Low response of BALB/c macrophages to priming and activating signals

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Abstract: Trehalose dimycolate (TDM), a mycobacterial glycolipid, is a powerful macrophage-priming agent. However, its efficiency seems limited in the case of BALB/c mice. Peritoneal macrophages harvested from TDM-treated BALB/c mice did not control BCG growth in vitro as efficiently as similar macrophages from two other mouse strains, (B6 × D2)F1 and C57BL/6, which are respectively *Bcg*^{r} and *Bcg*^{s}. BALB/c macrophages elicited by TDM also exhibited a low capacity to produce hydrogen peroxide and, after activation by lipopolysaccharide (LPS), weak cytostatic activity against P815 mastocytoma cells. Finally, alkaline phosphodiesterase, a marker of resident and inflammatory macrophages, was still expressed at a high level in macrophages of BALB/c mice treated with TDM. Low responsiveness of BALB/c macrophages to stimuli was not observed with TDM only; activation for tumor cytotoxicity of thioglycolate-elicited macrophages from BALB/c mice required also higher doses of interferon-γ, and LPS. L-Arginine-dependent production of nitric oxide was inducible in macrophages from BALB/c mice, but the conditions required for its induction were more stringent. Thus, the reduced antiproliferative effects of BALB/c macrophages may be due to uncomplete induction of NO synthase after suboptimal stimulation. *J. Leukoc. Biol.* 52: 315–322; 1992.

Key Words: *antibacterial activity • antitumor activity • nitric oxide • trehalose dimycolate • lipopolysaccharide • interferon-γ*

INTRODUCTION

For several elements of the macrophage response to stimuli, differences have been observed among various strains of mice. Genetic control has been demonstrated for the inflammatory response [1], monocytopoiesis [2], production of interleukin-1 [3], response to interferons [4], resistance to some intracellular pathogens [5], and cytotoxicity against tumor cells [6]. In most cases, it is not known whether differences are due to defects in the perception of stimuli, in the transduction machinery, or in the effector mechanism. A defect in the stimulus-response coupling has been suspected in some cases. For example, macrophages (and B lymphocytes) of C3H/HeJ mice are defective in their response to the lipid A moiety of lipopolysaccharide (LPS) [7], but attempts to correlate deficiency with missing receptors have been unsuccessful [8]. Macrophages from A/J mice are inefficiently activated by bacille Calmette-Guérin (BCG) or interferon-γ but respond to 12-0-tetradecanoylphorbol13-acetate + A23187 and have receptors for interferon-γ [9].

The initial phase of resistance to infection with a large group of parasites (*Salmonella typhimurium, Leishmania donovani, Mycobacterium bovis* BCG etc) is determined in the mouse by the expression of a single dominant autosomal gene on chromosome 1 (*Ity/Lsh/Bcg*) (referred in this report as *Bcg*) [10, 11]. Although the *Bcg* gene product has not yet been identified, most investigators agree that its effects are macrophage mediated [12–15]. To focus on a possible gene product, several groups have extensively studied the multiple functional defects of macrophages harvested from mouse strains that expressed the susceptible phenotype (*Bcg*^{s}) compared to resistant strains (*Bcg*^{r}). Macrophages from *Bcg*^{s} animals are characterized by a diminished respiratory burst, reduced hexose monophosphate shunt activity, impaired production of TNF, and depressed expression of AcM.1 and Ia antigens but manifest stronger 5'-nucleotidase activity [12, 16–19]. Such an accumulation of dysfunctions in *Bcg*^{s} macrophages suggested a defect in their capacity to respond to activation stimuli [16, 20].

In a previous study, we confirmed that a difference in the anti-BCG activity of resident macrophages from *Bcg*^{r} and *Bcg*^{s} strains can be demonstrated in vitro and showed that such a difference is also detectable in macrophages primed in vivo by the action of a bacterial glycolipid, trehalose dimycolate (TDM) [21]. Macrophages develop the capacity to inhibit the growth of extracellular targets or intracellular parasites during the activation process in response to lymphokines, bacterial products, or synthetic immunomodulators. In most experimental models, macrophage activation is a two-step process: (1) young responsive macrophages must first be primed by interferon-γ, pyran copolymers, TDM, etc., and (2) primed macrophages are highly sensitive to bacterial products [LPS, muramyl dipeptide (MDP)] that achieve their activation [22]. Functional and biochemical markers can be used to characterize responsive, primed, and fully activated macrophages [22]. Using some of these markers, we have examined how macrophages obtained from BALB/c mice (a strain in which the anti-BCG activity of macrophages measured in vitro was found to be especially weak) responded to priming signals (TDM, interferon-γ) and to activation signals (LPS).

It appears that the antimicrobial and antitumoral effects of activated mouse peritoneal macrophages depend largely on their capacity to produce nitric oxide from L-arginine [23–30]. Mouse strains in which the activation leads to macrophages with weak antiproliferative capacity may thus present defects either in the transduction mechanisms controlling the expression of NO synthase or in one step of the metabolic pathway allowing the generation of nitric oxide.

Abbreviations: APD, alkaline phosphodiesterase; BCG, bacille Calmette-Guérin; FCS, fetal calf serum; i.p., intraperitoneal; LPS, lipopolysaccharide; MDP, muramyl dipeptide; MEM, minimum essential medium; TDM, trehalose dimycolate; TNF, tumor necrosis factor.

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We have compared the conditions required for the induction of NO synthase in macrophages harvested either from (B6 × D2)F1 or from BALB/c mice.

MATERIALS AND METHODS

Mice

Pathogen-free mice, 7 to 10 weeks old, female or male, were used. They were raised under barrier conditions and received sterile food and water. (C57BL6 × DBA/2)F1, thereafter called (B6 × D2)F1, DBA/2, and C57BL/6 mice were obtained from Iffa Credo (France). BALB/cAnN mice were from Charles River (France). BALB/cBy mice were raised in the INRA facilities (Nouzilly, France), from progenitors purchased at Iffa Credo.

Reagents

Trehalose dimycolate from *Mycobacterium tuberculosis* strain Peurois was prepared as described previously [31]. Lipopolysaccharide, prepared from *Salmonella enteritidis* by the method of Westphal, was purchased from Difco (Detroit, MI), dissolved in saline at 1 mg/ml, and boiled for 30 min. Murine recombinant interferon-γ, with a specific activity of 1×10^7 antiviral units/mg, was a kind gift of Dr. G.R. Adolf (Ernst-Boehringer Institut for Arzneimittel Forschung, Vienna, Austria). Concentrated Eagle's minimum essential medium (MEM) and thioglycolate broth were obtained from Pasteur Production (France); endotoxin-free fetal calf serum (endotoxin < 0.1 ng/ml) was purchased from J. Boy (France); RPMI 1640 supplemented with HEPES and glutamine and RPMI 1640 select-amine kit were from Gibco.

Middlebrook 7H9 broth and Middlebrook ADC enrichment were purchased from Difco; penicillin G was obtained from Serva (Heidelberg, FRG). [^{3}H]thymidine (1 Ci/mmol) was obtained from C.E.A. (France) and [^{3}H]uracil (26 Ci/mmol) was from Du Pont de Nemours (France).

Macrophages

Resident macrophages were obtained from untreated mice. Inflammatory macrophages were harvested 4 days after an intraperitoneal (i.p.) injection of 1 ml of thioglycolate broth. Primed macrophages were harvested 7 days after i.p. injection of 50 μg of TDM.

Peritoneal cells, obtained by washing the peritoneal cavity of mice with 5 ml of MEM, were centrifuged and counted, the number of macrophages being estimated after neutral red uptake. The cells were resuspended at the desired concentration in MEM supplemented with 5% fetal calf serum (FCS) and 100 U/ml of penicillin G, distributed in culture dishes, and allowed to adhere for 4 h at 37°C, in a 5% CO_2 atmosphere. Nonadherent cells were removed by three washes with phosphate-buffered saline.

Assay of anti-BCG activity

M. bovis BCG strain Pasteur 1173P2 was prepared as previously described [21]. Aggregated microorganisms were removed from BCG suspension by centrifugation (800*g*, 4 min) and the number of bacilli present in the supernatant was estimated by measuring the absorbance at 560 nm. BCG (2×10^6), in 150 μl of RPMI 1640 supplemented with FCS and penicillin, were distributed in each well. After various time intervals (from 24 to 96 h), 100 μl of Middlebrook's medium (Middlebrook 7H9 broth enriched with ADC) containing 0.3% saponin and 50 μl of a solution of [^{3}H]uracil (1 μCi) were added to each well. Viable BCG, present extracellularly or released after macrophage lysis by saponin, were estimated by the level of [^{3}H]uracil incorporation into bacterial nucleic acids during a 16-h pulse. BCG growth rate was measured in each experiment from the [^{3}H]uracil incorporation at 3, 24, and 48 h in control wells. The level of uracil incorporation was recorded on a log scale as a function of time in culture. The apparent multiplication index of BCG after 72 h in the presence of macrophages was calculated by dividing [^{3}H]uracil uptake (in cpm) by BCG cocultured for 72 h with macrophages by the [^{3}H]uracil uptake by BCG in control wells at the beginning of the experiment (after 3 h). When the multiplication index equaled 1, it was considered that zero population growth was obtained. A figure greater (or smaller) than 1 meant that the number of viable BCG was increasing (or decreasing) with time, the apparent multiplication index for BCG in the absence of macrophages being 16.7 after 72 h.

Assay of cytostatic activity against tumor cells

Expression of cytostatic activity was obtained (1) after a treatment of TDM-primed macrophages with 10 ng/ml LPS during the 4-h adherence period and (2) after a treatment of thioglycolate-elicited macrophages during 24 h with 10 U/ml interferon-γ and 10 ng/ml LPS. Cytostatic activity of macrophages against P815 mastocytoma cells was measured as previously described, by a [^{3}H]thymidine cumulative incorporation assay [31]. Briefly, P815 cells (0.3×10^6 cells/ml) were added to macrophage monolayers (1.2×10^6 macrophages/ml) in MEM supplemented with 5% FCS and antibiotics and containing 1.2 μM [^{3}H]thymidine (1 Ci/mmol). After 18 h, P815 cells were collected on glass fiber filters and radioactivity was determined by liquid scintillation spectrometry. The results were expressed as P815 residual growth, growth of P815 cells cocultured in the presence of macrophages being compared to the growth of P815 cells cultured alone.

H_2O_2 Production

The rate of H_2O_2 release was calculated according to the method of Pick and Keisari [32]. After adherence, macrophages (1×10^6 macrophages/ml) were incubated in Hanks' balanced salt solution containing 28 μM phenol red, 0.16 μM horseradish peroxidase (Sigma), and 0.1 μM TPA (Sigma). After various time intervals (10, 20, 40 min) supernatants were collected and alkalized with NaOH and the absorbance at 560 nm was recorded.

Alkaline phosphodiesterase activity

Peritoneal cells (1.2×10^6 macrophages/ml) were plated in 35-mm dishes After 4 h at 37°C, nonadherent cells were washed out. Macrophage monolayers were lysed with 1 ml of 0.1% Triton X-100. A 100-μl lysate aliquot was added in a spectrophotometer cuvette containing 1.5 mM *p*-nitrophenyl-thymidine-5'-monophosphate (Sigma) in 100 mM glycine-NaOH buffer (pH 9.6) and 2 mM zinc acetate. The variation of the absorbance was recorded at 420 nm (37°C).

Nitrite and citrulline release

Macrophage supernatants were collected and centrifuged. Nitrite was assayed by the Griess reaction. The reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in

1 N HCl) and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.15% in H_2O). A volume of 600 μl of reagent was mixed with 100 μl of sample and incubated 30 min in the dark. The absorbance was measured at 540 nm.

Urea was eliminated prior to citrulline determination by pretreating the samples with 0.5 mg/ml urease (Sigma) for 1 h at 37°C. Samples (400 μl) were then added with 100 μl of 40% trichloroacetic acid and centrifuged; 400 μl of supernatant was added to 2 ml of a mixture, in equal proportions, of 59.3 mM diacetylmonoxine (in H_2O) and 31.9 mM antipyrine + 55 μM ferric sulfate (in 6 N H_2SO_4). Samples were heated for 15 min at 95°C. Absorbance was measured at 465 nm.

Protein in cell lysates was measured by the method of Lowry with bovine serum albumin used as a standard.

RESULTS

TDM-elicited macrophages harvested from BALB/c mice have low anti-BCG activity

In several strains of mice, injection i.p. of TDM elicited macrophages that expressed strong anti-BCG activity in vitro; the apparent multiplication index of BCG cocultured 72 h with these macrophages was always less than 1 [0.43 ± 0.13 in the case of (B6 × D2)F1 and 0.54 ± 0.14 in the case of C57BL/6] [21]. In contrast, peritoneal macrophages elicited by TDM in BALB/c mice did not block BCG growth efficiently during long-term cultures; apparent multiplication indices of BCG calculated at 72 h were 2.25 ± 0.37 and 2.89 ± 0.78 with macrophages from BALB/cAnN and BALB/cBy, respectively [21].

The cytostatic activity induced by LPS in macrophages primed by TDM in BALB/c mice is lower than in several other strains

Mouse peritoneal macrophages are not spontaneously cytostatic against tumor cells; to develop strong cytostatic activity, macrophages must first be primed (with BCG, interferon-γ, pyran copolymers, TDM, etc.) and then activated (with LPS, MDP, TNF-α, etc.) [33–35]. The efficiency of LPS as an activating agent is impressive: a short exposure (4 h) to low doses of LPS (<10 ng/ml) was generally sufficient to induce TDM-primed macrophages to express strong cytostatic activity; with macrophages from (B6×D2)F1 and C57Bl/6, the residual growth of P815 mastocytoma cells was routinely less than 5% (with an effector-to-target ratio of 4 at the beginning of the coculture). As shown in **Table 1**, in the case of macrophages harvested from BALB/c mice, the cytostatic activity against P815 cells was significantly lower than in the two other mouse strains tested.

TABLE 1. Cytostatic Activity Induced by LPS in Peritoneal Macrophages Elicited by Trehalose Dimycolate in Various Mouse Strains[a]

Mouse strain	Tumor residual growth (% of control)	Statistical data: *n*	Statistical data: *P*
BALB/cAnN	23.1 ± 4.2	8	0.003
BALB/cBy	22.3 ± 7.6	5	0.03
(B6 × D2)F1	4.2 ± 1.4	5	
C57BL/6	7.1 ± 2.9	6	0.37

[a]TDM-elicited macrophages were treated with 10 ng/ml LPS for 4 h; then P815 mastocytoma cells were added on macrophage monolayers at a macrophage/P815 ratio of 4 and P815 cells and macrophages were cocultured for 20 h, in the presence of [^{3}H]thymidine, in LPS-free medium. Tumor residual growth was measured by comparison to control wells containing P815 cells only ([^{3}H]thymidine incorporation in control wells = 10^5 cpm). Results are expressed as mean of *n* independent experiments ± SEM. The significance, *P*, of the differences observed between the different mouse strains and (B6 × D2)F1 mice was calculated using Student's unpaired *t*-test.

TABLE 2. Rate of Hydrogen Peroxide Production after TPA Triggering of Peritoneal Macrophages Elicited by Trehalose Dimycolate in Various Mouse Strains[a]

Mouse strain	Hydrogen peroxide production (nmol/min/100 μg protein)	Statistical data: *n*	Statistical data: *P*
BALB/cBy	82 ± 2.1	3	
(B6 × D2)F1	178 ± 1.4	3	0.004
C57BL/6	182 ± 37.0	3	0.029

[a]Results are expressed as mean of *n* independent experiments ± SEM. The significance, *P*, of the differences observed between BALB/cBy mice and the two other mouse strains was calculated using Student's unpaired *t*-test.

The efficiency of TDM as a priming agent is reduced in BALB/c mice

Primed macrophages can be distinguished by two biochemical markers: unlike resident and inflammatory macrophages, they produce high levels of hydrogen peroxide upon triggering and they have low alkaline phosphodiesterase (APD) activity. The figures found for hydrogen peroxide production and APD activity for macrophages elicited by TDM in various mouse strains are given in **Tables 2 and 3.** The rate of hydrogen peroxide production is twice as high in (B6 × D2)F1 and C57BL/6 as in BALB/c. The level of APD is reduced 8- to 10-fold after priming in (B6 × D2)F1 and C57BL/6 but only 3-fold in BALB/c. By four criteria—induction of NADPH oxidase, increase of anti-BCG activity, increase of sensitivity to LPS, and down-regulation of APD activity—the efficiency of TDM as a priming agent is reduced in BALB/c mice.

Higher doses of interferon-γ and LPS are required to activate thioglycolate-elicited macrophages from BALB/c mice

Cytostatic activity against P815 mastocytoma cells can be induced in thioglycolate-elicited macrophages by a 24-h treatment with interferon-γ and LPS. Thioglycolate macrophages harvested from BALB/c mice were activated for cytotoxicity against tumor cells when incubated with high doses of interferon-γ (10 U/ml) and LPS (5 ng/ml). However, macrophages harvested from (B6 × D2)F1 responded to fivefold lower doses of interferon-γ and of LPS. As shown in **Figure 1,** thioglycolate macrophages from (B6 × D2)F1 were fully activated (P815 residual growth = 4%) in the presence of 0.5 U/ml interferon-γ and 10 ng/ml LPS or 1 U/ml interferon-γ and 2 ng/ml LPS. In such conditions thioglycolate macrophages from BALB/c were devoid of antitumor action.

Anti-BCG activity of macrophages from BALB/c and (B6 × D2)F1 mice is partly dependent on arginine and NO synthase activity

Activation of macrophages is correlated with the induction of NO synthase and L-arginine–dependent production of nitric oxide, which has antiproliferative effects on tumor cells, parasites, and intracellular pathogens [23–30]. We thus exa-

TABLE 3. Alkaline Phosphodiesterase Activity in Peritoneal Macrophages Elicited by Trehalose Dimycolate in Various Mouse Strains[a]

Mouse strain	APD activity (nmol substrate hydrolyzed/h/100 μg protein)	Statistical data	
		n	*P*
BALB/cBy	27.5 ± 4.5	6	
(B6 × D2)F1	9.3 ± 0.9	6	0.003
C57BL/6	14.8 ± 4.7	4	0.097

[a]The activity of alkaline phosphodiesterase in resident macrophages was equivalent to 75 nmol of substrate hydrolyzed/h/100 μg protein, whatever the strain of mice used. Results are as expressed in Table 2.

mined the L-arginine and NO synthase dependence of the anti-BCG activity of TDM-primed macrophages; this functional test was selected because it showed large differences between macrophages obtained from (B6 × D2)F1 and BALB/c mice. With both macrophage populations, anti-BCG activity was partly dependent on NO synthase activity: BCG growth increased in the absence of arginine or in the presence of NO synthase inhibitor (**Table 4**). However, diagrams of variations of anti-BCG activity of TDM-elicited macrophages as a function of arginine concentration in the culture medium were clearly different for (B6 × D2)F1 and BALB/c mice (**Fig. 2**). The optimal expression of anti-BCG activity of (B6 × D2)F1 macrophages required large quantities of exogenous arginine (3.45 mM) and thus probably high and continuous activity of NO synthase. By contrast, in the case of macrophages harvested from BALB/c, the anti-BCG activity observed did not increase further when the arginine concentration was made greater than 0.38 mM. Arginine did not seem to be the factor limiting the antimycobacterial activity of BALB/c macrophages. The differences in arginine dependence shown in Figure 2 for the two mouse strains were not due to differences in arginase activity; similar amounts of ornithine accumulated in 24 h as measured by radio-HPLC [36] of the macrophage supernatants (data not shown). It has been demonstrated that NO synthase activity is associated with a reduction of macrophage viability in long-term cultures [37]. However, when estimated by the release of lactate dehydrogenase into supernatants [37], the viabilities after full activation of the two types of macrophages were similar (data not shown).

NO synthase is difficult to induce in BALB/c macrophages

With optimally activated macrophages, we did not find significant differences in the quantities of NO synthase metabolites accumulated after 24–48 h in macrophage culture medium. Activated macrophages from (B6 × D2)F1 and BALB/c were able to produce up to 250 nmol of citrulline and 100 nmol of nitrite per 10^6 cells (after 24 h, arginine concentration was probably limiting, because of the high arginase activity of macrophages). However, the rate of production of NO synthase metabolites by macrophages (elicited by TDM or by thioglycollate) from BALB/c was slightly lower that observed with (B6 × D2)F1 macrophages (**Fig. 3**).

By contrast, after suboptimal activation, differences in the NO synthase activity of macrophages harvested from (B6×D2)F1 and BALB/c mice became apparent. As shown in Figure 3A, when LPS was not continuously present but was applied only over a limited period of time (4 h), the production of NO synthase metabolites was extremely limited in the case of BALB/c macrophages; in the case of (B6×D2)F1 macrophages, the rate of production during the first 24 h was only slightly reduced and the accumulation of nitrite or citrulline in the medium after 24 h was at least three times higher than in BALB/c macrophage medium. The same phenomenon was observed after stimulation of thioglycolate-elicited macrophages by interferon-γ and LPS (Fig. 3B).

LPS treatment of TDM-primed macrophages was necessary in order to observe cytostatic activity against tumor cells (Table 1), but it was not required for anti-BCG activity (Fig. 2A). We verified that BCG bacilli were able to trigger NO synthase activity in TDM-primed macrophages; even at a ratio as low as one bacillus per macrophage, strong production of nitrite and citrulline was induced. For example, nitrite production was 148.6% ± 7.3 (n=6) that induced by 10 ng/ml LPS. BCG was also active on macrophages harvested from BALB/c; despite their lower accumulation of nitrite (63% ± 5, n=9) in the medium than that observed with (B6×D2)F1 macrophages, we did not find large enough differences in the kinetics or duration of NO synthase metabolite production to explain the limited anti-BCG activity of BALB/c macrophages.

A diminished antiproliferative activity observed in some circumstances in macrophages of BALB/c mice may be due

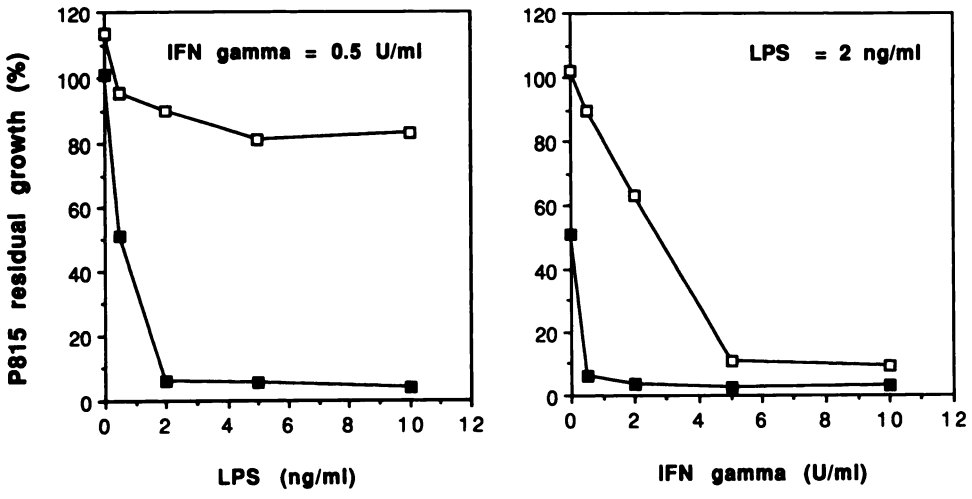

Fig. 1. Activation of thioglycolate-elicited macrophages for tumor cytotoxicity. Thioglycolate-elicited macrophages were treated during 24 h with various doses of interferon-γ (0, 0.5, 2, 5, and 10 U/ml) and of LPS (0, 0.5, 2, 5, and 10 ng/ml). After this treatment, macrophage monolayers were washed and P815 mastocytoma cells and [^{3}H]thymidine were added. Cytostatic activity was measured after 18 h in triplicates. Standard deviations were within 10% of the mean. The data are representative of three separate experiments. (□) BALB/c macrophages; (■) (B6×D2)F1 macrophages.

TABLE 4. Reduction of Anti-BCG Activity of TDM-Elicited Macrophages in the Presence of Arginase or *N*-Monomethyl-L-arginine[a]

Mouse strain	Anti-BCG activity of macrophages (apparent multiplication index of BCG after 72 h)		
	Control	+ NMMA	+ Arginase
BALB/cAnN	2.40	4.16	6.25
(B6 × D2)F1	0.40	0.74	1.25
C57BL/6	0.46	0.72	—

[a]Experimental procedure as in Figure 2; *N*-monomethyl-L-arginine (Calbiochem) was 0.1 mM and arginase (Sigma) 7 U/ml. The apparent multiplication index of BCG after 72 h was 16.7 in the absence of macrophages and 2.38, 9.58, and 12.08 in the presence of resident macrophages harvested from (B6 × D2)F1, C57BL6, and BALB/c, respectively.

to incomplete or transient induction of NO synthase when activating signals are suboptimal. Because BCG bacilli delivered especially strong stimulation, this explanation cannot be applied to the anti-BCG activity.

DISCUSSION

Macrophages recovered from peritoneal exudates of mice pretreated with TDM exhibit several functional and biochemical properties characterizing preactivated (or primed) macrophages: a low level of alkaline phosphodiesterase activity, a high rate of hydrogen peroxide production upon TPA or zymosan triggering, and high susceptibility to LPS as an activating agent [34]. These markers have been described in macrophages obtained after infection of animals by BCG or after stimulation by pyran copolymers or interferon-γ [33, 35, 38]. In this report, we demonstrated that TDM was not an efficient priming agent in BALB/c mice in contrast to other mouse strains [(B6×D2)F1, C57BL/6]. In macrophage monolayers prepared from BALB/c mice, the rate of hydrogen peroxide production after addition of TPA was lower than in monolayers prepared from (B6×D2)F1 or C57BL/6 mice. By contrast, the level of alkaline phosphodiesterase, an enzyme strongly expressed in resident and inflammatory macrophages, was higher in macrophage cultures from BALB/c mice. Finally, TDM macrophages harvested from BALB/c mice developed limited cytostatic activity against tumor cells in response to LPS. Differences in the level of anti-BCG and antitumor activities that can be induced in macrophages from BALB/c and from other mouse strains have been observed over a period of 3 years, with two different sublines of BALB/c mice from two different breeding facilities. Thus, the adherent cell population elicited by TDM in BALB/c mice seems to contain incompletely primed macrophages or a reduced proportion of

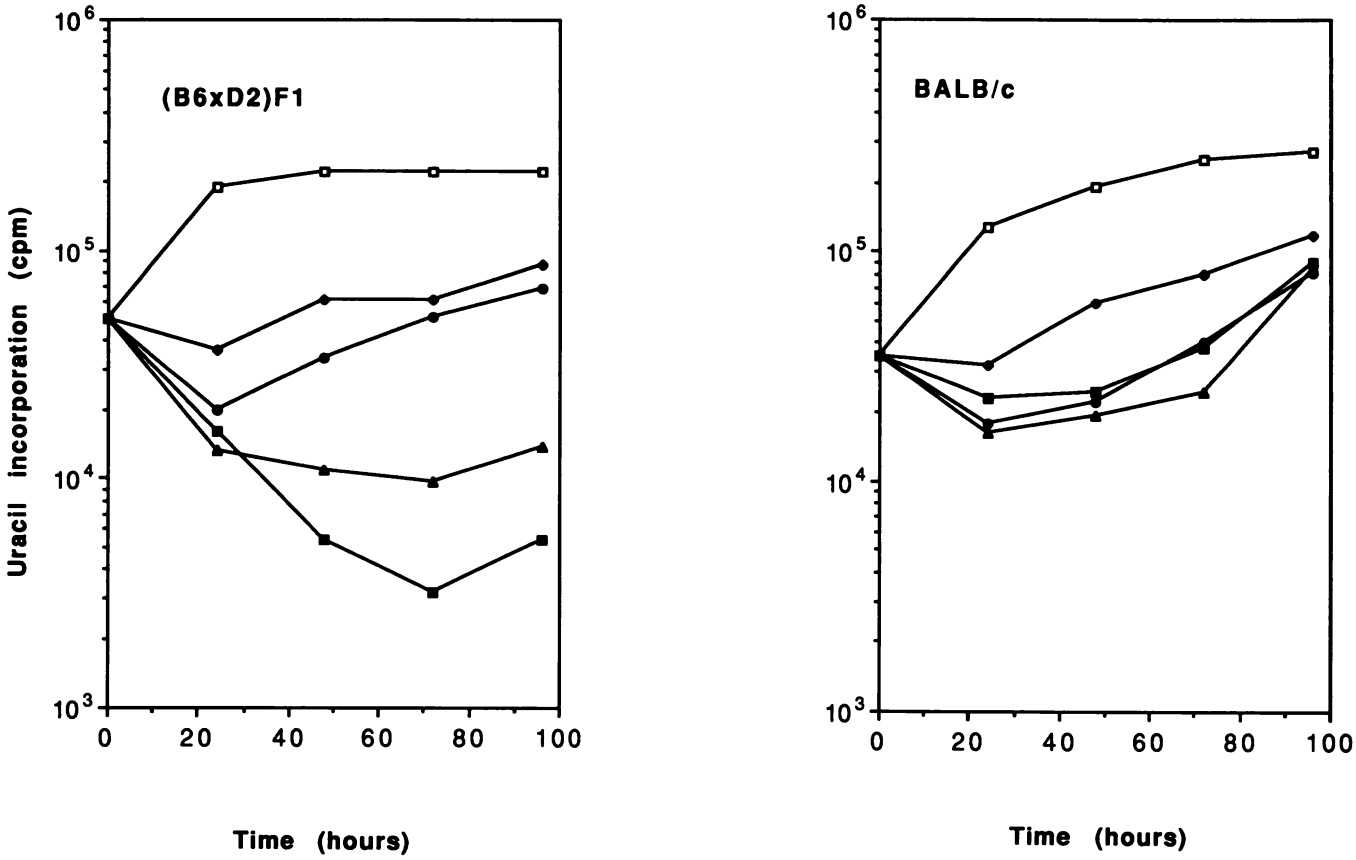

Fig. 2. Arginine dependence of the anti-BCG activity of TDM-elicited macrophages. Peritoneal cells collected from TDM-treated mice [(B6×D2)F1 or BALB/c strain] were plated in microtest wells (0.4 × 10^6 macrophages/well) in MEM supplemented with 5% FCS and penicillin. After 4 h, nonadherent cells were washed out; wells received 150 μl of RPMI 1640 medium reconstituted with various concentrations of arginine and 0.2 × 10^7 BCG bacilli. After 3, 24, 48, 72, and 96 h, 100 μl of Middlebrook medium supplemented with ADC and 0.3% saponin and 50 μl [^{3}H]uracil were added. After 16 h, radioactivity incorporated by BCG was measured. Results are mean uracil incorporation for triplicates, SD values being within 10% of the mean. The data are representative of three separate experiments; taking into account the results of these three experiments, uracil incorporation measured after 72 h in cultures containing 3.45 mM arginine was reduced to 4.3% ± 0.6 of the incorporation measured with no arginine added in the case of (B6×D2)F1 macrophages and to 46.8% ± 0.2 in the case of BALB/c macrophages. BCG added to TDM-elicited macrophages cultured in the presence of (♦) no added arginine; (●) 0.38 mM arginine; (▲) 1.15 mM arginine; (■) 3.45 mM arginine. (□) BCG alone in control wells.

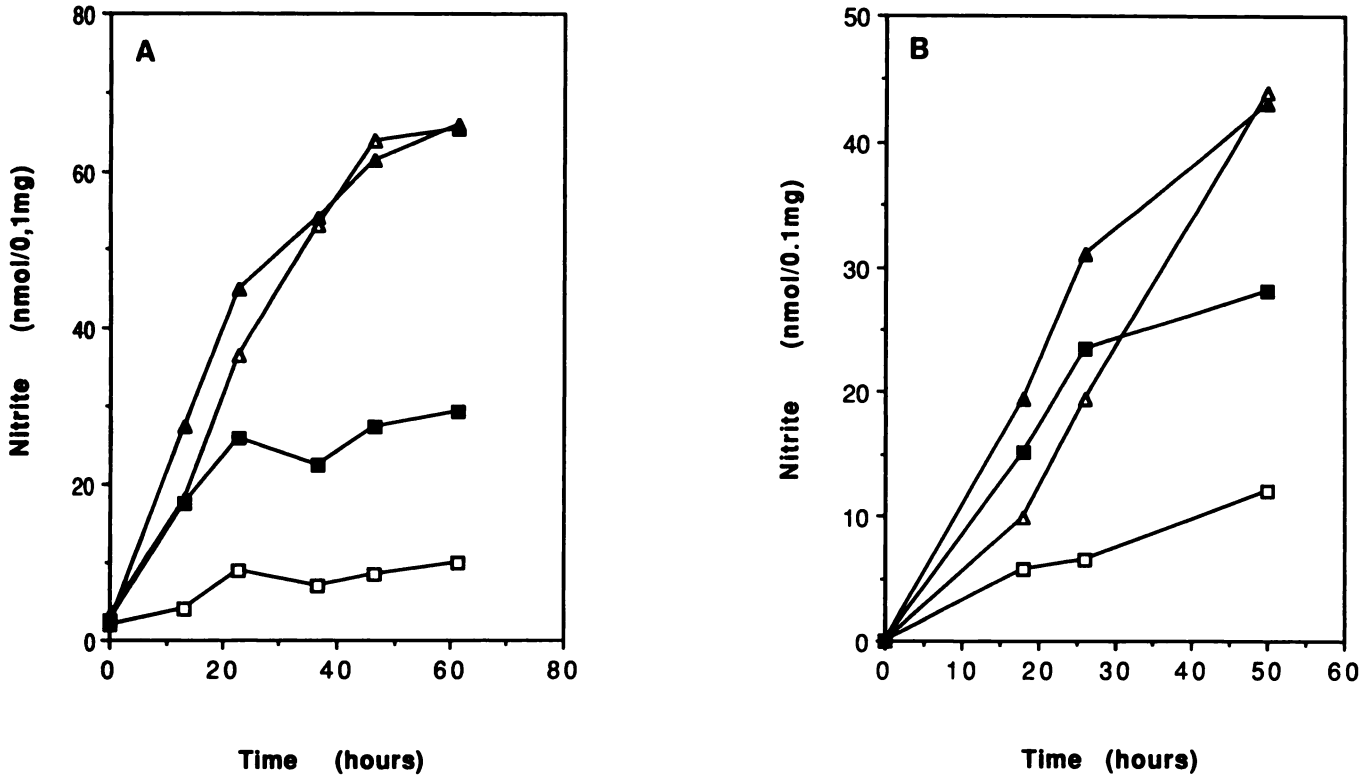

Fig. 3. Kinetics of nitrite production by activated macrophages: (A) TDM-elicited macrophages treated with LPS; (B) thioglycolate-elicited macrophages treated with interferon-γ and LPS. Macrophages were obtained from BALB/c mice (open symbols) or from (B6×D2)F1 mice (filled symbols). The production of nitrite was measured as a function of time. (A) TDM-elicited macrophages were incubated 4 h with 5 ng/ml LPS, washed, and placed in RPMI 1640 supplemented with 5% FCS and antibiotics without LPS (■, □) or with 5 ng/ml LPS (▲, △). (B) Thioglycolate-elicited macrophages were cultured 24 h in the presence of 5 U/ml interferon-γ and 1 ng/ml LPS. After the incubation, macrophage monolayers were washed and placed in RPMI 1640 supplemented with 5% FCS and antibiotics without interferon-γ and LPS (■, □) or with 5 U/ml interferon-γ and 1 ng/ml LPS (▲, △). The data are representative of three (A) or two (B) separate experiments. In each sample, nitrite (this figure) and citrulline (data not shown) were assayed, both assays leading to identical conclusions.

primed macrophages. It has been reported that the inflammatory response is limited in BALB/c mice. Such a defect does not explain our observations: the number of peritoneal cells and the percentage of macrophages in the exudates were similar in the various strains of mice tested (data not shown). The enzymatic activities (alkaline phosphodiesterase, NADPH oxidase, NO synthase) were calculated with reference to the protein content of macrophage monolayers.

The low responsiveness of BALB/c macrophages to stimulation was observed with stimuli other than TDM and thus may be a general phenomenon. Macrophages elicited by a sterile inflammatory agent such as thioglycolate broth required higher doses of interferon-γ and LPS to become activated for tumor cytotoxicity when they originated from BALB/c mice instead of (B6 × D2)F1; after optimal stimulation, similar responses were observed in the two strains. Our results are in agreement with the observations reported by Roach et al. on the induction of antileishmania activity in *Lsh*^{r} and *Lsh*^{s} bone marrow-derived macrophages [29] but clearly different from those reported by Liew et al., who found large differences in the ability of *Lsh*^{r} and *Lsh*^{s} to produce NO, even after optimal stimulation [39]. Inhibition of tumor cell growth and of BCG multiplication depends, at least in part, on the same biochemical mechanism: the production of nitric oxide from L-arginine by an inducible enzyme, NO synthase. The NO synthase pathway was not defective per se in BALB/c macrophages: after optimal stimulation, the levels of enzymatic activity measured were similar in macrophages from BALB/c and from (B6×D2)F1. However, when suboptimal stimulation was applied, the induction of the NO synthase was always lower in BALB/c macrophages. Suboptimal stimulation can be generated by using low concentrations of LPS (1 to 3 ng/ml), by limiting the duration of the stimulation, or by replacing LPS with a less potent agent such as muramyl dipeptide (MDP) (data not shown).

The low antitumor or antibacterial activities observed in BALB/c macrophages may be due principally to a defective response to priming and activating signals. Neither a limitation in the number of cells nor absence of an effector mechanism has been observed. Because the intensity of macrophage priming and activation was inferior in the case of BALB/c mice independently of the stimulus used (TDM, interferon-γ, LPS, MDP, or viable BCG), it seems that a common intracellular mechanism (controlling the production of a second messenger, gene activation, or mRNA stability) is partly defective in BALB/c macrophages. The defect may be related to the *Bcg* gene if one supposes that the anti-BCG activity is controlled, even in resident macrophages, by the intensity of the response to BCG bacilli as a stimulation signal. It may also be superimposed in BALB/c mice on the defect depending on the susceptible allele of the *BCG* gene, because in several parameters defects of BALB/c macrophages appeared more pronounced than those in macrophages collected from C57BL/6 mice, another *Bcg*^{s} strain [21].

In the experimental conditions we used routinely to induce cytostatic activity against tumor cells in TDM-primed

macrophages, LPS was present only during the 4-h adherence period. Therefore, sluggish induction of NO synthase after suboptimal stimulation may explain the reduced antitumor activity of macrophages elicited by TDM in BALB/c mice. However, it is more difficult to explain the low anti-BCG activity of these macrophages, which were able to produce reduced but still reasonable amounts of NO synthase metabolites after addition of BCG.

Several observations suggest that the capacity to produce NO from L-arginine is an important part of macrophage anti-BCG activity [24, 25]. However, other anti-BCG mechanisms may act in parallel or even in synergy with NO: the restoration of BCG growth in the presence of *N*-monomethyl-L-arginine was partial (Table 4) and resident macrophages harvested from *Bcg*^{r}-mice were bacteriostatic [21]. Macrophages from BALB/c mice were able to generate NO if they were stimulated continuously but did not express NO synthase activity after withdrawal of activating signals. In fact, they present a reduced sensitivity to multiple stimuli resulting in multiple functional defects. From the literature, it appears that BALB/c mice, in addition to their susceptibility to several intracellular pathogens [5], present several defects compared to other mouse strains: a defective inflammatory response [1], low interferon-γ production in response to BCG/purified protein derivative [40], but high sensitivity of some hematopoietic precursors to interferon-γ [4]. The defects described in this report were never complete and thus they may not be physiologically important under in vivo conditions. However, our observations show that the macrophage activation process may be different in different mouse strains and that BALB/c mice, a frequently used experimental model, in fact have peculiar properties.

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Low response of BALB/c macrophages to priming and activating signals.

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