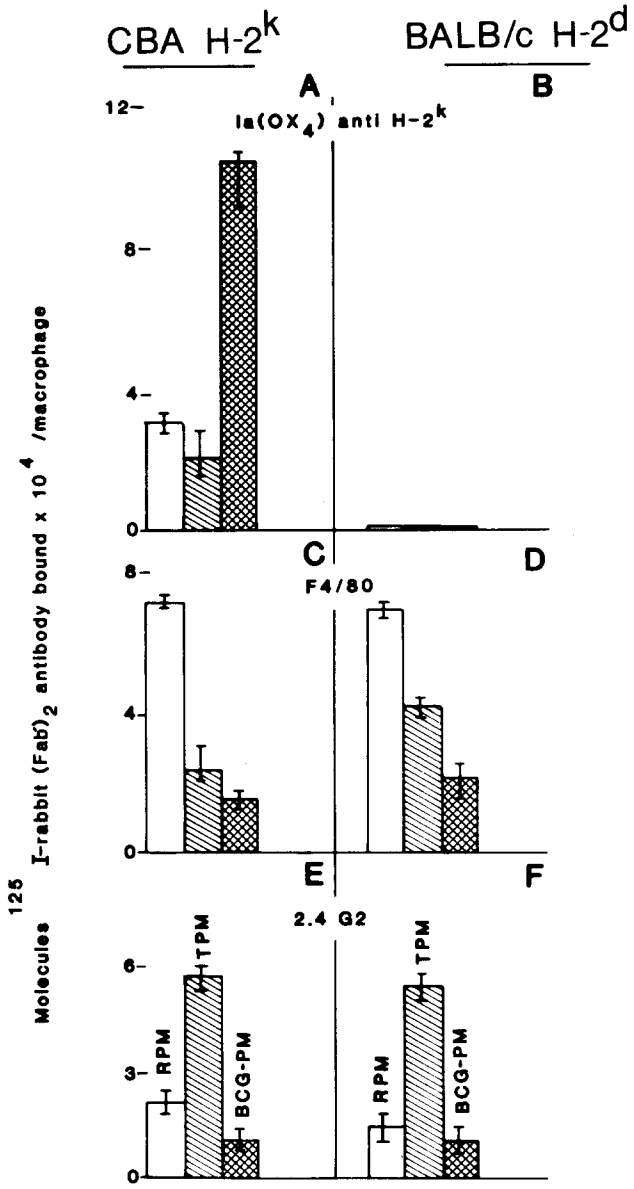

FIG. 5. Expression of antigens on peritoneal macrophages measured by saturation binding assays. BCG-PM, RPM, and TPM were obtained from both CBA (panels A, C, and E) and BALB/c mice (panels B, D, and F). 3×10^5 peritoneal macrophages were cultured in 96-well microtiter plates in DMEM + 5% FBS for 4 h, washed, and assayed as described in Materials and Methods. The rabbit $F(ab')_2$ antibody used to detect binding of rat (anti-F4/80, FcR) and mouse (anti-Ia H-2^k) antibodies was used at 7.6×10^3 cpm/μg.

strains spread rapidly and displayed reduced mannose-receptor activity similar to that observed with macrophages from PO mice.

BCG-activated CBA macrophages assayed after 4-h adherence and washing showed a striking threefold increase in expression of Ia antigen compared with TPM and

RPM from the same strain (Fig. 5). Binding of antibody was <1% in the BALB/c strain control and was therefore a result of Ia antigen on CBA cells and not to FcR or cytophilic antibody. The enhanced expression of Ia antigen was stable after 3 d of cultivation in DMEM + 10% FBS.

In contrast, BCG-PM showed reduced expression of F4/80 and of FcR, with no difference between strains. F4/80 expression was greatest on resident macrophages (71,000 molecules RAR bound), reduced in TPM (28,000–40,000), and markedly reduced (18,000–20,000) in BCG-PM, in agreement with previous results.[2] FcR expression on BCG-PM was also clearly reduced compared with TPM (12,000 vs. 56,000), but RPM expressed rather less antigen (15,000–22,000), relative to TPM. Because FcR is not confined to macrophages, it should be noted that there were <10% lymphocytes and PMN in any of these preparations.

Reduced expression of F4/80 and FcR combined with enhanced Ia antigen was a consistent finding in at least five independent experiments.

Single-Cell Analysis. To identify the cells labeled by each antibody and to detect possible heterogeneity, we next examined the adherent populations by radioautography. Results are shown in Table III and Fig. 6. F4/80 antigen was readily detectable on >90% of TPM (>50 grains/cell) and was reduced on BCG-PM (40% of cells heavily labeled), but 83% of cells were still clearly, though less heavily, labeled (>20 grains/cell) compared with control preparations without first-stage antibody. Because the small number (<10%) of remaining lymphocytes, PMN, and dendritic cells were all unlabeled by F4/80, these results confirm that the majority of adherent BCG-PM express this macrophage-specific antigen.

In contrast, expression of Ia antigen increased from 16% (TPM) to 64% (BCG-PM) and was restricted to cells from the CBA strain. Most cells showed heavy labeling, resembled the cells labeled by F4/80, and were therefore macrophages. No morpho-

TABLE III

*Radioautographic Analysis of Antigens on BCG-PM and TPM**

Strain	Cells‡	Control without first-stage antibody	Cells labeled by antibody		
			F4/80	OX-4	2.4G2
				%	
CBA	TPM (A)	1	90 ± 2	12 ± 3	88 ± 4
	TPM (B)	1	93 ± 3	16 ± 2	92 ± 2
CBA	BCG-PM (A)	1	40 ± 3	59 ± 3	20 ± 7
	BCG-PM (B)	1	85 ± 2	64 ± 4	89 ± 2
BALB/c	BCG-PM (A)	1	41 ± 3	1	ND
	BCG-PM (B)	1	83 ± 5	1	ND

* Peritoneal cells were cultured for 4 h in DMEM + 5% FBS on glass cover slips, washed well, and incubated with saturating concentrations of monoclonal antibody followed by ^{125}I rabbit F(ab')$_2$ antibody at trace levels (3×10^5 cpm/0.5 μg).

‡ 300 cells counted in triplicate preparations. (A) and (B) indicate the percent of adherent cells with >50 and >20 grains, respectively. Mean ± SD shown. Differential count: 90% macrophages, 6% lymphocytes, 3% PMN, and 1% dendritic cells by morphological criteria.

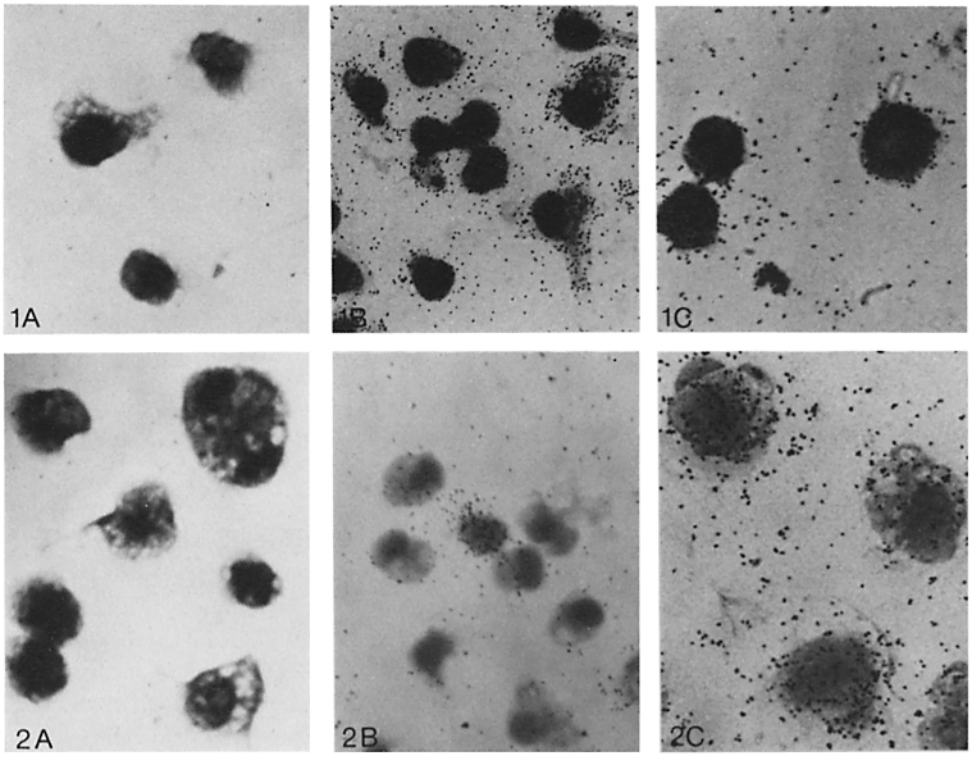

FIG. 6. Radioautographic analysis of antigen F4/80 and Ia on BCG-PM and TPM. 4-h adherent macrophages were incubated with saturating concentrations of monoclonal antibody and binding detected with trace-labeled ^{125}I-RAR as described in Materials and Methods. Antigen F4/80 on CBA-PM. (1 A) Control without antibody F4/80. (Similar results obtained for TPM or BCG-PM.) (1 B) TPM with F4/80. (1 C) BCG-PM with F4/80. Ia antigen, all with antibody OX-4 (2 A) BALB/c BCG-PM, strain control. (2 B) CBA TPM. (2 C) CBA BCG-PM. × 300.

logic difference was noted between Ia-positive and Ia-negative macrophages. Small numbers of dendritic cells seen also expressed Ia antigen strongly (26).

Expression of FcR was similar to that of F4/80. ~90% of TPM were heavily labeled by 2.4G2, whereas most BCG-PM showed reduced labeling. Because it is known from other studies that BCG-activated macrophages have functional Fc receptors (27), experiments were performed to examine binding and uptake of sheep erythrocytes opsonized with rabbit antibody (IgG). The results, not shown, confirmed that FcR could be detected on most of the BCG-PM cells by a rosetting assay, but that these cells bound and ingested fewer antibody-coated erythrocytes than TPM.

It can therefore be concluded that the decreased expression of surface determinants and of endocytosis by BCG-activated macrophages was not restricted to mannose-specific receptors, but was also evident with two independent markers, F4/80 and FcR. Moreover, the same population of cells expressed increased levels of Ia antigen, and most of the cells that displayed this altered phenotype were macrophages.

Phagocytosis of BCG Organisms by RPM. The effect of phagocytosis of BCG organisms on the expression of F4/80, 2.4G2, Ia antigens, and on the uptake of ^{125}I-mannose-BSA was studied as described in Materials and Methods. Phagocytosis of organisms in vitro failed to induce the changes in antigen expression seen after infection in vivo.

Stained preparations revealed that there were five to six organisms per macrophage and that 80% of the adherent cells had intracellular organisms. 2% of the adherent cells had intracellular organisms after BCG infection in vivo 10 d before harvest. We concluded that phagocytosis of organisms could not by itself account for the altered surface phenotype of BCG-PM (23).

Discussion

The plasma membrane of the activated macrophage must play an important role in its ability to recognize and destroy invading microorganisms. The activated cell spreads more rapidly on a foreign surface; the killing of an extracellular target depends on close contact, e.g., via antibody and Fc receptors (27), and its ability to release H_2O_2 is triggered by surface-active agents such as PMA or lectins, which have little effect on nonactivated macrophages (4, 6). Yet, we know little about changes in surface properties of macrophages upon activation or how secretory, microbicidal, or cytotoxic activities are controlled by external stimuli.

In this study, we have demonstrated, with quantitative assays for several receptors and antigens, that mouse BCG-PM display markedly different surface properties compared with TPM or cells obtained from untreated animals. The BCG-PM showed a clear reduction in mannosyl receptor and FcR function and of antigen F4/80, but enhanced expression of Ia antigenic determinants. Only the BCG-PM released high levels of H_2O_2 after stimulation by PMA, and these cells were therefore regarded as activated. Changes in the surface markers studied here made it possible to discriminate between BCG-PM and nonactivated macrophages, unlike previous studies in which TPM also displayed a reduced level of 5′ nucleotidase activity (28) as well as enhanced secretion of plasminogen activator (25).

Single-cell analysis confirmed that the vast majority of BCG-PM under study still expressed the macrophage-specific markers F4/80 and the mannosyl receptor, and that Ia antigen expression had been induced on macrophages and was not being detected on B lymphocytes or dendritic cells. Adherence itself did not cause the loss in mannosyl receptor or F4/80 because freshly harvested cells displayed similarly reduced activity. Control experiments showed that cell injury by secretion products could not account for reduced endocytosis of mannose-BSA. Because altered expression of all surface markers persisted for 60 h in culture, we concluded that BCG infection had generated a population of macrophages with a stable reversal in surface phenotype. It remains to be shown that other activating agents bring about a similar change.

Because binding assays were performed on live cells at 4°C, the reduced binding of ligands and antibodies by BCG-PM indicates that expression of these receptors and antigens at the cell surface was diminished, and thus accounts for decreased uptake and degradation of mannose-terminated glycoconjugates. Reduced uptake at 37°C provides evidence against the presence of a substantial intracellular pool of the mannosyl receptor in BCG-PM. The M,FR (30,000 mol wt), FcR (47,000–60,000 mol wt) (15), F4/80 antigen (160,000 mol wt)[2] and Ia antigen (28,000–32,000 mol wt) (14) are on distinct molecules and the determinants are independent of one another, e.g., antibody F4/80 or mannan do not block any other marker (unpublished observation). The coordinate reduction of three determinants and induction of Ia could conceivably

be a result of a common biosynthetic event, e.g., glycosylation, but we feel that it is likely to reflect a more complex change during membrane biogenesis.

Studies with other antigens and ectoenzymes, e.g., 5′ nucleotidase and alkaline phosphodiesterase (29) might help to establish the selectivity of changes in the plasma membrane of the activated macrophage. Because receptor-mediated, and possibly-fluid phase, pinocytosis (30) are reduced in BCG-activated macrophages, it is unlikely that enhanced internalization alone could account for decreased expression at the surface. Quantitative assessments of the synthesis, internalization, turnover, and possible recycling of all the molecules concerned should yield further insights into the cellular mechanisms responsible. It should also be noted that uptake of BCG organisms is known to inhibit phagolysosomal fusion (31) and to reduce phagocytosis of various particulates, with or without opsonins (23). In our studies, the peritoneal macrophages obtained after infection contained few detectable organisms and the surface changes in these cells could not be mimicked in uninfected cells by uptake of BCG organisms in vitro.

Two hypotheses can be proposed to account for the changes in plasma membrane observed, viz., that the cell population is changed and represents an earlier stage or alternate pathway of macrophage differentiation or that the mature macrophage is able to modulate its surface phenotype. In this regard, it is known that Ia-like antigens may be expressed during earlier stages of myeloid differentiation (32), but that these antigens can also be induced on inflammatory peritoneal macrophages by treatment with lymphokines (11). It remains to be seen whether lymphokines can also induce the loss of F4/80 and other markers in nonactivated macrophages in vitro.

Several other studies have noted reduced FcR-mediated phagocytosis in some macrophages obtained after immunization with BCG (30) or infection by *Mycobacterium leprae* (33). It has also been argued for some time that epithelioid cells in BCG-induced granulomata show little morphologic evidence of endocytic activity (34). The present studies indicate that reduced ingestion of antibody-coated tumor cells (27) and resultant extracellular lysis could be a result of the decreased number of FcR on the surface of the BCG-PM. Although an extracellular mode of killing resembles a phagocytic mechanism in many respects, the known ability of an activated macrophage to destroy intracellular pathogens needs to be considered in the light of its poor phagocytic activity. Moreover, diminished clearance of β-glucuronidase and other lysosomal hydrolases via mannosyl receptors may exacerbate extracellular catabolism and tissue injury.

The possible role of Ia antigens in interactions between macrophages and other cells or in control of their effector functions remains unclear. Nussenzweig et al. (26) have provided evidence that Ia positive macrophages do not actively sensitize T cells in a model immune response, unlike small numbers of dendritic cells that induced strong responses. Because activated macrophages express Ia antigens in common with other cells and because expression of the currently available macrophage-specific markers decreases during activation, it is necessary to produce new markers specific for the activated macrophage to study its contribution to immune responses. Studies to this end are in progress in our laboratory.

Summary

Infection of the mouse peritoneal cavity by bacillus Calmette-Guérin (BCG) markedly alters the surface properties of the macrophages induced, compared with

cells obtained from uninfected control animals or after injection of thioglycollate broth. Quantitative binding assays with radiolabeled ligands or antibodies showed that BCG-activated peritoneal macrophages (BCG-PM) expressed one-fourth or less receptor activity for mannose-terminal glycoconjugates as well as reduced levels of Fc receptors and of antigen F4/80 compared with nonactivated macrophages. Endocytosis mediated by mannose-specific receptors was reduced in parallel. In contrast, surface Ia antigen was increased threefold in the same adherent cell population.

Radioautographic analysis confirmed that >80% of adherent cells still expressed low levels of the macrophage-specific mannosyl receptor and antigen F4/80, and that Ia antigens had been induced on 64% of macrophages rather than on other cells. Control experiments established that only the BCG-PM macrophages released H_2O_2 after stimulation with phorbol myristate acetate, whereas both BCG-PM and thioglycollate-induced macrophages produced superoxide anion and plasminogen activator. The BCG-PM were viable, secreted normal levels of lysozyme, and displayed a stable phenotype after cultivation for 60 h. Inhibitors of oxygen products, prostaglandins, and proteases did not alter reduced endocytosis by BCG-PM.

These studies indicated that expression of macrophage surface markers is reversed by BCG-activation, and that their known enhanced ability to lyse target cells extracellularly is associated with decreased endocytosis via specific receptors. Whether these changes are a result of an altered cell population or of modulation of selective surface properties is not known.

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