INFECTION AND IMMUNITY, Aug. 1994, p. 3472–3478
0019-9567/94/$04.00+0

Vol. 62, No. 8

Defective Antigen Presentation by *Mycobacterium tuberculosis*-Infected Monocytes

JENS GERCKEN,[1] JULIUSZ PRYJMA,[2] MARTIN ERNST,[1]* AND HANS-DIETER FLAD[1]

Department of Immunology and Cell Biology, Forschungsinstitut Borstel, D-23845 Borstel, Germany,[1] and Department of Immunology and Microbiology, Institute of Paediatrics, Jagiellonian University, Collegium Medicum, PL-30-663 Cracow, Poland[2]

Received 6 January 1994/Returned for modification 3 March 1994/Accepted 4 May 1994

In this study we investigated the effect of an in vitro infection with *Mycobacterium tuberculosis* on the ability of human monocytes to present the soluble antigen tetanus toxoid to T cells. We observed that tetanus toxoid-specific T-cell proliferation was markedly reduced when monocytes were infected with large numbers (bacterium-to-monocyte ratio, 50:1) of both viable and heat-killed mycobacteria. The level of antigen-induced gamma interferon release also was decreased when *M. tuberculosis*-containing monocytes were used as antigen-presenting cells. However, mycobacterium-infected monocytes did not show or trigger suppressive activity, because the presence of mycobacterium-infected monocytes did not affect the T-cell response induced by tetanus toxoid-pulsed control monocytes. When *M. tuberculosis*-infected monocytes were fixed with paraformaldehyde, they were not able to serve as antigen-presenting cells even in the presence of untreated accessory monocytes. Moreover, the uptake of both viable and heat-killed *M. tuberculosis* cells reduced the expression of human leukocyte antigen DR on monocytes. With regard to accessory function, monocytes infected with large numbers of mycobacteria were less efficient as accessory cells than were control monocytes in cultures of T cells activated with pokeweed mitogen. These results indicate that infection with large numbers of *M. tuberculosis* cells impairs the ability of monocytes to process and/or present soluble antigen and to serve as accessory cells in T-cell activation.

Mycobacterial infections are often associated with impairment of the immune response (3, 25). Animal studies and in vitro experiments suggest that mycobacteria or their products can induce suppression of T-cell responses not only to mycobacterial antigens but also to unrelated antigens and polyclonal T-cell activators (7, 8, 13, 18, 21, 31, 33). The generation of suppressor T cells and/or macrophages was usually found to be associated with a reduced response of T lymphocytes in vitro, and in some cases the involvement of prostaglandins was documented (18). Recently, more attention has been paid to the possible alteration of the antigen-presenting and accessory functions of monocytes/macrophages. The reduced antigen-presenting and accessory functions of monocytes/macrophages after infections with viruses and intracellular parasites have already been documented (9, 14, 16). A reduction in the number of major histocompatibility complex (MHC) class II antigens in humans infected with *Mycobacterium leprae* (5) and in mice infected with *M. microti* or *M. kansasii* (15, 24) and a decrease in the expression of CD11b and CD14 on monocytes infected in vitro with *M. avium-M. intracellulare* complex have been reported (31). Antigen presentation requires antigen processing and association of immunogenic peptides with MHC class II molecules, which involves translocation and fusion of intracellular vesicles (for a review, see reference 12). Because mycobacteria and mycobacterial products are known to interfere with phagosome-lysosome fusion and influence the cellular environment (1, 4, 19, 29), it seems conceivable that intracellular mechanisms necessary for antigen processing and association of immunogenic peptides with MHC class II molecules might be affected in monocytes/macrophages harboring parasites. Here we report that monocytes containing large numbers of *M. tuberculosis* cells cannot present tetanus toxoid (TT) antigen and that their ability to support lectin-induced T-cell proliferation is diminished.

* Corresponding author. Mailing address: Department of Immunology and Cell Biology, Forschungsinstitut Borstel, Parkallee 22, D-23845 Borstel, Germany. Phone: 49-4537-10-453. Fax: 49-4537-10-404.

MATERIALS AND METHODS

Cell populations. Peripheral blood mononuclear cells were isolated from heparin-treated blood of healthy donors by using a standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient. They were suspended in Hanks balanced salt solution supplemented with 0.1% bovine serum albumin and underwent countercurrent centrifugal elutriation (Beckman JE-6B-Elutriation System) to obtain lymphocytes and monocytes. The separation was performed at 10°C and at a constant rotor speed (3,500 rpm). The cells (2×10^8 to 5×10^8 in 50 ml) were loaded at a flow rate of 25 ml/min and subsequently eluted by gradually increasing the flow rate to 50 ml/min. Twenty cell fractions (50 ml each) were collected; the last of these was collected while the rotor was turned off. Monocyte enrichment in the last fraction was confirmed by nonspecific esterase staining (85 to 95% positive) and/or expression of CD14 antigen (80 to 90% LeuM3 positive).

Monocytes were washed once with cold RPMI medium and kept in an ice bath at a concentration of 5×10^6/ml in incomplete medium (RPMI 1640 medium supplemented with L-glutamine and 10% fetal calf serum; all reagents were from GIBCO, Grand Island, N.Y.) until use. Cells collected in fractions 5 to 10 (which contained 80 to 90% CD3-positive cells) were pooled and rosetted with AET-pretreated sheep erythrocytes to isolate T cells (17). Rosetting (T lymphocytes) and nonrosetting (non-T lymphocytes) cells were separated by Ficoll-Paque density gradient centrifugation. Cells recovered from the pellet by osmotic shock (T cells) were washed twice with Hanks balanced salt solution and finally adjusted to a

concentration of 10^6/ml in complete medium (incomplete medium to which 100 U of penicillin per ml and 100 μg of streptomycin per ml were added). Non-T cells were washed in Hanks balanced salt solution and also adjusted to a concentration of 10^6/ml in complete medium. In some experiments elutriated lymphocytes were depleted of B lymphocytes and human leukocyte antigen (HLA)-DR-positive cells by using magnetic cell sorting (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) (20). Briefly, lymphocytes (10^7 cells per ml of phosphate-buffered saline [PBS]) were labeled with anti-CD20 (Dako, Hamburg, Germany) and anti-HLA-DR (Becton Dickinson, Heidelberg, Germany) followed by biotinylated goat anti-mouse immunoglobulin G (Dianova, Hamburg, Germany) and finally by streptavidin-conjugated magnetic microbeads (Miltenyi Biotech GmbH). Following each incubation step, the cells were washed with PBS and centrifuged down through a fetal calf serum cushion. Finally, the cells were adjusted to 10^7 cells per ml in PBS and passed through a magnetized steel wool column to separate unlabeled lymphocytes from the magnetically labeled cells. The depleted lymphocyte fraction contained less than 1% B cells or HLA-DR-positive cells as monitored by flow cytometry.

Bacteria. *M. tuberculosis* H37Rv (kindly provided by S. Rüsch-Gerdes, Division of Medical Microbiology, Forschungsinstitut Borstel) were cultured on Löwenstein-Jensen medium at 37°C for 20 to 25 days. The bacteria were harvested and homogenized in PBS, and clumps were removed by low-speed centrifugation. The supernatant, containing predominantly single bacterial cells, was then centrifuged, and the bacteria were washed twice with PBS. The concentration of bacteria was determined by counting in a Neubauer chamber. Heat-killed *M. tuberculosis* cells were prepared by autoclaving the bacteria for 20 min. Live and heat-killed bacteria were from the same batch of *M. tuberculosis*. Bacterial preparations were stored at −70°C until use. In some experiments strains of *Escherichia coli* (NCTC 10418) or *Salmonella enteritidis* (clinical isolate) were used. These strains were grown for 18 h on sugar broth, washed twice with a large volume of saline, and opsonized (30 min at 37°C) in the presence of 5% fresh human serum. After additional washing, the density of bacterial cells was measured spectrophotometrically (at 540 nm), and the cell number was calculated by using previously determined standard curves (based on CFU counts).

To evaluate the uptake of *M. tuberculosis* by monocytes, we labeled viable bacteria with fluorescein isothiocyanate (FITC; Sigma, Deisenhofen, Germany), as described by Drevets and Campbell (6), by incubation with 0.1 mg of FITC per ml in PBS (pH 7.2) at 37°C for 2 h. Thereafter FITC-labeled bacteria were washed twice with PBS to remove unbound FITC.

Phagocytosis of bacteria. Monocytes were suspended in RPMI supplemented with 10% fetal calf serum and 2 mM L-glutamine (incomplete medium) at a concentration of 2×10^6 to 5×10^6/ml and incubated for 1 h at 37°C under 5% CO_2 in a polypropylene tube with a 10- or 50-fold-larger number of *M. tuberculosis* cells. In some experiments 10% human AB serum was added as a source of opsonins. After incubation, cells were washed twice and resuspended in medium supplemented with antibiotics (complete medium). Latex particles with a diameter of 0.94 ± 0.08 μm (Serva, Heidelberg, Germany) were washed twice with 70% ethanol and suspended in medium at a concentration of 6×10^9/ml. These were added to the suspension of monocytes to obtain a 100:1 particle/monocyte ratio, and the mixture was incubated for 15 min before being washed. When *E. coli* or *S. enteritidis* was used for phagocytosis, bacteria were always opsonized and the bacterium/monocyte ratio during incubation was also 100:1. Control monocytes to which bacteria were not added were incubated and washed in parallel.

Flow cytometry. To determine the effect of stimulation with *M. tuberculosis* on monocyte viability and the expression of HLA-DR, we pulsed monocytes with various concentrations of live or heat-killed mycobacteria as described above. To determine HLA-DR expression at given time points, we harvested cells and labeled them with phycoerythrin-conjugated anti-HLA-DR (Becton Dickinson). Isotype-specific phycoerythrin-conjugated immunoglobulin G2a was used as the control antibody. All staining and washing procedures were performed at 4°C in PBS supplemented with 0.1% NaN_3. Finally, samples were fixed in 1.5% paraformaldehyde–PBS and analyzed after 24 h by using a FACStarPlus flow cytometer (Becton Dickinson). (Channel numbers in Table 3 are given in linear scale converted from originally logarithmically amplified signals by using LYSIS II software [Becton Dickinson].)

Cell viability was assessed as described by Sasaki et al. (30). Briefly, control and mycobacterium-pulsed monocytes were incubated in PBS plus propidium iodide (0.5 μg/ml; Dianova) for 10 min. Thereafter the cells were washed twice to remove free dye and fixed with paraformaldehyde as described above.

To determine the uptake of mycobacteria, we incubated FITC-labeled microorganisms together with monocytes as described above. Thereafter, samples of cells were fixed with 1.5% paraformaldehyde and analyzed by flow cytometry.

Antigen pulse. Control and bacterium-containing monocytes (2×10^6 to 5×10^6/ml in complete medium) were incubated for 3 h with TT (Behring, Marburg, Germany) at a concentration of 10 Lf/ml (Lf = limit of flocculation). Thereafter the cells were washed four times and adjusted to a concentration of 10^6/ml in complete medium. Control nonpulsed monocytes were treated the same way in parallel. In some experiments, after the antigen pulse the cells were fixed with paraformaldehyde, essentially as described by Moreno and Lipsky (23). Briefly, after antigen-pulsed or control monocytes were washed with PBS, the cells were exposed to 1% paraformaldehyde in PBS for 2 min at 37°C. Thereafter the reaction was stopped by addition of 0.06% cold glycylglycine in PBS, and cells were washed three times in PBS. Such cells were subsequently incubated in complete medium for 1 h, and after an additional final wash they were used as antigen-presenting cells.

Evaluation of T-lymphocyte proliferative response. T-cell proliferative response was determined by [^{3}H]thymidine incorporation. Unless otherwise stated, cultures were pulsed with [^{3}H]thymidine (specific activity, 1 μCi/mM; Amersham Buchler, Brunswick, Germany) after 6 days of culture, and incorporated radioactivity was determined in a beta-counter. Supernatants were collected after 4 or 5 days of culture, and the concentration of gamma interferon (IFN-γ) was determined by an enzyme-linked immunosorbent assay (reagents kindly provided by H. Gallati, Hoffmann-La Roche, Basel, Switzerland).

RESULTS

Evaluation of bacterial uptake. To assess the uptake of bacteria by monocytes, live FITC-labeled bacteria were used for pulse stimulation (1-h phagocytosis period) and the number of monocytes loaded with bacteria was measured by flow cytometry. As expected, the proportion of phagocytic cells depended on the bacterium/monocyte ratio during phagocytosis and was usually between 25 and 50% at a 10:1 bacterium/monocyte ratio and over 80% at a 50:1 bacterium/monocyte ratio. As judged from fluorescence intensity, a 1-h incubation of mycobacteria with monocytes at a ratio of 50:1 resulted in an uptake of 5 to 10 times more microorganisms per cell (10 to 50

TABLE 1. T-cell proliferation and IFN-γ release by T lymphocytes activated with TT presented by *M. tuberculosis*-infected monocytes

Treatment of monocytes	Mean T-cell proliferation on day 6 (10^3 cpm) ± SD	Mean IFN-γ release at 96 h (ng/ml) ± SD
Expt 1		
None	2.12 ± 0.09	
M. tuberculosis (10:1)	1.61 ± 0.04	
M. tuberculosis (50:1)	0.94 ± 0.01	
TT pulse	5.76 ± 0.05	
M. tuberculosis (10:1), TT pulse	4.21 ± 0.11	
M. tuberculosis (50:1), TT pulse	1.91 ± 0.03	
Expt 2		
TT pulse	21.65 ± 0.13	
M. tuberculosis (50:1), TT pulse	2.87 ± 0.28	
M. tuberculosis (50:1, ops.[a]), TT pulse	2.65 ± 0.11	
M. tuberculosis (10:1, ops.[a]), TT pulse	7.03 ± 0.11	
Expt 3[b]		
None	0.41 ± 0.02	0.412 ± 0.022
TT pulse	5.49 ± 0.13	5.363 ± 0.056
M. tuberculosis (50:1)	0.72 ± 0.09	0.842 ± 0.010
M. tuberculosis (50:1), TT pulse	0.83 ± 0.12	1.487 ± 0.015
M. tuberculosis (autoclaved) (50:1)	0.95 ± 0.04	
M. tuberculosis (autoclaved) (50:1), TT pulse	1.61 ± 0.09	0.647 ± 0.028

[a] Bacteria were opsonized since pooled human AB serum (10%) was present during incubation of monocytes with mycobacteria.

[b] Lymphocytes depleted of CD19 and HLA-DR positive cells by magnetic cell sorting were used as a source of responding T lymphocytes.

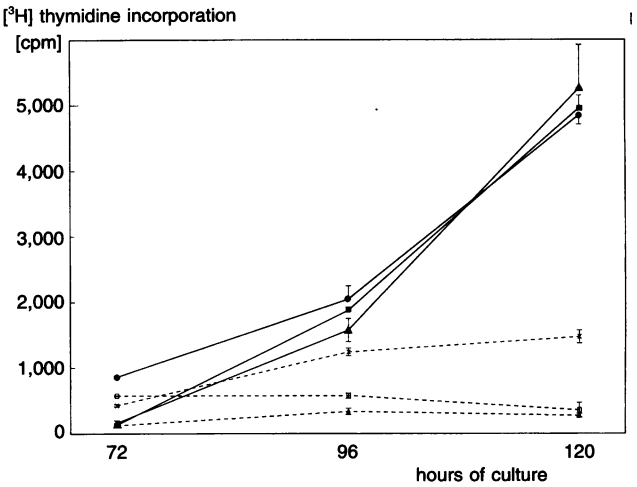

FIG. 1. Kinetics of the T-cell proliferative response (mean counts per minute [cpm] of triplicate cultures at indicated time points) in the presence of 10^4 intact monocytes (△), monocytes infected with *M. tuberculosis* (□), TT-pulsed *M. tuberculosis*-infected monocytes (×), TT-pulsed control monocytes (■), or combinations of equal numbers of untreated monocytes plus TT-pulsed monocytes (▲) or *M. tuberculosis*-infected monocytes plus TT-pulsed monocytes (●). The results shown are representative of two independent experiments.

bacteria per phagocytically active monocyte) than did the use of a low (10:1) ratio (1 to 5 bacteria per phagocytically active monocyte) (data not shown).

Cell viability. The effects of phagocytosis of *M. tuberculosis* on cell viability were similar when live and heat-killed organisms were used. In general, when monocytes were incubated with a large number of *M. tuberculosis* cells for 16 h (incubation ratio, 50:1), more than 70% of the cells remained viable, as judged from flow cytometry measurement of propidium iodide exclusion. Incubation of monocytes with fewer mycobacteria resulted in a viability of 80 to 90%, whereas in the control less than 5% of the cells were stained with propidium iodide.

After phagocytosis of *M. tuberculosis*, monocytes have a reduced ability to trigger antigen-specific T-cell response. Monocytes isolated from donors whose T cells did not respond to purified protein derivative of tuberculin were incubated with a suspension of *M. tuberculosis*. The bacterium/monocyte ratio was approximately 10:1 or 50:1. In some experiments, pooled fresh human serum was present during incubation as a source of opsonins, or bacteria were autoclaved before use. After 1 h of incubation, cells were washed free of bacteria, pulsed with TT, and added to the cultures of autologous T lymphocytes.

As shown in Table 1, the ability of monocytes to trigger TT-specific T-cell proliferation was absent or markedly reduced when monocytes were allowed to phagocytose mycobacteria. This reduction depended on the concentration of mycobacteria during incubation with monocytes. When monocytes were incubated with a small number of bacteria, their ability to trigger T-cell responses was much less markedly affected. The presence of human serum during phagocytosis had no influence on the subsequent function of monocytes. The inefficient antigen presentation by mycobacterium-infected cells could not be overcome by an increased concentration of monocytes and was also seen when monocytes phagocytosed heat-killed microorganisms. In parallel to the reduced proliferative response, the amount of antigen-induced IFN-γ secretion was considerably reduced in cultures in which the antigen was presented by *M. tuberculosis*-containing monocytes. Furthermore, the reduced T-cell proliferative response was not due to the shift of the response kinetics (Fig. 1). From the above data, it was concluded that monocytes infected with a large number of mycobacteria have reduced antigen-presenting capacity.

Monocytes infected with *M. tuberculosis* are not suppressive and do not trigger suppressive activity. It could be argued that the reduced T-cell response to antigen-pulsed monocytes infected with *M. tuberculosis* was due to the induction of suppressor mechanisms. Such mechanisms were detected ex vivo during mycobacterial infections, and ingestion of mycobacteria has been demonstrated to trigger the production of prostaglandin E_2, which is known to possess suppressive and immunoregulatory properties (28). To exclude such a possibility, control monocytes or monocytes infected with viable mycobacteria were added to T cells activated by antigen-pulsed control monocytes. As shown in Fig. 1, the addition of monocytes containing mycobacteria had no influence on the response triggered by antigen-pulsed control monocytes. Similarly, no suppressive circuits were triggered by antigen-pulsed mycobacterium-containing cells (Fig. 2). We therefore conclude that monocytes infected with mycobacteria are not directly suppressive and do not trigger suppressive activity of other cells (such as T lymphocytes) in culture.

Impaired antigen presentation by *M. tuberculosis*-infected monocytes. The ineffective triggering of T-cell responses by antigen-pulsed, mycobacterium-infected monocytes could be due to a disturbance of antigen processing (including association with MHC class II molecules) or to changes in accessory functions provided to T lymphocytes by monocytes during

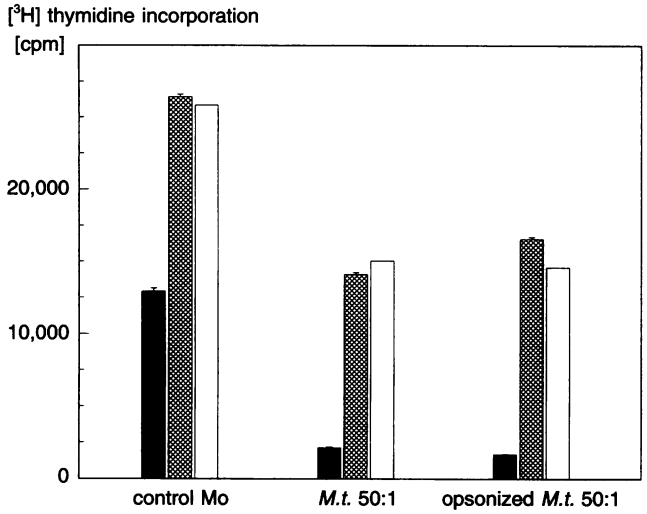

FIG. 2. T-lymphocyte (10^5 per well) proliferation (mean counts per minute [cpm] of culture triplicates) in the presence of 10^4 (10%) TT-pulsed control monocytes (solid bar, left panel) or *M. tuberculosis* (*M.t.*)-infected TT-pulsed monocytes (solid bars, middle and right panels) and in the presence of an additional 10^4 (10% + 10%) TT-pulsed control monocytes (cross-hatched bars). Open bars represent the expected values for the cross-hatched bars. These values were calculated as the sum of cpm induced by 10% pretreated TT-pulsed monocytes plus cpm induced by 10% TT-pulsed control monocytes. Data shown are from one representative experiment of four performed.

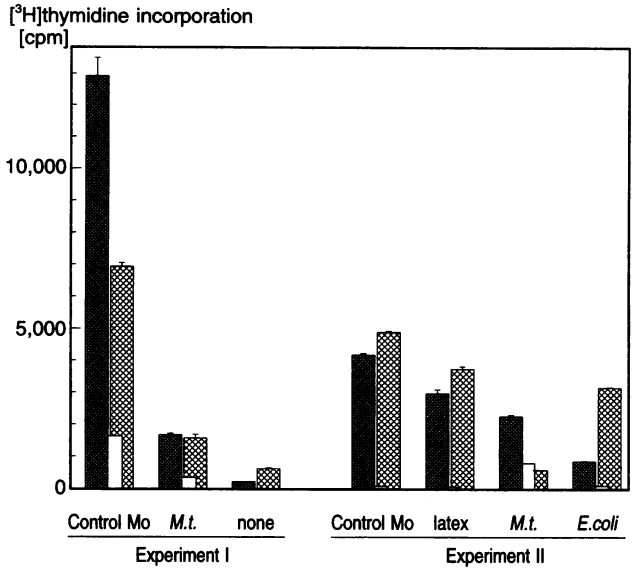

FIG. 3. T-cell proliferation. Control monocytes or cells which engulfed *M. tuberculosis* (*M.t.*), *E. coli*, or latex particles were pulsed with TT and added (10%) to cultured T lymphocytes before (▩) or after (□, ▨) paraformaldehyde fixation. To some cultures, 5% intact monocytes (adherent cells) were added (▨). The figure shows [^{3}H]thymidine incorporation (mean of culture triplicates) of two independent experiments.

culture or could result from the impairment of both functions mentioned above. To differentiate between these possibilities, control and infected monocytes were pulsed with antigen, washed, fixed with paraformaldehyde, and then added to T cells supplemented with intact monocytes serving the function of accessory cells. As shown in Fig. 3, antigen-pulsed control monocytes or monocytes which phagocytosed latex beads had lost their antigen-presenting capacity after treatment with paraformaldehyde but could trigger a significant response when added to T cells supplemented with intact monocytes. TT-pulsed monocytes which took up extracellular bacteria (*E. coli*) did not induce significant T-cell proliferation but, after paraformaldehyde fixation, could do so in the presence of control monocytes. In contrast, cells infected with a large number of mycobacteria were ineffective as antigen-presenting cells even in the presence of accessory monocytes. Similarly, the inability of mycobacterium-containing and TT-pulsed monocytes to trigger T-cell proliferation and IFN-γ secretion was not corrected by the addition to the culture of intact monocytes which could function as accessory cells in the presence of pokeweed mitogen (PWM) (Table 2). The data in Table 2 also indicate that no substantial leakage of antigen, which could be picked up and presented by intact monocytes, from either control or mycobacterium-containing cells occurred. From these data we conclude that infection of monocytes with *M. tuberculosis* interferes with antigen processing and/or its association with MHC class II molecules.

Reduced expression of HLA-DR on monocytes infected with *M. tuberculosis*. Recently we observed that phagocytosis of extracellular bacteria does not change the HLA-DR expression on monocytes (2) and that such cells, when fixed with paraformaldehyde, can present antigen in the presence of accessory cells (27). Since the expression of HLA-DR is of primary importance for antigen presentation, we asked whether the expression of HLA-DR would change after phagocytosis of *M. tuberculosis* cells. When monocytes were incubated with a large number of *M. tuberculosis* cells, the expression of HLA-DR was substantially reduced (Table 3). The reduction was seen after phagocytosis of both viable and autoclaved microorganisms; it could be observed shortly after phagocytosis and was still present after 16 h of incubation. In contrast, phagocytosis of *S. enteritidis* had no effect on HLA-DR expression.

Accessory function of mycobacterium-infected monocytes is diminished. The data presented above did not exclude the possibility that the accessory function of monocytes could also be reduced after phagocytosis of *M. tuberculosis*. In keeping with this notion, we have recently shown that after ingestion of extracellular bacteria, monocytes have a reduced ability to

TABLE 2. Inability of control monocytes to restore the reduced antigen-presenting capacity of *M. tuberculosis*-infected monocytes

TT-pulsed monocytes	Intact monocytes added	Mean T-cell proliferation (10^3 cpm) ± SD	Mean IFN-γ release (ng/ml) ± SD
Control	−	4.52 ± 0.06	7.430 ± 0.010
	+	4.45 ± 0.04	7.900 ± 0.028
Infected[a]	−	2.20 ± 0.02	2.326 ± 0.002
	+	2.08 ± 0.01	2.681 ± 0.004
None	−[b]	0.12 ± 0.01	<0.039
	+[b]	12.34 ± 0.23	10.945 ± 0.056

[a] Infected with *M. tuberculosis* (50:1).
[b] PWM was added to these cultures. The results of the last row of the table demonstrate the functional capacity for accessory functions of the intact monocytes added.

TABLE 3. Reduction of HLA-DR expression on monocytes after phagocytosis of *M. tuberculosis* H37Rv[a]

Treatment	Expression of HLA-DR after phagocytosis of bacteria			
	After 3 h		After 16 h	
	% Positive	Mean channel of fluorescence intensity	% Positive	Mean channel of fluorescence intensity
None	94.3	83	96.3	158
M. tuberculosis				
5:1	93.4	86	89.4	166
25:1	63.4	55	73.6	103
50:1	43.5	34	55.8	59
Autoclaved *M. tuberculosis*				
5:1	93.6	83	87.8	164
25:1	72.1	44	65.7	83
50:1	57.3	48	44.8	58
S. enteritidis, 100:1	91.2	85	88.3	112

[a] Representative results of three independent experiments; mean channel numbers are given in linear scale.

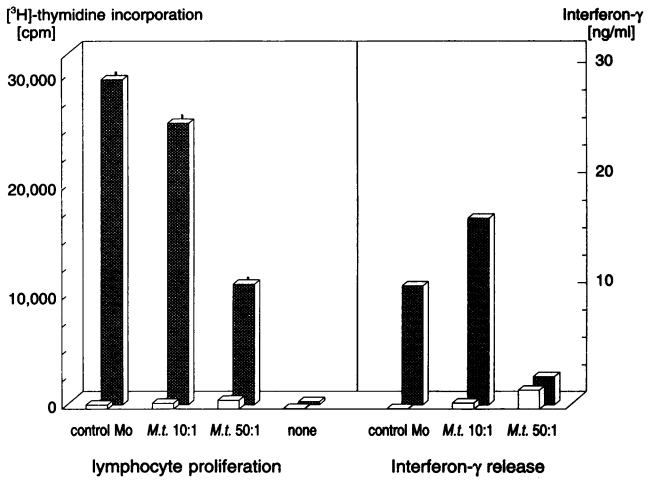

FIG. 4. PWM-induced proliferation and IFN-γ release by T lymphocytes in the presence of control monocytes and monocytes which were preincubated with large or small numbers of *M. tuberculosis* (*M.t.*). The figure shows data from one representative experiment of five in which thymidine incorporation was measured and one of two in which IFN-γ secretion was measured. Symbols: ■, +PWM; □, −PWM.

function as accessory cells in cultures of T lymphocytes activated with PWM (27). As shown in Fig. 4, monocytes infected with large numbers of *M. tuberculosis* are less efficient as accessory cells in PWM-activated cultures than are control monocytes.

DISCUSSION

Our data indicate that phagocytosis of *M. tuberculosis* results in an impaired ability of monocytes to process and/or present soluble antigen and to provide costimulatory signals to T lymphocytes. Monocytes which were allowed to phagocytose mycobacteria and were later pulsed with TT could not trigger antigen-specific T-cell proliferation or IFN-γ secretion, and this observation was not caused by a shift in the response kinetics. The phenomenon was reproducibly observed only when monocytes were incubated with a large number of mycobacteria before the pulse with antigen. Preincubation of monocytes with a small number of bacteria had no effect on their antigen-presenting and accessory functions, or the observed reduction was much lower than that observed with the large number of bacteria. Because monocyte populations incubated with large and small numbers of FITC-labeled microorganisms differed much more in intensity of fluorescence than in the percentage of cells containing mycobacteria, we think that the bacterial load is important for the observed functional changes. The impairment of the monocyte function was unlikely to be caused by a cytotoxic effect exerted by the microorganisms, because more than 70% of the cells were viable under these conditions. To clarify the mechanism responsible for the above observation, we considered three possibilities, namely, direct or indirect suppression of the T-cell response, inefficient accessory (costimulatory) function, and impairment of antigen presentation.

Our data exclude a direct suppressive effect of prostaglandins on T-lymphocyte proliferation or induction of suppressive circuits (e.g., release of prostaglandins). As shown above, the response induced by antigen-pulsed control monocytes was not affected by the addition to cultures of monocytes containing mycobacteria. Furthermore, when TT-pulsed monocytes with and without mycobacteria were added to T lymphocytes, the response was close to that expected from an additive effect. In contrast, we cannot exclude the possibility that the release of prostaglandins or other soluble factors induced by phagocytosis of mycobacteria (e.g., interleukin-10) interferes with the ability of monocytes to process antigen and express immunogenic peptides in the context of MHC class II antigen or that it influences their accessory function. In fact, our data suggest that, as a consequence of phagocytosis of mycobacteria, these functions of monocytes are impaired. Control, antigen-pulsed, and paraformaldehyde-fixed monocytes could induce T-cell proliferation in the presence of accessory cells. In contrast, similarly treated antigen-pulsed monocytes containing phagocytosed mycobacteria were ineffective, which indicates that such cells did not have the antigen-MHC class II complex available for the interaction with the T-cell receptor. In addition, the antigen-presenting capacity of unfixed, mycobacterium-containing monocytes was not restored in the presence of untreated monocytes. Such findings suggest that antigen processing, association with MHC class II molecules, and/or transport to the cell surface is impaired. It is conceivable that the impairment of antigen presentation, which we observed after phagocytosis of *M. tuberculosis*, was due to changes in the intracellular traffic of TT-derived peptides and of MHC class II molecules, because it is known that mycobacteria effectively inhibit phagosome-lysosome fusion in the phagocytic cells and may modulate the pH of macrophage lysosomes (1, 4, 19, 29). These alterations within the phagocytic cell may also be relevant for the reduced expression of MHC class II antigens on macrophages, which was reported to occur after ingestion of *M. leprae*, *M. kansasii*, and *M. microti* (5, 15, 24). In keeping with this notion, we observed a reduction of HLA-DR expression on monocytes after phagocytosis of *M. tuberculosis* but not after phagocytosis of *S. enteritidis*. Similarly, phagocytosis of other extracellular bacteria does not influence HLA-DR expression (2) and does not affect the ability of monocytes to present antigen when intact monocytes are present in culture (27) (Fig. 3). The reduction of HLA-DR expression on monocytes was seen only when cells were incubated with large

numbers of bacteria, and this parallels the reduction of their antigen-presenting capacity. The reduction in expression of MHC class II antigens after phagocytosis of large but not small numbers of mycobacteria was also reported by others in studies with mice (24). Interactions between antigen-presenting cells and T lymphocytes, which lead to a proliferative response, require costimulatory signals, which can also be provided by bystander accessory cells (for a review, see reference 10). The costimulatory signals include direct cell-cell interactions and cytokines. It is known that mycobacteria and their products can induce cytokine secretion (22, 32), and we observed substantial production of interleukin-1, tumor necrosis factor alpha, and interleukin-6 by monocytes after phagocytosis of *M. tuberculosis* (11a). In contrast, cell surface expression of adhesion molecules and CD14 antigen is down-regulated in monocytes after phagocytosis of extracellular bacteria and mycobacteria (2, 11a, 31). Furthermore, induction of the surface expression of the costimulatory B7/BB1 molecule is markedly reduced on *M. tuberculosis*-infected monocytes (11). In line with these observations, the accessory function of monocytes in lectin-activated cultures was reduced after phagocytosis of *M. tuberculosis*. However, the diminished accessory function of monocytes after phagocytosis of *M. tuberculosis* was not responsible for the inefficient triggering of T-cell proliferation and IFN-γ secretion, since the addition of intact monocytes to such cultures had no effect on the response.

In conclusion, our data indicate that after phagocytosis of *M. tuberculosis*, the antigen-presenting capacity and accessory function of monocytes are altered. This finding is in contrast to recently published data of Pancholi et al., who showed that monocytes/macrophages which harbor *M. bovis* BCG may sequester mycobacterial antigens from recognition by T cells but that their ability to present other antigens may not be affected (26). In our opinion, the reason for this apparent discrepancy lies primarily in the dose of mycobacteria used for monocyte infection. The dose used by Pancholi et al. was an order of magnitude lower than that used in our studies and had no effect on the HLA-DR expression on monocytes. In addition, as shown by McDonough et al., this strain does not influence phagosome-lysosome fusion, in contrast to *M. tuberculosis* H37Rv (19). These authors have found, however, that the impairment of phagosome-lysosome fusion occurs 3 days after infection of monocytes with the small number (bacterium/phagocytic cell ratio, 10:1) of *M. tuberculosis* H37Rv cells. From these data it seems conceivable that the impairment of lysosome-phagosome fusion depends on the bacterial load within the phagocytic cell and the strain of mycobacteria used. This would correspond to our data, which show that cells infected with large but not small numbers of mycobacteria are inefficient as antigen-presenting cells. In contrast to the experimental conditions used by Pancholi et al. (26), which more closely resemble a primary infection in vivo, the conditions which we chose may be more relevant to the situation of a localized infection and to the anergic status seen in disseminated mycobacterial infections.

ACKNOWLEDGMENTS

We thank E. Kaltenhäuser and R. Bergmann for excellent technical assistance and R. Hinz for secretarial assistance.

This work was supported by Bundesministerium für Forschung und Technologie, grant 01K18827/6, and grant KBN 2040/4/91 of the Polish Research Committee.

REFERENCES

1. **Armstrong, J. A., and D. Hart.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J. Exp. Med. **134**:713–740.
2. **Baran, J., and J. Pryjma.** The reduced expression of HLA-class II antigens and adhesion molecules on monocyte surface after phagocytosis of bacteria. Arch. Immunol. Ther. Exp., in press.
3. **Bhatnagar, R., A. N. Malaviya, S. Narayanan, P. Rajgopalan, R. Kumar, and O. P. Bharadwaj.** 1977. Spectrum of immune response abnormalities in different clinical forms of tuberculosis. Am. Rev. Respir. Dis. **115**:207–212.
4. **Chicurel, M., E. Garcia, and F. Goodsaid.** 1988. Modulation of macrophage lysosomal pH by *Mycobacterium tuberculosis*-derived proteins. Infect. Immun. **56**:479–483.
5. **Collings, L. A., N. Tidman, and L. W. Poulter.** 1985. Quantitation of HLA-DR expression by cells involved in the skin lesions of tuberculoid and lepromatous leprosy. Clin. Exp. Immunol. **61**:58–66.
6. **Drevets, D. A., and P. A. Campbell.** 1991. Roles of complement and complement receptor type 3 in phagocytosis of *Listeria monocytogenes* by inflammatory peritoneal macrophages. Infect. Immun. **59**:2645–2652.
7. **Ellner, J. J., and T. M. Daniel.** 1979. Immunosuppression by mycobacterial arabinomannan. Clin. Exp. Immunol. **35**:250–257.
8. **Fournie, J., E. Adams, R. J. Mullins, and A. Basten.** 1989. Inhibition of human lymphoproliferative responses by mycobacterial phenolic glycolipids. Infect. Immun. **57**:3653–3659.
9. **Fruth, U., N. Solioz, and J. A. Louis.** 1993. *Leishmania major* interferes with antigen presentation by infected macrophages. J. Immunol. **150**:1857–1864.
10. **Geppert, T. D., L. S. Davis, H. Gur, M. C. Wacholtz, and P. E. Lipsky.** 1990. Accessory cell signals involved in T-cell activation. Immunol. Rev. **117**:5–65.
11. **Gercken, J., H.-D. Flad, and M. Ernst.** 1993. Modulation of T-cell costimulatory antigens on human monocytes following stimulation with *Mycobacterium tuberculosis*. Immunobiology **189**:172–173.

11a. **Gercken, J., and M. Ernst.** Unpublished data.

12. **Germain, R. N., and D. H. Margulies.** 1993. The biochemistry and cell biology of antigen processing and presentation. Annu. Rev. Immunol. **11**:403–450.
13. **Harada, Y., S. Harada, A. Kajiki, Y. Kitahara, M. Takamoto, T. Ishibashi, and A. Shinoda.** 1992. Clinico-immunological study of the patients infected with *Mycobacterium avium* complex. Kekkaku **67**:71–81.
14. **Hayward, A. R., G. S. Read, and M. Cosyns.** 1993. Herpes simplex virus interferes with monocyte accessory function. J. Immunol. **150**:190–196.
15. **Kaye, P. M., M. Sims, and M. Feldmann.** 1986. Regulation of macrophage accessory cell activity by mycobacteria. II. In vitro inhibition of Ia expression by *Mycobacterium microti*. Clin. Exp. Immunol. **64**:28–34.
16. **Leopardi, R., J. Ilonen, L. Mattila, and A. A. Salmi.** 1993. Effect of measles virus infection on MHC class II expression and antigen presentation in human monocytes. Cell. Immunol. **147**:388–396.
17. **Madsen, M., and H. E. Johnsen.** 1979. A methodological study of E-rosette formation using AET-treated sheep red blood cells. J. Immunol. Methods **27**:61–74.
18. **Makonkawkeyoon, S., and W. Kasinrerk.** 1989. In vitro suppression of interleukin 2 production by *Mycobacterium leprae* antigen. Clin. Exp. Immunol. **76**:398–403.
19. **McDonough, K. A., Y. Kress, and B. R. Bloom.** 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. Infect. Immun. **61**:2763–2773.
20. **Miltenyi, S., W. Müller, W. Weichel, and A. Radbruch.** 1990. High gradient magnetic cell separation with MACS. Cytometry **11**:231–238.
21. **Molloy, A., G. Gaudernack, W. R. Levis, Z. A. Cohn, and G. Kaplan.** 1990. Suppression of T-cell proliferation by *Mycobacterium leprae* and its products: the role of lipopolysaccharide. Proc. Natl. Acad. Sci. USA **87**:973–977.
22. **Moreno, C., J. Taverne, A. Mehlert, C. A. W. Bate, R. J. Brealey, A. Meager, G. A. W. Rook, and J. H. L. Playfair.** 1989. Lipoarabinomannan from *Mycobacterium tuberculosis* induces the production of tumor necrosis factor from human and murine macrophages. Clin. Exp. Immunol. **76**:240–245.
23. **Moreno, J., and P. E. Lipsky.** 1986. Differential ability of fixed antigen-presenting cells to stimulate nominal antigen-reactive and

alloreactive T4 lymphocytes. J. Immunol. **136**:3579–3587.
24. **Mshana, R. N., R. C. Hastings, and J. L. Krahenbuhl.** 1988. Infection with live *Mycobacteria* inhibits in vitro detection of Ia antigen on macrophages. Immunobiology **177**:40–54.
25. **Nash, D. R., and J. E. Douglass.** 1980. Anergy in active pulmonary tuberculosis. A comparison between positive and negative reactors and an evaluation of 5 TU and 250 TU skin test doses. Chest **77**:32–37.
26. **Pancholi, P., A. Mirza, N. Bhardwaj, and R. M. Steinman.** 1993. Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. Science **260**:984–986.
27. **Pryjma, J., J. Baran, M. Ernst, M. Woloszyn, and H.-D. Flad.** 1994. Altered antigen-presenting capacity of human monocytes after phagocytosis of bacteria. Infect. Immun. **62**:1961–1967.
28. **Pryjma, J., W. Uracz, and M. Zembala.** 1989. Immunoregulatory function of monocytes, p. 327–334. *In* M. Zembala and G. L. Asherson (ed.), Human monocytes. Academic Press Ltd., London.
29. **Rastogi, N., M. Bachelet, and J. P. Carvalho de Sousa.** 1992. Intracellular growth of *Mycobacterium avium* in human macrophages is linked to the increased synthesis of prostaglandin E2 and inhibition of the phagosome-lysosome fusions. FEMS Microbiol. Immunol. **89**:273–280.
30. **Sasaki, D. T., S. E. Dumas, and E. G. Engleman.** 1987. Discrimination of viable and non-viable cells using propidium iodide in two color immunofluorescence. Cytometry **8**:413–420.
31. **Tsuyuguchi, I., H. Kawasumi, T. Takashima, T. Tsuyuguchi, and S. Kishimoto.** 1990. *Mycobacterium avium-Mycobacterium intracellulare* complex-induced suppression of T-cell proliferation in vitro by regulation of monocyte accessory cell activity. Infect. Immun. **58**:1369–1378.
32. **Valone, S. E., E. A. Rich, R. S. Wallis, and J. J. Ellner.** 1988. Expression of tumor necrosis factor in vitro by human mononuclear phagocytes stimulated with whole *Mycobacterium bovis* BCG and mycobacterial antigens. Infect. Immun. **56**:3313–3315.
33. **Wadee, A. A., R. Sher, and A. R. Rabson.** 1980. Production of a suppressor factor by human adherent cells treated with mycobacteria. J. Immunol. **125**:1380–1386.