DOWN-REGULATION OF MANNOSYL RECEPTOR-MEDIATED ENDOCYTOSIS AND ANTIGEN F4/80 IN BACILLUS CALMETTE-GUÉRIN-ACTIVATED MOUSE MACROPHAGES

Role of T Lymphocytes and Lymphokines*

By R. ALAN B. EZEKOWITZ‡ and SIAMON GORDON

From the Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, England

Infection of the mouse peritoneal cavity by bacillus Calmette-Guérin (BCG)[1] markedly alters the surface properties of macrophages (mϕ), compard with cells obtained from uninfected control animals or after injection of thioglycollate broth. BCG-activated peritoneal mϕ (BCG-PM) express enhanced Ia antigens (Ag), but reduced receptors for mannose-terminated glycoconjugates (MFR), Fc receptors, and mϕ-specific surface Ag F4/80 (1). Decreased receptor-mediated endocytosis is associated with active secretion of plasminogen activator (PA) (2), the ability to release O_2^- and H_2O_2 after surface stimulation (3, 4), and enhanced activity against various organisms and target cells (5, 6). The increase in Ia Ag (7–9) and in secretory (2, 10) and anti-microbial activity (11, 12) can depend on specifically sensitized T lymphocytes, and some of these effects are induced in nonactivated macrophages by treatment with lymphokines.

Surface antigens and receptors control recognition and effector functions of activated mϕ and provide ideal markers to study the complex effects of lymphocyte products on macrophages. We show here that down-regulation of mannose-specific endocytosis and Ag F4/80 accompanies induction of mϕ Ia by Ag-stimulated T lymphocytes and lymphokines. These alterations in the plasma membrane make it possible to distinguish between activated and nonactivated macrophages and permit study of the mechanism of cell activation in vitro.

Materials and Methods

Animals. CBAT$_6$T$_6$ (H-2K) mice were bred at the Sir William Dunn School of Pathology, and both sexes used weighed 20–30 g. CBA nude mice were obtained from Olac Laboratories, Bicester, Oxon, England.

Media and Reagents. Iscove's modification of Dulbecco's medium (IM) 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 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 mM) and magnesium (1.2 mM) were added. LPS from *Salmonella abortus equi*, prepared by the phenol extraction procedure, was a gift from Dr. M.-L. Lohmann-Matthes, Max Planck Institut, Freiburg, (Federal Republic of Germany). *N*-acetylmuramyl-L-alanyl-D-isoglutamine · 2H$_2$O (MDP) was bought from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA, and *Corynebacterium parvum* obtained as a formol-killed suspension from Dr. James Howard, Wellcome Reagents Ltd., Beckenham, England. Thioglycollate broth and proteose peptone were bought from Difco Laboratories, Detroit, MI.

Ligands. A glycoconjugate of mannose-bovine serum albumin (mannose-BSA) with 33–37 mol of sugar/mol protein was a gift from Dr. P. Stahl, Washington University, St. Louis, MO. This material was trace labeled with ^{125}I (1) and used at 4 × 10^6 cpm/μg. Mannan from bakers yeast (M-7504; Sigma Chemical Co., St. Louis, MO) was used at a concentration between 5 and 10 mg/ml to measure specific uptake of this ligand (1).

Antibodies. OX6, a monoclonal mouse anti-Ia K,S antibody (Ab) (13) was a generous gift of Dr. A. F. Williams, University of Oxford. F4/80, a rat monoclonal Ab specific for mature mouse mϕ, was used as a concentrated supernatant (14). The rat anti-mouse Fc receptor antibody, 2.4G2 (15), produced by Dr. J. Unkeless, The Rockefeller University, New York was used as a concentrated supernatant. Mac-I/70 (16), which binds to mϕ and polymorphonuclear leukocytes, was obtained from Dr. T. Springer, Harvard Medical School, Boston, MA, and used as a concentrated supernatant. An affinity-purified F(ab')$_2$ fragment of rabbit anti-rat Fab (RAR) was trace-labeled for indirect binding assays. This reagent cross-reacts with mouse immunoglobulin (Ig). A rat anti-Thy-1 (monomorphic) cytotoxic antibody (YBM 29.2.1) was obtained as an ascites fluid from Dr. H. Waldmann, Department of Pathology, Cambridge, England.

Peritoneal Cells. Resident peritoneal mϕ (RPM) were obtained from untreated animals, and elicited mϕ obtained 4 d after intraperitoneal injection of thioglycollate-broth (1 ml), endotoxin (30 μg), MDP (200 μg), or proteose peptone (1 ml of 1% solution). BCG-PM were obtained 8–21 d after intraperitoneal infection with ~1 × 10^7 live organisms, Pasteur strain 1011, obtained from Dr. R. North, Trudeau Institute, Saranac Lake, NY (1). *C. parvum*-activated PM were obtained 14 d after intraperitoneal injection of 0.2 ml of PBS containing 1.4 mg dry weight of organisms.

Peritoneal cells were washed, resuspended in medium (IM + 5% FBS), and plated in 24- or 96-well tissue culture trays (Linbro Chemical Co., Flow Laboratories, Irvine, Scotland) at 5 × 10^5 or 1 × 10^5 mϕ/well, respectively. The number of mϕ in the peritoneal washouts was determined in a haemocytometer after staining with Turk's solution. Adherent cell monolayers (>95% F4/80 positive) will be referred to as mϕ and were used in various assays after 4 h incubation at 37°C or after cultivation for up to 5 d in IM + 5% FBS. Cell viability was estimated by trypan blue dye exclusion, phase-contrast microscopy, and lysozyme production. Adherent cells from all preparations showed >95% viability by these criteria. The recovery of adherent cells was estimated after lysis with 1 N NaOH and cell protein analysis by the method of Lowry et al. (17).

Nylon Wool Separation of Lymphocytes. BCG-primed peritoneal cells were incubated in IM + 5% FBS for 2 h at 37°C, 5 × 10^7 cells/100-mm tissue culture dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA). The nonadherent cells were removed by two gentle washes of the adherent monolayer. Cells were collected by centrifugation and resuspended in 2 ml IM + 5% FBS and overlayed on a nylon-wool column (500 mg; Fenwal Inc., Ashland, MA), which had been preincubated with IM + 10% FBS for 30 min at 37°C (18). The column with cells was incubated at 37°C for 30 min and the nonadherent cells eluted with IM at 37°C. The cells were then washed, counted, suspended in IM + 5% FBS, and either injected intraperitoneally into mice at 1–5 × 10^5 in 0.2 ml saline, with or without 50 μg of purified protein derivative (PPD, Connaught Medical Research Laboratories, Toronto, Canada) or added to monolayers of RPM or TPM with or without 50 μg/ml PPD. 30% of nonadherent cells, of which <1% were mϕ, were recovered after passage over the nylon wool columns. Cell viability by trypan blue exclusion was >97%.

Depletion of Thy-1-positive Lymphocytes. 1 × 10^6 nylon wool-purified lymphocytes were incu-

bated with a 1:200 dilution of anti-Thy-1 Ab at 4°C for 60 min, washed twice, incubated with low toxicity guinea pig complement (Sera Laboratories, Crawley) at 1 in 10 dilution for 45 min at 37°C, and washed twice in PBS. The 40% viable cells remaining were added to adherent TPM with PPD and cultivated for 2 d. 90% of the lymphocytes were viable after incubation with complement alone.

Lymphokine Preparation. Immune lymphokine was prepared from spleens removed from CBA mice infected 14–21 d previously with live BCG, intraperitoneally. A single-cell suspension was washed twice in PBS and the cells cultured in IM + 5% FBS with 50 μg/ml PPD for 72 h at 37°C in a T-75 flask (Falcon Labware). Supernatants were centrifuged for 10 min at 450 g and passed through a 0.22-μm Millex filter (Millipore Corp., Bedford, MA). Control lymphokine was prepared by culturing BCG spleen cells in the absence of PPD, which was added to the supernatant before filtration. The immune lymphokine was stored for up to 1 wk at 4°C and was active in the range 8–30% vol/vol. 10% was used routinely and fresh medium and lymphokine were added daily to cells cultivated >24 h.

Concanavalin A (Con A) Lymphokine. Suspensions of spleen cells from untreated animals were cultivated in IM + 2% FBS with 3 μg/ml Con A for 2 d (Pharmacia Fine Chemicals, Uppsala, Sweden). Con A was absorbed by passing the supernatant down a Sephadex G10 column. The Con A lymphokine contained nondialyzable inhibitor(s) of growth, determined by [^{3}H]thymidine incorporation, and of PA secretion by mφ. These inhibitory activities could be removed by concentrating the supernatant fivefold in an Amicon filtration cell across a PM-10 membrane (19).

Assays of Mannose-specific Endocytosis. This was assayed at saturation by incubating mφ in 300 μl of IM + 5% FBS with 18 μg ^{125}I-mannose-BSA, with or without mannan (1). After 20–30 min at 37°C, the cells were washed five times, lysed with IN NaOH, and radioactivity measured in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers grove, IL). Results were expressed as ng mannose-BSA specifically taken up per 5×10^{-5} macrophages plated. Nonspecific uptake was always <5% of specific uptake.

Antigens. Binding of monoclonal Ab to mφ, which had been fixed for 10 min with 0.25% glutaraldehyde, was detected with ^{125}I-labeled RAR. Both first- and second-stage Ab were at saturation, so that the number of RAR molecules bound is proportional to the number of molecules of first stage Ab and thus a measure of Ag expression (14). Assays were performed at 4°C in the presence of sodium azide. Control preparations without first Ab showed <10% of specific binding. Results were expressed as the number of RAR molecules bound per mφ.

Single Cell Analysis Ag. Cover slip preparations of TPM and RPM were prepared after 48 h cultivation with immune or control lymphokine (10% vol/vol). The cover slips were then incubated with saturating concentrations of monoclonal Ab and ^{125}I RAR (5×10^5 cpm/cover slip) and processed for autoradiography.

Uptake of ^{125}I-Mannose BSA. Similar preparations were incubated with 18 μg/ml ^{125}I-mannose-BSA with or without mannan, for 20 min at 37°C. Mannan-treated controls showed no labeling.

Secretion Products. PA secretion was assayed on ^{125}I-fibrin plates (20). Different concentrations of mφ (6×10^4–5×10^5/well) were cultivated 4–48 h, and plasminogen dependent fibrinolytic activity assayed with acid-treated dog serum (ATDS) as the source of plasminogen. Fibrinolysis in serum free medium was <5% of that in ATDS. Superoxide anion was assayed after addition of 20 ng phorbol myristate acetate (PMA, Sigma Chemical Co.) (3, 21). Results were expressed as nmol O_2^- released/mg cell protein/60 min. Superoxide dismutase, 25 μg/ml (SOD, Sigma Chemical Co.) inhibited >80% of cytochrome C reduction. O_2^- production by lymphokine-activated mφ was assayed after 24 h treatment. Lysozyme was assayed by the lysoplate method (20) with chick egg lysozyme as standard.

Results

Effects of the Route and Time Course of BCG Infection on Macrophage Surface Properties and Secretion. This was examined as a basis for further studies. After intraperitoneal infection with BCG under standard conditions, the surface, endocytic, and secretory properties of mφ showed highly reproducible changes compared with RPM or TPM

(Table I). As reported previously (1), mannose-specific endocytosis, Ag F4/80, and Fc receptor Ag 2.4G2 were reduced to 20–50% of control values, whereas Ia (OX 6) expression was increased by 250–400%. BCG-PM released large amounts of O_2^- after PMA stimulation, and also PA, whereas TPM released high levels of PA, but less O_2^-. Expression of Mac-1 ag, not studied previously, was relatively stable and showed only minor changes after BCG infection, usually <10%. Comparable surface and other properties were found whether results were expressed in terms of mϕ number or cell protein. These changes were observed 6–42 d after BCG infection, but not 2 or 4 d after intraperitoneal infection with twice the standard dose. The effects of more prolonged infection were not studied. Intravenous infection induced the same properties in PM and further intraperitoneal treatment of these animals with PPD or heat-killed BCG did not accentuate the changes (not shown).

Role of Sensitized Lymphocytes and Antigen

ADOPTIVE TRANSFER IN VIVO. To examine the role of specifically sensitised lymphocytes in modulating the surface properties of macrophages, nylon wool-enriched BCG-primed peritoneal cells were first injected intraperitoneally into uninfected syngeneic mice, with or without PPD. PM harvested 48 h after injection of sensitized lymphocytes with PPD showed a similar pattern of surface changes and secretion to BCG infection (Table 1 and Fig. 1). Specific uptake of ^{125}I-mannose-BSA was reduced by 55–60% and Ag F4/80 to a lesser extent, by 35–50%, whereas Ia Ag and secretion of PA and O_2^- were enhanced 2–4-fold relative to mock-injected or untreated controls. Injection of sensitized lymphocytes without PPD failed to induce any change in the PM, whereas PPD alone contributed up to one-third to the increase in Ia. PPD alone decreased mannose-specific endocytosis by 40%, but did not diminish Ag F4/80. The effective range of lymphocytes was 1–10 × 10^5 per animal, the minimal effective concentration of PPD 25–50 μg.

We concluded that adoptive transfer of sensitized lymphocytes with specific antigen was able to induce the complete activation phenotype in PM from uninfected animals, but less efficiently than infection with BCG for markers such as Ag F4/80. PPD by itself had an intermediate effect which will be discussed further below.

TABLE I

Macrophage Properties after Infection with BCG or Injection of BCG-primed Lymphocytes Plus PPD into the Peritoneal Cavity *

Treatment	MFR (ng uptake of ^{125}I-mannose-BSA/5 × 10^{-5} mϕ/30 min/37°C)	Ag expression (mol/mϕ × 10^{-4})				Release of‡ O_2^- (nmol/mg/60 min)	Fibrinolysis (Percent solubilized/180 min/2 × 10^5 mϕ)
		F4/80	OX 6	2.4G2	Mac-1		
BCG infection	25 ± 8	7 ± 2	20 ± 4	8 ± 1	50 ± 12	180 ± 20	55 ± 10
Thioglycollate broth	90 ± 12	36 ± 6	8 ± 2	23 ± 6	60 ± 15	80 ± 30	66 ± 5
RPM	80 ± 10	51 ± 8	4 ± 3	14 ± 5	56 ± 10	26 ± 8	12 ± 5
BCG-sensitized lymphocytes and PPD	38 ± 4	25 ± 5	15 ± 1	8 ± 4	42 ± 18	150 ± 10	60 ± 5
Mock transfer, medium alone	83 ± 12	38 ± 2	8 ± 4	ND§	48 ± 16	34 ± 6	35 ± 3

* Mice were either infected as described in Materials and Methods or injected with 5 × 10^5 BCG-primed lymphocytes with 50 μg PPD. Peritoneal cells were harvested after 2 d, and adherent mϕ assayed after 4 h. Results show a representative experiment mean ± SD, of triplicate (Ag) or duplicate assays. Similar results were obtained in at least three independent experiments.
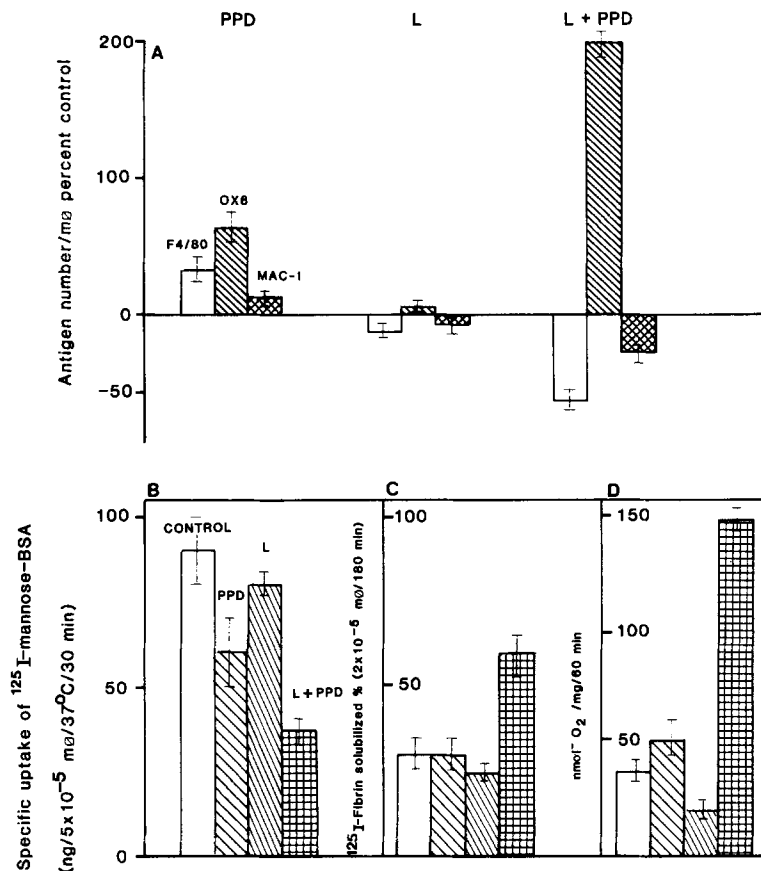‡ 20 ng PMA added for 60 min. Dimethyl sulfoxide control had no effect.
§ Not determined.

FIG. 1. Macrophage properties after injection of PPD, BCG-primed lymphocytes (L), or both into uninfected animals. (A) Antigen expression. Mϕ were harvested 2 d after injection of 1 × 10^6 lymphocytes with or without 50 μg PPD, cultivated for 4 h in IM + 5% FBS, washed, and fixed in glutaraldehyde before assay. 1 × 10^5 mϕ were plated per well and RAR used to detect binding of rat (anti-F4/80 and Mac-1) and mouse (anti-Ia H-2K) Ab at 8 × 10^3 cpm/μg. Results of triplicate assays are expressed as a percentage change from controls (see Table I) injected with medium alone, ± SD. Similar results were obtained in three independent experiments. (B) Specific uptake of ^{125}I-mannose-BSA by mϕ obtained and cultivated for 4 h as above. 5 × 10^5 live mϕ per well were incubated with 4 × 10^6 cpm/μg ligand with or without mannan, 10 mg/well. Results show mean ± SD of pooled results of three independent experiments. (C) Fibrinolytic activity. Mϕ were incubated with 5% vol/vol acid-treated dog serum after cultivation for 4 h in IM + 5% FBS. Results show average ± SD of duplicate assays for one of three similar experiments. (D) Release of superoxide after 60-min incubation with PMA, 20 ng/well. Results show average ± SD of duplicate assays for one of three similar experiments.

CO-CULTIVATION OF MACROPHAGES WITH LYMPHOCYTES AND ANTIGEN. To study the role of antigen and lymphocytes more directly, mϕ from uninfected animals were next exposed in culture to BCG-sensitized lymphocytes with or without PPD for 2 d before assay. Untreated and thioglycollate-elicited macrophages were compared as targets to evaluate the response of resident and newly recruited cell populations. Results are shown in Table II and Fig. 2. These indicate that the differences between BCG-activated mϕ and nonactivated RPM and TPM remained stable after cultivation for 2 d (cf. Tables I and II), that BCG-sensitized lymphocytes plus PPD efficiently

TABLE II

Macrophage Properties after Addition of Sensitized Lymphocytes, Antigen, and Lymphokine in Culture

Treatment	MFR (ng uptake/5 × 10⁻⁵ mφ/30 min 37°C)	Ag number			Superoxide
		F4/80	0X 6	Mac-1	
		$(molecules/m\phi) \times 10^{-4}$			$nmol/mg/60\ min$
BCG-PM	34 ± 4	8 ± 3	23 ± 5	50 ± 10	ND*
TPM	91 ± 29	73 ± 10	8 ± 2	90 ± 22	65 ± 10
RPM	77 ± 7	60 ± 24	7 ± 2	77 ± 11	12 ± 3
BCG Lyc + PPD: RPM‡	29 ± 7	39 ± 14	16 ± 1	72 ± 4	ND
BCG Lyc + PPD TPM	36 ± 8	45 ± 6	23 ± 4	79 ± 20	ND
Lymphokine: RPM‡	26 ± 3	40 ± 15	15 ± 1	80 ± 10	55 ± 6
Lymphokine: TPM	34 ± 6	55 ± 6	23 ± 3	72 ± 6	124 ± 12

* Not done.
‡ Adherent mφ from uninfected mice were incubated with BCG-primed lymphocytes (ratio 1:1) plus 50 μg PPD, with lymphokine (10% vol/vol) or with control medium for 2 d before assay, except for superoxide release, which was measured after 1 d.

induced several of the parameters of mφ activation in vitro, and that resident and thioglycollate-elicited mφ provided similar targets. The combination of lymphocytes and PPD induced a two- to threefold increase in Ia and a 60–70% decrease in mannose-receptor activity. Ag F4/80 was again diminished to a lesser extent than after infection in vivo (35% vs. 85%), whereas mac-1 levels remained unaltered. PPD by itself retained some ability to induce Ia and decrease mannose-specific endocytosis in vitro, but the effect was variable and less marked than after intraperitoneal injection. Sensitized lymphocytes alone had no effect (not shown).

Other experiments (not shown) indicated that BCG-primed lymphocytes and PPD induced fibrinolysis by RPM, as reported previously (2). The optimal ratio of sensitized lymphocytes to adherent mφ was 1 × 10⁵:1 × 10⁵. A ratio >1:1 in the presence of PPD was often cytotoxic to the macrophage target. PPD was effective in the concentration range of 25–50 μg/ml; 50 μg/ml was used routinely.

Role of Lymphokines. Addition of lymphokine-rich supernatants to cultivated PM induces spreading, secretion of PA (2, 22), the capacity to release H_2O_2 (10), enhanced Ia expression (7–9), and the ability to kill intracellular organisms (23) and tumor cell targets (24). Lymphokine was prepared by stimulating BCG-primed spleen cells with PPD and added to RPM and TPM for different periods of time before assay of mφ markers (Table II). These lymphokines were able to enhance Ia two- to threefold, and MFR activity was reduced by two-thirds in both targets. Ag F4/80 again fell to a lesser extent, by one-third, whereas Mac-1 levels remained unchanged. Lymphokine controls with PPD showed <20% of the efficacy of active supernatants (not shown). Although lymphokines induced similar antigenic and endocytic changes in TPM and RPM, TPM secreted twice as much O_2^- when challenged with PMA subsequently. Con A-induced lymphokines gave similar results to immune lymphokine. However, some batches contained inhibitory activity(ies) that was nondialyzable, but removable by concentration across a PM-10 membrane.

Kinetics and Stability. The time course of altered antigen and receptor expression and its reversibility are shown in Fig. 3, in which TPM were exposed to immune lymphokines continuously for 5 or 2 d before withdrawal. Although lymphokine stimulation affected several markers coordinately, the rate of change for each differed
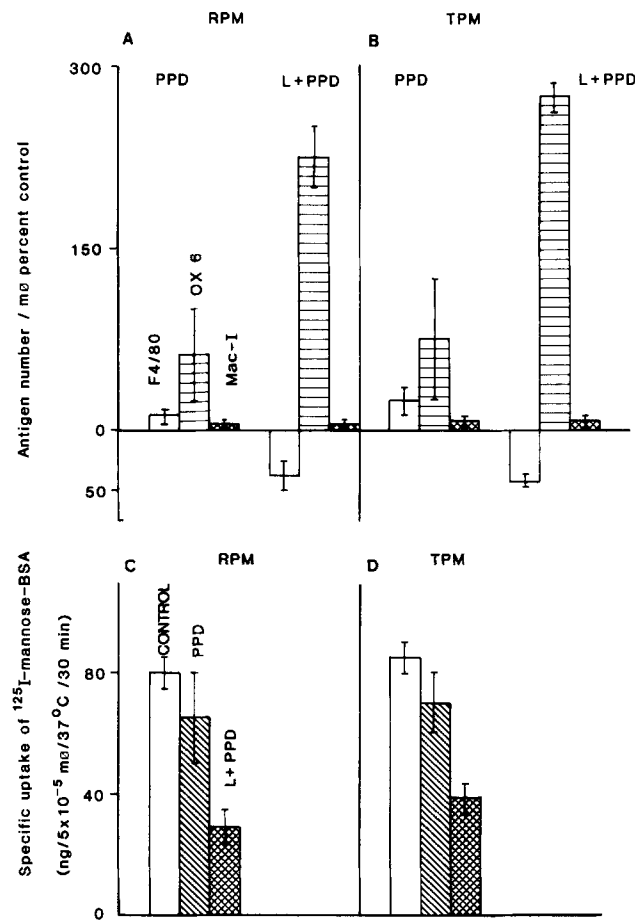
FIG. 2. Mφ properties after co-cultivation with BCG-primed lymphocytes (L) and PPD. (A, B) Ag. 1 × 10⁵ RPM or TPM were co-cultivated with 1 × 10⁵ sensitized lymphocytes and 50 μg PPD for 48 h, washed, fixed in glutaraldehyde, and assayed as in Fig. 1. Pooled results of three experiments, expressed as percentage change of control (see Table II), mean ± SD. (C, D) Mannose-specific endocytosis by 5 × 10⁵ RPM or TPM.

substantially. Reduction of MFR activity could be detected 4 h after exposure to lymphokine and decreased progressively until a plateau level of ~30% of control activity was attained after 1 d. Reduction in F4/80 was more gradual and less extensive, to 70% of control values. Induction of Ia Ag showed a lag period of 1 d and reached a maximum at day 3. These changes depended upon daily addition of fresh lymphokine, and after its removal Ia levels returned towards control values. Recovery of MFR activity and F4/80 was incomplete compared with control TPM in which receptor and Ag levels increased with cultivation. This was not due to cell loss or diminished viability.

For comparison, mφ activated in vivo by infection were examined under similar conditions in culture. Enhanced Ia expression was stable for 2-3 d and was then lost by day 5 (not shown). The addition of lymphokine on day 3, or daily addition of lymphokine from first plating, failed to reverse this fall, as did addition of PPD, even
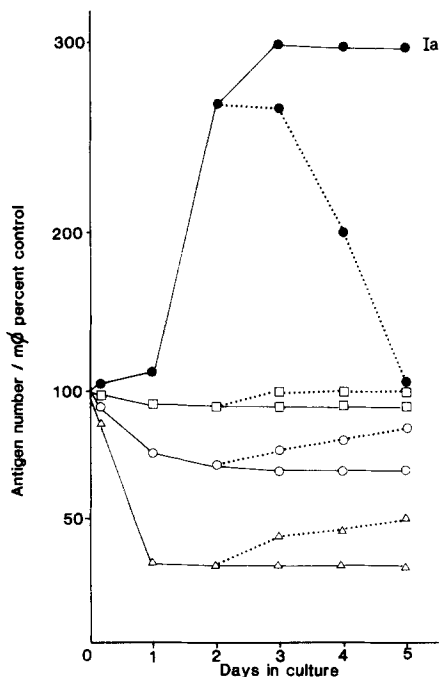
Fig. 3. Activation of TPM by Ag-induced lymphokine. Time course and stability of altered expression of Ag and MFR by lymphokine-activated macrophages. TPM were treated with lymphokine continuously by daily addition of 10% vol/vol, or for 2 d before washout. Surface Ag and specific uptake of ^{125}I-mannose BSA were measured as in Fig. 1. Results of replicate assays (range <10%) are shown as a percentage of those for untreated TPM cultivated and refed with control medium for the same period in culture. Prolonged treatment with lymphokine (—); removal of lymphokine (- - -). Control values for TPM cultivated for 4 h, 1, 2, 3, 4, and 5 d, respectively: Ag (number of sites/mϕ × 10^{-4}) F4/80 (O): 36, 71, 73, 100, 120, 111; OX 6 (●): 8, 9, 7, 6, 7; Mac-1 (□): 60, 61, 90, 120, 140, 130; MFR (ng/5 × 10^{-5} mϕ) (△): 90, 93, 91, 83, 86, 90. SD is not shown, <20% in all cases.

in the presence of BCG-sensitized lymphocytes. MFR and Ag F4/80 remained low for several days and then recovered to 40–50% of the values of untreated TPM after 5 d in culture. There was no obvious cell loss and lysozyme poduction, as an index of cell viability, was comparable to that of populations that had been cultivated for 2 d.

Single-cell Analysis. Lymphokine-treated mϕ were examined by autoradiography to establish whether the altered expression of surface markers was uniform within the population. Ia Ag induction was heterogeneous (60% of TPM and 40% of RPM heavily labeled by OX 6), but the reduction in MFR activity and F4/80 reflected a generalized decrease in all cells (>90% of mϕ labeled, though less heavily than untreated controls), rather than a subpopulation of unlabeled cells.

Role of T Lymphocytes; Depletion of Thy-1-positive Cells. Table III shows that the alteration of mϕ Ag and MFR activity by BCG-sensitized lymphocytes and PPD depends on Thy-1 positive cells. After ablation with anti-Thy-1 Ab and complement, but not complement alone, mϕ targets retained high levels of MFR and F4/80, and showed no increase in Ia Ag when co-cultivated with surviving lymphocytes and PPD.

Nude Mice. The properties of BCG-PM obtained from infected CBA *nu/nu* mice were compared with RPM and TPM from similar animals. Table IV shows that BCG

TABLE III

Alteration of Macrophage Surface Markers by BCG-sensitized Lymphocytes and PPD Depends on Thy-1 Positive Cells*

Treatment of lymphocytes	Antigen expression			MFR (ng uptake/ 5 × 10^5 mϕ/30 min/37°C)
	F4/80	OX 6	Mac-I	
	(molecules/mϕ) × 10^4			
Anti-Thy-1 + C'	83 ± 10	8 ± 1	90 ± 5	72 ± 8
C'	37 ± 15	13 ± 2	93 ± 2	46 ± 6
none	40 ± 6	18 ± 4	88 ± 6	38 ± 10

* BCG-primed lymphocytes were treated with anti-Thy-1 plus complement (C') or C' alone, as described, and then incubated with TPM plus 50 μg PPD for 48 h before assay. Results of one experiment done in triplicate representative of three independent experiments. Mean ± SD.

TABLE IV

Macrophages from Nude Mice Infected with BCG Express Activation Phenotype*

Treatment	MFR (ng uptake/5 × 10^{-5} mϕ/30 min/37°C)	Antigen expression			Superoxide‡	PA (percent fibrinolysis/180 min/2 × 10^5 mϕ)
		F4/80	OX 6	Mac-I		
		(molecules/mϕ) × 10^{-4}			nmol/mg/60 min	
BCG-PM nu/nu	27 ± 4	8 ± 2	11 ± 2	55 ± 2	172 ± 7	65 ± 3
RPM nu/nu	80 ± 6	46 ± 4	2 ± 2	80 ± 15	80 ± 40	10 ± 6
TPM nu/nu	68 ± 7	27 ± 5	5 ± 2	68 ± 20	33 ± 7	68 ± 7

* Macrophages from CBA nude mice (three mice pooled per group) harvested 8 d after intraperitoneal infected with BCG or 4 d after thioglycollate injection were plated and assayed after 4 h. Results of triplicate assays except for PA, which was performed in duplicate.

TABLE V

Effects of Corynebacterium parvum and Other Agents on Macrophage Properties*

Treatment	MFR (ng uptake/5 × 10^{-5} mϕ/30 min/37°C)	Antigen expression			Superoxide	PA (percent fibrinolysis/180 min/2 × 10^5 mϕ)
		F4/80	OX 6	Mac-I		
		(molecules/mϕ) × 10^{-4}			nmol/mg/60 min	
C. parvum	24 ± 3	4 ± 2	18 ± 3	36 ± 10	126 ± 12	62 ± 8
LPS	62 ± 8	42 ± 5	6 ± 3	81 ± 5	49 ± 5	18 ± 5
MDP	40 ± 15	62 ± 10	15 ± 3	80 ± 12	80 ± 1	16 ± 4
Proteose peptone	76 ± 12	45 ± 12	9 ± 3	70 ± 20	50 ± 24	22 ± 6

* Cells adhered for 4 h, washed and assayed. 0.2 ml C. parvum was injected intraperitoneally 14 d before harvest; 30 μg of LPS, 200 μg of MDP, and 1 ml of 1% proteose peptone were injected intraperitoneally 4 d before harvest. Representative of three independent experiments. Mean ± SD.

infection of nude mice reproduced all the changes associated with activation in normal mice. TPM from nudes resembled their normal counterparts, as did RPM from nudes, except for an exceptionally high level of O$_2^-$ release in the latter. Similar results were obtained in three separate experiments and established that the mϕ of nude animals are readily activatable according to the present criteria.

Other stimuli

C. PARVUM. Macrophages obtained after injection of *C. parvum* resemble BCG-PM in their ability to secrete H_2O_2, PA, and their tumoricidal and antimicrobial properties. 4-h-adherent PM from *C. parvum*-injected animals have similar surface, endocytic, and secretory properties to BCG-PM (Table V). These changes were seen 2–14 d after intraperitoneal injection. However, an in vitro pulse of *C. parvum* to a 4-h adherent monolayer of RPM failed to induce the surface changes seen after in vivo injection. Phase-contrast microscopy of cover slip preparations confirmed that the mϕ had phagocytised organisms.

INFLAMMATORY AGENTS. The effects of intraperitoneal injection of endotoxin, proteose peptone, and MDP were also studied. Table V shows that MDP-elicited mϕ display properties that are intermediate between RPM and BCG-PM, in that there is induction of Ia expression, some decrease in MFR activity (50% of RPM), and, as reported by others (25), enhanced secretion of superoxide, whereas fibrinolytic activity was not increased. Endotoxin- and proteose peptone-elicited PM, however, were similar to RPM. Addition of MDP or LPS in vitro to 4-h-adherent RPM, and further cultivation for 24 or 48 h, failed to induce any change in macrophage surface or secretory properties. Although not exhaustive, these studies confirmed that the activation phenotype described was not unique to BCG infection, but was confined to agents such as BCG and *C. parvum*, with known ability to induce enhanced antimicrobial/cytocidal activity.

Discussion

These, and earlier (1) studies, establish that BCG-activated mouse macrophages display complex changes in surface and endocytic function that accompany enhanced secretion of reactive intermediates of oxygen and PA. Some plasma membrane antigens (e.g., Ia) are increased in number, other receptors (MFR, FcR) and Ag (F4/80) are reduced, whereas Mac-1 Ag levels show less consistent change. We show further that (a) this phenotype is not unique to BCG but can also be induced by another mϕ-activating agent, *C. parvum*. Macrophages elicited by thioglycollate broth, LPS, or proteose peptone do not display these changes in Ia, MFR, or F4/80, which therefore discriminate between activated and nonactivated cells; (b) all changes characteristic of mϕ activation by BCG can be shown to depend on sensitized T lymphocytes and specific antigen. However, studies with nude mice indicate that the activation phenotype may also arise by an independent pathway; and (c) the altered surface properties are stable, occur in a coordinate manner, independent of a particular agent, and can be induced in vivo and in vitro. These studies confirm the central role of lymphocyte products as extrinsic regulators of mϕ activation. Down-regulation of the MFR, in particular, provides an attractive new marker to study heterogeneity of lymphokines and the mechanism of mϕ activation.

Others have also reported changes in Ia antigens (7–9), lactoperoxidase-labeled surface proteins (26), and of ectoenzymes such as 5' nucleotidase and alkaline phosphodiesterase (27) in BCG and lymphokine-activated macrophages. The plasma membrane of the mϕ therefore undergoes extensive, but highly selective remodeling upon activation. It is not known if lipids (28) and carbohydrate constituents are modified to a similar extent.

Although the various markers changed expression coordinately, differences in

extent, kinetics, and reversibility were noted under various conditions. Intact micro-organisms, e.g., BCG, *C. Parvum*, and, in other studies, *Listeria monocytogenes* (29), are efficient activating agents, but phagocytosis of these agents per se does not provide a sufficient stimulus. Soluble constituents derived from mycobacteria, e.g., MDP and PPD, were less effective by themselves, perhaps because of diffusion or more ready degradation within the animal. Partial activation observed with PPD in the absence of specifically sensitized T lymphocytes could be due to a direct effect on macrophages or via stimulation of residual B lymphocytes (30). LPS does not bring about the surface changes characteristic of BCG activation in vivo or in vitro, although it is able to stimulate other mϕ functions. Phagocytosis of latex particles by LPS-primed macrophages, a procedure designed to enhance their fibrinolytic activity (31), does not induce the surface properties characteristic of activation (R. A. B. Ezekowitz, unpublished observation).

T lymphocytes play an important role in cellular immunity to BCG (32) and *Listeria* (12), and have been implicated in the activation of mϕ by *C. parvum* (33). Evidence that Ag-stimulated T lymphocytes influence the surface and endocytic properties of mϕ, as well as their secretory activity, was obtained by adoptive transfer experiments in vivo and in vitro and by depletion of Thy-1-positive cells with specific Ab and complement. These studies made it possible to compare the efficiency of activation by infection and by lymphocytes and Ag. Reduction in mannose-specific endocytosis was marked in all situations, whereas Ag F4/80 decreased to a lesser extent during activation by adoptive transfer in vivo or in vitro, compared with infection in the animal. Such differences in marker expression could be due to heterogeneity in the mϕ population induced by different treatments or to additional pathways for activation during infection. Activation in culture represents a closed system, and similar surface changes were observed whether TPM or RPM were used as targets. However, during infection newly recruited mϕ contribute to the activated cell population (34), and it is known that less mature mϕ express lower levels of surface markers such as Ag F4/80 (35).

The present studies with nude mice indicate that mϕ may also be activated by pathways independent of mature T lymphocytes, although residual T lymphocyte function cannot be completely excluded (36). Macrophages from nude animals are able to display some of the parameters of activation (37–39), but it has also been reported (29) that mϕ Ia is not induced in nude mice infected with *Listeria*. Activation of complement could contribute to mϕ activation by *C. parvum* and other microorganisms in nude and normal mice (40).

Lymphokines obtained after Ag or mitogen stimulation were able to modulate all activation markers in peritoneal mϕ from uninfected animals. These effects were specific for lymphokines and have not been observed with LPS-, PMA-, or L cell-conditioned medium, a source of macrophage colony-stimulating factor (unpublished observation). Crude lymphokine preparations contain inhibitors as well as stimulatory activities, and it will be important to characterize the active mediator(s) and its mechanism of action. The MFR provides a sensitive, quantitative, and cell-specific marker for such purposes (41).

Mϕ surface changes initiated by lymphokine vary in their stability. Ia Ag is readily lost upon removal of lymphokine, but recovery of reduced receptors and surface antigens is incomplete. Similar results were obtained after cultivation of BCG-PM

from infected mice. The role of sensitized lymphocytes and of specific Ag in main-taining the activated state in vitro should be examined further. In our experience, BCG-PM become refractory to restimulation by lymphocytes, Ag, or lymphokines, unlike reports on reinduction of Ia Ag on mϕ from *Listeria*-infected mice (42).

The mechanisms by which lymphocyte products alter mϕ properties are not understood. Decreased endocytosis via mannosyl receptors is associated with reduced expression of receptors at the cell surface (1). Down-regulation of MFR by lymphokine is rapid, time 1/2 $\cong$ 16 h, independent of known specific ligands, and reaches a plateau level, ~25% of control, within 1 d. Residual activity remains stable subsequently and is not due to a lymphokine resistant subpopulation. Loss of receptors could be due to decreased synthesis and/or increased turnover or shedding, until a new steady state is attained. Other workers have shown that different mϕ plasma membrane constit-uents vary in their rate of turnover, time 1/2 = 2 to >80 h (43–46), depending on mϕ stimulation, interaction with specific ligands, and molecular size. Differences in the kinetics of loss of mannosyl receptors and Ag F4/80 and of induction of Ia Ag are compatible with a common mechanism, e.g., one by which lymphokines selectively activate or repress a number of mϕ gene products.

Although activated mϕ express reduced levels of MFR and FcR compared with elicited or resident mϕ, and endocytosis via these receptors is reduced, the receptors remain functional at the cell surface. The FcR mediates extracellular lysis of Ab-coated target cells by stimulating release of H_2O_2 (47), and the MFR effectively triggers the respiratory burst upon contact with zymosan particles that have not been opsonized (G. Berton, unpublished observation). Whereas both BCG-activated and thioglycollate-elicited mϕ readily release O_2^- after treatment with PMA, more selec-tive surface stimuli trigger only the activated cells (G. Berton, unpublished observa-tion). These observations indicate that alterations in plasma membrane structure and function plays a crucial role in controlling the effector mechanisms of the activated mϕ.

Summary

Bacillus Calmette-Guérin (BCG) infection alters the surface and endocytic prop-erties of mouse peritoneal macrophages (PM) compared with thioglycollate-elicited (TPM) or resident PM (RPM). Expression of Ia antigen (Ag) is enhanced up to fourfold, but plasma membrane receptors that mediate binding and uptake of mannosyl/fucosyl-terminated glycoconjugates (MFR), Fc receptors, and the macro-phage (mϕ)-specific Ag F4/80 are reduced by 50–80%. Levels of Mac-1 remain relatively stable. These changes are accompanied by enhanced secretion of O_2^-, after further stimulation with phorbyl myristate acetate, and of plasminogen activator. Both these products are released by TPM, but not RPM. The characteristic surface phenotype of BCG-PM can also be induced by injection of *C. parvum*, another mϕ-activating agent, but not by thioglycollate broth, lipopolysaccharide, or proteose peptone. Purified protein derivative (PPD) and N-acetylmuramyl-L-alanyl-D-isoglu-tamine·$2H_2O$ are soluble agents with partial activity.

Alteration of mϕ markers by BCG infection depends on T lymphocyte function, although studies with nude mice indicate that other pathways may also serve to modify the surface of the mϕ. Mϕ from uninfected animals displayed all markers of activation after adoptive transfer of specifically-sensitised lymphocytes with PPD,

intraperitoneally, or after co-cultivation. Treatment of primed lymphocytes with anti-Thy-1 antibody and complement ablated this effect. Lymphokines obtaned by Ag or mitogen stimulation induced similar changes in TPM and RPM. Mannose-specific endocytosis decayed rapidly, time $1/2 \cong 16$ h and stabilized at ~25% of control values. Single-cell analysis showed that residual MFR activity was uniform in the target population. Loss of Ag F4/80 after activation by lymphocyte and PPD was less marked than after infection (35% vs 80%), unlike MFR activity, which declined to a similar extent. Induction of mϕ Ia by lymphokine reached a peak after 2-3 d and was lost within 2 d of its removal. Recovery of MFR and F4/80 was incomplete under these conditions.

These studies establish that activated mϕ known to display enhanced antimicrobial/anticellular activity express markedly different surface properties distinct from elicited or resident cells. The role of antigen-stimulated T cell products in regulating mϕ function is confirmed, and down-regulation of mannosyl-receptor-mediated endocytosis provides a sensitive, quantitative, and cell-specific new marker to study their properties and mechanism of action. Extensive, but selective remodeling of mϕ plasma membrane structure could play an important role in controlling recognition and effector mechanisms of the activated mϕ.

References

1. Ezekowitz, R. A. B., J. Austyn, P. D. Stahl, and S. Gordon. 1981. 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. *J. Exp. Med.* **154:**60.

2. Gordon, S., and Z. A. Cohn. 1978. Bacille Calmette Guérin infection in the mouse. Regulation of macrophage plasminogen activator by T lymphocytes and specific antigen. *J. Exp. Med.* **147:**1175.

3. Johnston, R. B. Jr., C. A. Godzik, and Z. A. Cohn. 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. Exp. Med.* **148:**115.

4. Nathan, C. F., and R. K. Root. 1977. Hydrogen peroxide release from mouse peritoneal macrophages. Dependance on sequential activation and triggering. *J. Exp. Med.* **146:**1648.

5. Hibbs, J. B. 1975. Activated macrophages as cytotoxic effector cells. *Transplantation (Baltimore).* **19:**77.

6. Freedman, V. H., T. A. Calvelli, S. Silagi, and S. C. Silverstein. 1980. Macrophages elicited with heat-killed bacillus Calmette-Guérin protect C57BL/6J mice against a syngeneic melanoma. *J. Exp. Med.* **152:**657.

7. Steinman, R. M., N. Nogueira, M. D. Witmer, J. D. Tydings, and I. S. Mellman. 1980. Lymphokine enhances the expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. *J. Exp. Med.* **152:**1248.

8. Steeg, P. S., R. N. Moore, and J. J. Oppenheim. 1980. Regulation of murine macrophage Ia-antigen expression by products of activated spleen cells. *J. Exp. Med.* **152:**1734.

9. Scher, M., D. I. Beller, and E. R. Unanue. 1980. Demonstration of a soluble mediator that induces exudates rich in Ia-positive macrophages. *J. Exp. Med.* **152:**1684.

10. Nathan, C., N. Nogueira, C. Juangbhanich, J. Ellis, and Z. A. Cohn. 1979. Activation of

macrophages *in vivo* and *in vitro*. Correlation between hydrogen peroxide release and killing of *Trypanosoma cruzi*. *J. Exp. Med.* **149:**1056.

11. North, R. J. 1973. Importance of thymus-derived lymphocytes in cell-mediated immunity of infection. *Cell. Immunol.* **7:**166.

12. Lane, F. C., and E. R. Unanue. 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. *J. Exp. Med.* **135:**1104.

13. McMaster, W. R., and A. F. Williams. 1979. Monoclonal antibodies to Ia antigens from rat thymus: cross reactions with mouse and human and use in purification of rat Ia glycoproteins. *Immunol. Rev.* **47:**117.

14. Austyn, J. A., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11:**805.

15. Unkeless, J. C. 1979. Characterization of monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* **150:**580.

16. Springer, T., G. Galfre, D. S. Secher, and C. Milstein. 1979. Mac-I: a macrophage differentiation antigen identified by monoclonal antibody. *Eur. J. Immunol.* **9:**301.

17. Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193:**265.

18. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3:**645.

19. Lafferty, K. J., L. Andrus, and S. J. Prowse. 1980. The role of lymphokine and antigen in the control of specific T cell responses. *Immunol. Rev.* **51:**279.

20. Gordon, S. 1978. Assays for macrophage activation and secretion products. *In* Handbook of Experimental Immunology. 3rd edition. D. M. Weir, editor. Blackwell Scientific Publications, Oxford. **33:**1–14.

21. Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1978. Biological defense mechanisms. The production by leukocytes of superoxide, a potent bactericidal agent. *J. Clin. Invest.* **56:**1035.

22. Nogueira, M., S. Gordon, and Z. Cohn. 1977. Trypanosoma cruzi: the immunological induction of macrophage plasminogen activator requires Thymus-derived lymphocytes. *J. Exp. Med.* **146:**172.

23. Sharma, S. D., and J. S. Remington. 1981. Macrophage activation and resistance to intracellular infection. *Lymphokines.* **3:**181.

24. Meltzer, M. S. 1981. Tumor cytotoxicity by lymphokine-activated macrophages: development of macrophage tumoricidal activity requires a sequence of reactions. *Lymphokines.* **3:**319.

25. Cummings, N. P., M. J. Pabst, and R. B. Johnston, Jr. 1980. Activation of macrophages for enhanced release of superoxide anion and greater killing of *Candida albicans* by injection of muramyl dipeptide. *J. Exp. Med.* **152:**1659.

26. Yin, H., S. Aley, C. Bianco, and Z. A. Cohn. 1980. Plasma membrane polypeptides of resident and activated mouse peritoneal macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **77:**2188.

27. Morahan, P. S., P. J. Edelson, and K. Gass. 1980. Changes in macrophages ectoenzymes associated with anti-tumor activity. *J. Immunol.* **125:**1312.

28. Mahoney, E. M., W. A. Scott, F. R. Landsberger, A. L. Hamill, and Z. A. Cohn. 1980. Influence of fatty acyl substitution on the composition and function of macrophage membranes. *J. Biol. Chem.* **255:**4910.

29. Lu, C. Y., E. Peters, and E. R. Unanue. 1981. Ia bearing macrophages in athymic mice: antigen presentation and regulation. *J. Immunol.* **126:**2496.

30. Sultzer, B. M., and K. Nilsson. 1972. PPD tuberculin-a B cell mitogen. *Nat. New Biol.* **240:**198.

31. Gordon, S., Unkeless, J. C. and Cohn, Z. A. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis. Evidence for a two-stage process. *J. Exp. Med.* **140:**995.

32. Lefford, M. J. 1975. Transfer of adoptive immunity to tuberculosis in mice. *Infect. Immun.* **7:**1174.

33. Sljivic, V. S., and S. R. Watson. 1977. The adjuvant effect of *Corynebacterium parvum*: T-cell dependence of macrophage activation. *J. Exp. Med.* **145:**45.

34. Spector, W. G., and G. B. Ryan. 1970. The mononuclear phagocyte in inflammation. *In* Mononuclear Phagocytes. R. van Furth, editor. Blackwell Scientific Publications, Oxford. 219.

35. Hirsch, S., J. M. Austyn, and S. Gordon. 1981. Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture *J. Exp. Med.* **154:**713.

36. Hünig, T., and M. J. Bevan. 1980. Specificity of cytotoxic T cells from athymic mice. *J. Exp. Med.* **152:**688.

37. Cheers, C., and R. Waller. 1975. Activated macrophages in congenitally athymic 'nude' mice and in lethally irradiated mice. *J. Immunol.* **115:**844.

38. Cutler, J. E., and H. H. Poor. 1981. Effect of mouse phagocytes on *Candida albicans* in *in vivo* chambers. *Infect. Immun.* **31:**1110.

39. Johnson, W. J., and E. Balish. 1980. Macrophage function in germ-free, athymic (*nu/nu*) and conventional flora (*nu/+*) mice. *Res. J. Reticuloendothel. Soc.* **28:**55.

40. Ghaffar, A. 1980. The activation of macrophages by *Corynebacterium parvum*: effects of anticomplementary agents cobra venom factor and sodium cyanate. *Res. J. Reticuloendothel. Soc.* **27:**327.

41. Stahl, P., and S. Gordon. 1982. Expression of a mannosyl-fucosyl receptor for endocytosis on cultured primary macrophages and their hybrids. *J. Cell Biol.* **93:**49.

42. Beller, D. I., and E. R. Unanue. 1981. Regulation of macrophage Ia antigen expression. *In* Naito Symposium on Self Defence Mechanisms, Tokyo. Role of Macrophages. In press.

43. Edelson, P. J., and Z. A. Cohn. 1976. Nucleotidase activity of mouse peritoneal macrophages. I Synthesis and degradation in resident and inflammatory populations. *J. Exp. Med.* **144:**1581.

44. Werb, Z., and Z. A. Cohn. 1972. Plasma membrane synthesis in the macrophage following phagocytosis of polystyrene latex particles. *J. Biol. Chem.* **247:**2439.

45. Mellman, J., J. C. Unkeless, R. Steinman, and Z. A. Cohn. 1981. Internalization and fate of Fc receptors during endocytosis. *J. Cell Biol.* **91:**124a.

46. Kaplan, G., J. C. Unkeless, and Z. A. Cohn. 1979. Insertion and turnover of macrophage plasma membrane proteins. *Proc. Natl. Acad. Sci. U. S. A.* **76:**3824.

47. Nathan, C., L. Bruckner, G. Kaplan, J. Unkeless, and Z. Cohn. 1980. Role of activated macrophages in antibody-dependent lysis of tumor cells. *J. Exp. Med.* **152:**183.