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Reduced Replication of *Toxoplasma gondii* Is Necessary for Induction of Bradyzoite-Specific Antigens: a Possible Role for Nitric Oxide in Triggering Stage Conversion

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Stage conversion between tachyzoites and bradyzoites of *Toxoplasma gondii* was investigated in vitro by using murine bone marrow-derived macrophages (BMMs) as host cells. Following infection of untreated BMMs with tachyzoites, spontaneous expression of bradyzoite-specific antigens (Bsa) occurred at low frequency with *Toxoplasma* strain-dependent ratios from 0.03 to 2%. As previously described for peritoneal macrophages, activation of tachyzoite-infected BMMs with gamma interferon (IFN-γ) or lipopolysaccharide resulted in the induction of Bsa. When bradyzoites were used for infection, prolonged expression of Bsa could be observed in IFN-γ-activated BMMs. The induction of Bsa expression seemed to be closely linked to parasite multiplication and increased to maximal values of 50 to 70% in intermediately activated macrophages with nitric oxide (NO) levels that allowed reduced parasite replication. Identical results in stage conversion were obtained when sodium nitroprusside was used as a source of exogenous NO, indicating that NO might be a molecular trigger of stage conversion. NO is reactive with iron-sulfur centers in proteins, thereby inhibiting proteins involved in the mitochondrial respiratory chain. Using oligomycin and antimycin A as inhibitors of mitochondrial function, growth inhibition of parasites and induction of Bsa were obtained. Since microglia are the functional correlates of macrophages in the central nervous system and inhibit *T. gondii* upon activation with IFN-γ, a similar mechanism might be involved during cyst development in the brain.

The intracellular protozoan parasite *Toxoplasma gondii* is able to interconvert in the human host between two stages; acute infection is associated with rapid multiplication of tachyzoites and is regularly overcome by immunocompetent patients after several weeks. Gamma interferon (IFN-γ) seems to be the major mediator for resistance in acute infection (19, 26, 28, 29). During latent infection, cysts, containing slowly dividing bradyzoites, are formed mainly in the brain and, to a lesser extent, in muscle tissue and other organs. They persist and normally remain silent throughout life. However, reactivation of cysts accompanied by conversion of bradyzoites to tachyzoites is a major problem in immunocompromised hosts and may result in toxoplasmic encephalitis (18, 27). So far, factors that are responsible for establishing chronic infection and factors that promote reactivation of silent brain cysts rarely have been investigated. Elucidation of a trigger or triggers for interconversion should give more insight into the process of stage conversion and into the organotrophic behavior of cyst formation. In addition, identifying possible triggers might be valuable for developing therapies and drugs to prevent reactivation of brain cysts in immunocompromised patients. To analyze stage conversion in vitro, it is important to differentiate accurately between tachyzoites and bradyzoites; recently, bradyzoite-specific monoclonal antibodies (MAbs), in addition to preexisting tachyzoite-specific MAbs, have been developed (2, 21, 30, 31). By using these tools, we have been able to analyze stage conversion in vitro. Expression of bradyzoite-specific antigens (Bsa) seems to occur spontaneously at low frequency in tachyzoite-infected cell cultures (2, 25). In a previous study, we demonstrated that IFN-γ-activated peritoneal macrophages are able to induce the expression of Bsa to a significantly greater extent than nonactivated macrophages (2).

In this study, bone marrow-derived macrophages (BMMs) of mice were used instead of peritoneal macrophages because BMMs represent a more homogeneous population. Induction of Bsa was found to be associated with (i) an intermediate stage of activation of macrophages defined by nitric oxide (NO) release and with (ii) reduced replication of parasites. In addition, it was demonstrated that exogenous NO, as well as inhibiting mitochondrial function, is sufficient to inhibit parasite growth and to induce Bsa expression.

MATERIALS AND METHODS

Parasites. In this study, the mouse-virulent *T. gondii* strains RH and BK and the mouse-avirulent strains NTE, 561, and DX were used (12). Tachyzoites were maintained in L929 murine fibroblasts as host cells and were harvested when host cell lysis initiated. The culture medium consisted of RPMI medium (Biochrom, Berlin, Germany), supplemented with 1% fetal calf serum, 300 mg of glutamine per liter, and penicillin-streptomycin. Cysts of strain NTE were separated from mouse brain by continuous density gradient centrifugation as previously described (6). Bradyzoites were released from cysts by digestion with 300 U of pepsin per ml (Sigma, Deisenhofen, Germany) in 0.9% NaCl (pH 1.5) for 2 min, followed by washing with phosphate-buffered saline and centrifugation.

Infection assay. Bone marrow cells were isolated from 12- to 16-week-old NMRI mice (Zentralinstitut für Versuchstierzucht, Hannover, Germany) and seeded at a concentration of 2×10^5 cells per well on 13-mm cover slides located within 24-well microtiter plates. The medium consisted of RPMI-Click medium (Biochrom) supplemented with 10% fetal calf

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serum, 300 mg of L-glutamine per liter, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), penicillin-streptomycin, and 25% supernatant from L929 fibroblasts (conditioned medium) as a source of macrophage colony-stimulating factor (3). For most experiments, BMMs were infected with 10^5 tachyzoites per well on day 7. To avoid host cell lysis, the infection dose was 10^4 tachyzoites per well for a cultivation time of 4 days following infection in untreated macrophages. In cases in which no other time is mentioned, drugs and immunomodulators were added simultaneously during the time of infection. Before IFN-γ was added 3 h postinfection (p.i.), BMMs were washed three times with medium to remove extracellular parasites.

143B and 143B/260 human fibroblasts (kindly provided by M. P. King [13]) were also cultured on cover slides in microtiter plates and infected with 10^5 tachyzoites.

MAbs. The generation and characterization of bradyzoite-specific MAbs 4F8 and 7E5 have been described previously (2).

Immunofluorescence assay. Slides were fixed for 5 min in methanol on days 2 and 4 postinfection and incubated in turn with (i) bradyzoite-specific MAb 4F8 or 7E5, (ii) fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma), (iii) polyclonal mouse anti-toxoplasma serum, and (iv) tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma). Total and relative numbers of MAb 4F8-reactive parasitophorous vacuoles were determined by counting each vacuole as 1 unit, independent of the number of parasites within each vacuole. The replication rate of parasites was analyzed semiquantitatively and is given in numbers of parasites per vacuole. Each individual experiment was performed at least three times. Data given in the tables are from a representative experiment.

Determination of nitrite. Culture fluids were assayed for NO_2^- by the Griess reaction (16). Basically, 100 μl of supernatant was incubated for 5 min with 100 μl of Griess reagent and the optical density at 543 nm was measured with $NaNO_2$ as a standard.

Reagents. Lipopolysaccharide (LPS) (from *Salmonella typhimurium*), polymyxin, catalase (from bovine liver), antimycin A (from a *Streptomyces* sp.), and oligomycin were obtained from Sigma. N^G-monomethyl-L-arginine (NMMA) was obtained from Calbiochem, Frankfurt am Main, Germany. IFN-γ and aphidicolin were obtained from Boehringer Mannheim (Mannheim, Germany). Sodium nitroprusside (SNP) and potassium hexacyanoferrate(III) were obtained from Fluka (Neu-Ulm, Germany).

RESULTS

Kinetics of Bsa expression in IFN-γ-activated BMMs infected with tachyzoites. In a previous report, we demonstrated the inductive effect of IFN-γ on the expression of Bsa in peritoneal macrophages infected with tachyzoites. In this study, we used BMMs as host cells, because they represent a more homogeneous population than peritoneal macrophages. All experiments with activated BMMs were performed with *T. gondii* NTE, a recent clinical isolate with little virulence in mice (12). At the time of infection, BMMs were simultaneously activated with 100 U of IFN-γ per ml, resulting in total inhibition of parasite replication. Under these conditions, parasitophorous vacuoles contained single parasites. Reactivity with the bradyzoite-specific MAb 4F8 was found not to be enhanced above the normal background level of 1 to 2%. However, when the NO synthase inhibitor NMMA was added to cultures 24 h p.i., the complete block of parasitic growth was abolished and reduced parasite multiplication occurred. On day 4 p.i., parasitophorous vacuoles contained 2 to 16 parasites; the portion of MAb 4F8-reactive vacuoles increased to 42% (Table 1). A second Bsa, recognized by MAb 7E5, was expressed to the same extent (data not shown). Six days p.i., enhanced parasite growth in IFN-γ–NMMA-treated BMMs resulted in partial host cell lysis and the portion of MAb 4F8-reactive parasites decreased to less than 5%. To exclude a direct influence of IFN-γ on tachyzoites prior to invasion, IFN-γ was added (i) 3 h before infection and (ii) 3 h p.i. after removal of extracellular parasites by several washing steps. NMMA was added at a final concentration of 100 μM 24 h p.i. Independent of the time of infection, parasites expressed Bsa up to 50%, indicating that IFN-γ is not triggering stage conversion by direct interaction with tachyzoites. NMMA without IFN-γ has neither an effect on replication of parasites nor an effect on stage conversion.

TABLE 1. Kinetics of bradyzoite-specific antigen expression of *T. gondii* in activated BMMs infected with strain NTE tachyzoites

Day p.i.	% of MAb 4F8-reactive vacuoles		
	Control[a]	100-U/ml IFN-γ[b]	100-U/ml IFN-γ and NMMA[c]
2	<2	<2	<2
4	<2	<2	42 ± 7[d]
6	<2	<2	<5

[a] Samples for days 4 and 6 were infected with 10^4 tachyzoites per well instead of 10^5 tachyzoites per well to avoid host-cell lysis.
[b] No parasite multiplication occurred under these conditions. The number of individual parasites decreased from day 2 to day 6 p.i.
[c] NMMA was added 24 h p.i. at a final concentration of 100 μM.
[d] Mean ± standard error (n = 5).

Prolonged expression of Bsa in activated BMMs infected with bradyzoites. Using bradyzoites instead of tachyzoites for infection, the influence of activated BMMs on expression of Bsa could also be observed (Table 2). The conversion from bradyzoites to tachyzoites in untreated BMMs was completed within 4 days p.i., expression of Bsa decreased to 2%, and parasites had undergone rapid replication with the first foci of lysed host cells. In contrast, like in tachyzoite-infected cells, activation of BMMs with 100 U of IFN-γ per ml resulted in complete inhibition of bradyzoite replication. Four days p.i., only a few individual parasites resided within macrophages, indicating that most bradyzoites were killed by activated macrophages. Decreased NO release by adding NMMA or replacing medium 24 h p.i. enabled slow parasite multiplication, with an average of two to four parasites being located within a parasitophorous vacuole 4 days p.i. About 90% of these vacuoles were still strongly reactive with MAb 4F8. Six

TABLE 2. Prolonged expression of bradyzoite-specific antigens in activated BMMs infected with strain NTE bradyzoites

Treatment[a]	% of MAb 4F8-reactive vacuoles	No. of parasites/vacuole
IFN-γ (0–96 h)	100 (day 4)	1
IFN-γ (0–96 h)–NMMA (24–96 h)	93 (day 4)	2–4
Control	<2 (day 4)	2–50
IFN-γ (0–144 h)–NMMA (24–144 h)	86 (day 6)	2–20
Control	10 (day 3)	2–20

[a] The length of stimulation with IFN-γ and NMMA is given in parentheses. The IFN-γ concentration was 100 U/ml. NMMA was added at a final concentration of 100 μM.

TABLE 3. Influence of differently activated BMMs on expression of bradyzoite-specific antigens 4 days p.i. with strain NTE tachyzoites

Treatment	Duration of treatment[a]: 96 h: % of MAb 4F8-reactive vacuoles	96 h: No. of parasites/vacuole	96 h: Amt of NO_2^- (μM) on[b]: day 1	96 h: Amt of NO_2^- (μM) on[b]: day 3	24 h: % of MAb 4F8-reactive vacuoles	24 h: No. of parasites/vacuole	24 h: Amt of NO_2^- (μM) on[b]: day 1	24 h: Amt of NO_2^- (μM) on[b]: day 3
100-U/ml IFN-γ–10-ng/ml LPS	—[c]	—[c]	41.0	—[c]	<2	1	44.0	10.7
30-U/ml IFN-γ–10-ng/ml LPS	<2	1	39.2	37.0	69	1–4	36.5	8.3
10-U/ml IFN-γ–10-ng/ml LPS	<2	1	39.5	26.0	74	1–8	36.1	6.4
100-U/ml IFN-γ	<2	1	28.0	41.0	65	2–8	27.0	7.5
30-U/ml IFN-γ	<2	1	10.0	37.0	45	2–16	8.0	6.0
10-U/ml IFN-γ	<2	—[d]	3.0	9.0	<2	—[d]	3.2	2.0
1,000-ng/ml LPS	33	1–4	28.0	15.0	<5	2–50	26.0	3.0
100-ng/ml LPS	45	2–8	14.3	8.4	<2	—[d]	13.5	2.0
10-ng/ml LPS	15	2–16	10.6	4.8	<2	—[d]	11.0	2.0

[a] IFN-γ and LPS were added simultaneously with infection and were present in culture medium for (i) the entire cultivation time of 96 h or (ii) 24 h, followed by cultivation of BMMs in medium without IFN-γ and LPS for a further 72 h.
[b] NO_2^- was measured 24 h and 72 h p.i.
[c] BMMs had undergone self-destruction, probably as a result of toxic NO release.
[d] Host cell lysis by massive parasite multiplication occurred.

days p.i., parasitophorous vacuoles had increased in size in IFN-γ–NMMA-treated BMMs and contained 2 to 20 parasites per vacuole, 86% of which still reacted with MAb 4F8. Parasitophorous vacuoles in untreated BMMs on day 3 p.i. had the same size as those in IFN-γ–NMMA-treated BMMs on day 6 p.i. and were taken as a control. Only 10% of these vacuoles showed weak reactivity with MAb 4F8, excluding the possibility that the expression of Bsa was only dependent on the size of the vacuole. Instead, the loss of MAb 4F8 reactivity in vacuoles of the same size indicated that prolonged expression of Bsa occurred in parasites within IFN-γ–NMMA-treated BMMs.

Intermediate activation of BMMs results in maximal expression of Bsa. To define the optimal conditions for Bsa expression, BMMs were stimulated at the time of tachyzoite infection with different amounts of (i) IFN-γ, (ii) LPS, and (iii) IFN-γ–LPS and were analyzed on day 4 p.i. for expression of Bsa (Table 3). Mediators were present in the medium for the entire time of cultivation (0 to 96 h) or were replaced with fresh medium (without LPS and without IFN-γ) after 24 h of incubation. Activation of BMMs was monitored 24 and 72 h p.i. by measurement of NO_2^- in the supernatant. Medium was exchanged on day 2 p.i. Induction of Bsa occurred in intermediately activated BMMs (defined by intermediate values for NO release), allowing a reduced parasite replication. Maximal induction of Bsa expression was found in vacuoles that contained two to eight parasites. It was shown that this intermediate stage of activation was obtained either by activation of BMMs with only LPS (10 to 1,000 ng/ml) for the entire cultivation time or by activation with either IFN-γ (30 and 100 U/ml) or IFN-γ–LPS (30 and 10 U of IFN-γ per ml, 10 ng of LPS per ml) for 24 h.

Influence of endogenous NO on stage conversion. To further analyze whether retarded parasite growth is necessary for induction of Bsa, macrophages were treated with polymyxin and anti-tumor necrosis factor alpha (TNF-α) in combination with IFN-γ at the time of infection. These mediators were chosen because they are known to diminish the microbicidal activity of IFN-γ-treated macrophages by preventing NO release (16, 24). NO formation was completely inhibited, as confirmed by measurement of NO_2^-, and neither a parasitostatic effect nor enhanced expression of Bsa was observed in such treated BMMs (Table 4). These data indicate that induction of Bsa in activated BMMs is closely linked to inhibition of parasite growth which is suppressed by NO formation. IFN-γ-activated pathways that are not blocked by polymyxin or anti-TNF-α seemed not to be sufficient to induce expression of Bsa.

For successively decreasing the microbicidal potential of BMMs, stimulation with IFN-γ–LPS was followed by NMMA titration. Release of NO_2^- was taken as a parameter for monitoring macrophage activation. Increasing NMMA concentration resulted in continuously decreasing NO release accompanied by decreasing toxoplasmastatic activity of BMMs (Table 5). As was shown in LPS-activated BMMs, maximal expression of Bsa correlated with intermediately activated conditions and intermediate NO concentrations, resulting in weak parasite growth (two to eight parasites per vacuole) on day 4 p.i. The highest level of Bsa expression was obtained when NO release consisted of about 40 to 65% of the maximum level of NO release obtained by IFN-γ–LPS treatment without NMMA.

Exogenous NO inhibits *T. gondii* multiplication and induces expression of Bsa. SNP functions as an NO donor and can be used as a source of exogenous NO in cell culture, but the NO release did not linearly correlate with the SNP concentration

TABLE 4. Influence of NO-release-inhibiting mediators on expression of bradyzoite-specific antigens in BMMs infected with tachyzoites

Treatment[a]	% of MAb 4F8-reactive vacuoles	No. of parasites/vacuole	Amt of NO_2^- (μM)[b]
100-U/ml IFN-γ–NMMA	58	2–16	17
100-U/ml IFN-γ–polymyxin	<2	—[c]	<2
100-U/ml IFN-γ–anti-TNF-α	<2	—[c]	<2

[a] NMMA was added at a final concentration of 100 μM 24 h p.i. Polymyxin was added at a concentration of 25 μg/ml 2 h before infection. IFN-γ and 2×10^4 neutralizing units (NU) of anti-TNF-α per ml were added at the time of infection. All samples were analyzed 4 days p.i.
[b] NO_2^- was measured 24 h p.i.
[c] —, partial host-cell lysis caused by massive parasite multiplication.

TABLE 5. Influence of endogenous NO on expression of bradyzoite-specific antigens in activated BMMs infected with strain NTE tachyzoites

Amt of NMMA (μM)[a]	% of MAb 4F8-reactive vacuoles	No. of parasites/vacuole	Amt of NO_2^- (μM)[b]
300	<5	2–30	18.5
200	<5	2–30	21.5
150	16	2–20	25.0
100	24	2–16	28.9
70	33	2–8	42.4
50	59	2–8	42.0
30	22	2–4	48.9
0	<5	1	61.0

[a] NMMA was added simultaneously with activation of BMMs with 10-U/ml IFN-γ–10-ng/ml LPS and infection with 10^5 tachyzoites.
[b] NO_2^- release was measured 24 h p.i.

(9, 20). BMMs were incubated with SNP at concentrations from 10 to 500 μM at the time of infection. Weak suppression of *T. gondii* growth could be observed at 20 μM SNP, whereas 500 μM SNP resulted in nearly complete suppression of parasite growth. No morphological alteration of BMMs was observed when SNP was used at concentrations up to 500 μM. In addition to suppressing *T. gondii* growth, SNP treatment led to induction of Bsa (Table 6). Potassium hexacyanoferrate (III) in identical concentrations was used as a control to exclude possible influences of hexacyanoferrate during SNP treatment and was shown to have no influence on replication and conversion. Therefore, exogenous NO seemed to be sufficient to suppress *T. gondii* multiplication as well as to induce Bsa.

Inhibition of mitochondrial function is a trigger for stage conversion. Released NO is reactive with iron-sulfur centers in proteins, thereby inhibiting several proteins involved in mitochondrial electron transport (10, 15). It was essential to investigate whether inhibition of the respiratory chain might be a potential trigger for stage conversion. At the time of infection, BMMs were incubated with oligomycin, an inhibitor of mitochondrial ATP synthetase function, and antimycin A, an inhibitor of electron transport of the respiratory chain. Both drugs revealed effects similar to those of SNP: replication was dose-dependently decreased and was associated with induction of Bsa (Table 7). By using BMMs as host cells, it was not possible to differentiate whether the effect of oligomycin and

TABLE 6. Influence of SNP as a source of exogenous NO on expression of bradyzoite-specific antigens in BMMs infected with strain NTE tachyzoites

Amt of SNP (μM)[a]	% of MAb 4F8-reactive vacuoles	No. of parasites/vacuole	Amt of NO_2^- (μM)[b]
500	14.0	2–4	22.0
300	22.2	2–8	17.6
200	20.8	2–8	13.6
150	33.8	2–16	13.0
100	26.9	4–16	11.3
70	35.6	4–16	9.6
50	31.0	4–20	7.6
30	17.7	4–20	6.2
20	12.0	4–20	5.6
10	5.0	4–50	4.5
0	<2	4–50	<2

[a] SNP was dissolved in cell culture medium and added to BMMs at the time of infection. Medium with fresh SNP was exchanged with the old medium daily.
[b] NO_2^- was measured 24 h p.i.

TABLE 7. Influence of mitochondrial inhibition on expression of bradyzoite-specific antigens in BMMs infected with strain NTE tachyzoites

Amt of antimycin A (ng/ml)[a]	% of MAb 4F8-reactive vacuoles	No. of parasites/vacuole
30	48	1–4
20	21.6	1–4
15	23	1–8
12	12	2–16
10	5	2–50
7	<2	2–50

[a] Antimycin A was added at the time of infection.

antimycin A was solely by inhibition of host cell mitochondria or included inhibition of parasite mitochondria. To differentiate between these two possibilities, 143B/260 fibroblasts, a cell line possessing a nonfunctional mitochondrial respiratory chain, were used as host cells. These cells were generated from the human fibroblast line 143B (13). The lack of mitochondrial DNA in 143B/260 cells results in the inability to synthesize essential components necessary for ATP synthesis in mitochondria (13). Replication of parasites in oligomycin- and SNP-treated cells was inhibited significantly in both cell lines (Table 8). Inhibition of replication was stronger in 143B cells than in 143B/260 cells. In addition, Bsa expression as a result of oligomycin and SNP treatment was slightly enhanced in 143B cells compared with that in 143B/260 cells. Parasites in untreated controls replicated to the same extent in both cell types, and no enhanced Bsa expression was observed.

Influence of pyrimethamine and aphidicolin. Since it was not possible to induce Bsa without inhibition of parasite multiplication, it was important to know whether replication blocked by a mechanism other than NO and respiratory chain inhibition would also result in enhanced expression of Bsa. Aphidicolin, an inhibitor of eucaryotic DNA polymerase α, and pyrimethamine, a dihydrofolate reductase inhibitor, are drugs known to inhibit *T. gondii* growth (8, 22). With both drugs, the portion of 4F8-reactive vacuoles increased (15% for pyrimethamine [± 4.5%, $n = 3$], 22% for aphidicolin [± 4%, $n = 3$]), but to an extent less than that found in IFN-γ-activated macrophages.

Expression of Bsa in different strains of *T. gondii*. Spontaneous expression of Bsa in unstimulated BMMs among five *T. gondii* strains was compared (Table 9). The parasites of all strains, including mouse-virulent strains RH and BK, consisted of a small subpopulation expressing the MAb 4F8-reactive antigen. On day 4 p.i., strain-dependent differences were observed, with relative amounts of MAb 4F8-reactive parasites

TABLE 8. Influence of oligomycin and SNP on parasite replication and Bsa expression in 143B and 143B/260 cells

Treatment	Effect on:			
	143B cells		143B/260 cells	
	No. of parasites/vacuole	% of MAb 4F8-reactive vacuoles	No. of parasites/vacuole	% of MAb 4F8-reactive vacuoles
None (control)	2–50	<2	2–50	<2
Oligomycin (0.5 μg/ml)	2–8	15	2–20	10
SNP (500 μM)	2–8	28	2–20	15

TABLE 9. Strain-dependent expression of bradyzoite-specific antigens in activated and untreated BMMs

Strain	% of MAb 4F8-reactive vacuoles in:	
	Unstimulated BMMs	LPS-activated BMMs
BK	<0.03	3.6
RH	0.3	14.4
RKR	1	19.4
DX	0.7	54.4
NTE	2	48

ranging from less than 0.03% for highly mouse-virulent strain BK to 2% for strain NTE.

In addition, these five strains of *T. gondii* were compared for their ability to express Bsa in LPS-activated BMMs. On day 4 p.i. with tachyzoites, all strains had a 20 to 100 times larger portion of MAb 4F8-reactive parasites in activated BMMs than that in untreated cells (Table 8). Induced expression of Bsa varied strain dependently, with the highest rates for mouse-avirulent strains DX and NTE, and the lowest rates for the highly mouse-virulent strain BK.

The increasing portion of MAb 4F8-reactive parasites could be due to induction of the 4F8 antigen or to a selection process favoring the parasites that express this antigen. Total numbers of MAb 4F8-reactive vacuoles were compared on days 2 and 4 p.i. in BMMs treated with 100 ng of LPS per ml at time of infection with tachyzoites. On day 4 p.i., the total number of MAb 4F8-reactive vacuoles increased strain dependently 4- to 40-fold compared with day 2 p.i. To exclude the possibility that prolonged cell culture time from day 2 to day 4 is responsible for the increase in MAb 4F8-reactive vacuoles, total amounts of MAb 4F8-reactive vacuoles in LPS-treated and -untreated BMMs were compared on day 4 p.i.; strain dependently, up to 10-fold more MAb 4F8-reactive vacuoles were found in activated BMMs than in untreated BMMs, indicating that real induction of Bsa had occurred.

DISCUSSION

Bradyzoite-specific MAbs have recently been developed by several groups (2, 21, 30, 31). By using these tools, it has become possible to analyze stage conversion and to monitor the interconversion between tachyzoites and bradyzoites on a molecular level. The bradyzoite-specific antigens that are recognized by MAb 4F8 and 7E5 seemed to be coexpressed, as is the case for most of the known Bsa (2, 25). MAb 4F8 was therefore used as a general marker for Bsa expression in this study. Tachyzoites have been described as a heterogeneous population, including a small subpopulation of parasites that spontaneously expresses Bsa (2, 25). As was shown by comparison of the three mouse-avirulent and two mouse-virulent strains of *T. gondii* in this study, the formation of these Bsa-expressing subpopulations seems to be a general feature of all *T. gondii* strains. However, there are significant differences in the relative amounts of these subpopulations, which differ strain dependently from 0.03 to 2%. Whether spontaneous conversion from tachyzoites to bradyzoites under these conditions is stable or is a more dynamic process, in which reconversion to tachyzoites also occurs, cannot be answered yet.

Jones et al. (14) reported the in vitro cultivation of *T. gondii* cysts in astrocytes in the presence of IFN-γ. In this cell culture system, cyst formation seemed to occur spontaneously with no promotional effect of IFN-γ on cyst development. Instead, IFN-γ suppressed the division of tachyzoites and prevented host cells from rupturing. In contrast to Jones et al., we used activated BMMs as host cells and found a strongly enhanced conversion from tachyzoites to bradyzoites, given the condition that reduced parasite replication occurred. It was shown that the increase in the portion of 4F8-reactive parasites was not due to a selection process. Instead, enhancement of the absolute number of MAb 4F8-reactive vacuoles in activated BMMs compared with controls suggests that real induction took place. As was observed for spontaneous Bsa expression, the inducibility of Bsa expression seems to be a common feature of all *T. gondii* strains and also represents strain-dependent differences; mouse-virulent strain BK had the lowest potential to express Bsa. In strain BK, spontaneous as well as induced expression of Bsa was 5- to 10-fold lower than that of the other strains. The significant difference in Bsa expression between mouse-virulent strains BK and RH indicates that the inducibility for Bsa expression is not strictly linked to virulence.

In contrast to our previous study with mouse peritoneal macrophages as host cells (2), induction of Bsa expression in BMMs was not observed when replication of *T. gondii* was completely blocked by activating macrophages with 100 U of IFN-γ per ml. BMMs represent a more homogeneous population than peritoneal macrophages do; nevertheless, the reason for this difference remains unclear. Maximal expression of Bsa was strictly linked to reduced parasite multiplication and occurred in intermediately activated BMMs with about half-maximal NO release compared with fully activated macrophages. Intermediate activation was achieved (i) by adding the NO synthase inhibitor NMMA 24 h after activation with IFN-γ, (ii) by replacing the medium after 24 h of stimulation, or (iii) by LPS treatment without IFN-γ. MAb 4F8-reactive parasites were mostly located within vacuoles containing 2 to 16 parasites (3). Permanent activation of BMMs seems to be unnecessary for the triggering process, since a 24-h incubation period with IFN-γ is sufficient to induce Bsa. Activation of BMMs with IFN-γ–LPS is not the only trigger that can induce Bsa. Inhibiting parasite multiplication by using the drugs pyrimethamine and aphidicolin also resulted in weak expression of Bsa, further supporting the observation that stage conversion seems to be closely linked to parasite multiplication.

The promotional influence of activated macrophages on expression of Bsa is not only restricted to tachyzoites but is also extended to bradyzoites. Parasites continued expression of Bsa when bradyzoites were used for infection. In contrast, in vacuoles of comparable size in untreated cells, parasites lost reactivity for bradyzoite-specific MAbs and differentiated into tachyzoites (2).

In activated macrophages, NO formation is induced by an L-arginine-dependent pathway (1, 16). The critical role of NO for the toxoplasmastatic effect in activated macrophages is well documented by inhibiting its formation with the NO synthase inhibitor NMMA (16, 17, 24). It is generally accepted that minor amounts of LPS (probably including those in our culture media) are necessary to activate macrophages in combination with IFN-γ. Polymyxin is able to neutralize the effects of LPS and prevents NO release (16). In addition, it has also been shown that anti-TNF-α treatment prevents NO release in IFN-γ–LPS-treated macrophages (16, 24). The use of polymyxin, anti-TNF-α, and high concentrations of NMMA at the time of infection resulted in inhibition of NO release in IFN-γ-treated macrophages accompanied by normal parasite growth. Under these conditions, no conversion was found, indicating that retarded parasite growth seems to be necessary for induction of Bsa.

NO-liberating agents like SNP can be used as a source of exogenous NO in cell culture (9). Exogenous NO was found to be a potent inhibitor of *Toxoplasma* replication, confirming that endogenous NO is sufficient to exceed a toxoplasmastatic effect. The ability of exogenous NO to induce stage conversion leads to the hypothesis that NO might be a molecular trigger of Bsa induction in activated macrophages. Endogenous NO is reactive with iron-sulfur centers in proteins, thereby inhibiting several proteins that are involved in mitochondrial electron transport (10, 15). Indeed, inhibition of mitochondrial electron transport and ATP formation by oligomycin and antimycin, respectively, resulted in decreasing multiplication and induction of Bsa in BMMs. The influence of oligomycin seems not to be restricted only to host-cell mitochondria but also extends to parasite mitochondria, as was demonstrated with the cell line 143B/260. As a result of lacking mitochondrial DNA, these cells have lost their ability to synthesize ATP in their mitochondria (13). The decrease in parasite replication in oligomycin-treated 143B/260 cells therefore indicates inhibition of parasite mitochondria. Instead of their nonfunctional mitochondria, 143B/260 fibroblasts have a growth rate comparable to that of 143B cells, the cell line they were generated from. The reason is an enhanced glycolysis rate that seems to generate a sufficient level of ATP (13). The increased ATP synthesis from glycolysis in 143B/260 cells might also explain the weaker oligomycin-induced inhibition of parasite replication in this cell line than that of 143B cells. It might also be a reason for the identical parasite replication rates found in oligomycin-untreated 143B and 143B/260 cells. These studies suggest involvement of parasite mitochondria in energy delivery of intracellular parasites. It might also be possible that the intracellular ATP content might influence the induction of Bsa expression. However, the exact mechanism by which the inhibition of mitochondrial function promotes Bsa expression remains to be clarified. It is known that inhibitors of the respiratory chain, for example, also have an influence on the generation of reactive oxygen intermediates and on the formation of mitochondrion-derived signal transducers (23).

Since most tissue cysts are found in the brain, BMMs are not the natural host cells for cyst formation in vivo. However, microglial cells are the functional correlates of macrophages in the brain and are also able to inhibit *T. gondii* growth after stimulation with IFN-γ and to induce NO release (4, 5, 7). In a recent study, the reactivation of a chronic *T. gondii* infection was correlated with a down-regulated expression of inducible NO synthase and other markers of microglial and/or macrophage activity (11). The in vitro data presented in this study suggest a role for NO not only in inhibiting parasite replication but also in triggering induction of bradyzoite development. Whether microglial cells and/or macrophages might promote cyst development in vivo by their ability to release NO during establishment of a chronic infection would be interesting to investigate in the future.

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