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Serum Factors, Cell Membrane CD14, and β_2 Integrins Are Not Required for Activation of Bovine Macrophages by Lipopolysaccharide

THOMAS W. JUNGI,* HEINZ SAGER, HEIKO ADLER,† MARIJA BRCIC, AND HEDI PFISTER

Institute of Veterinary Virology, University of Berne, CH-3012 Berne, Switzerland

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The role of serum factors such as lipopolysaccharide (LPS)-binding protein (LBP) and of macrophage-expressed CD14 and β_2 integrins in the activation of bovine macrophages by LPS was investigated. Macrophage activation was determined by measuring tumor necrosis factor production, NO generation, and upregulation of procoagulant activity by LPS (*Escherichia coli* O55:B5) at concentrations of 100 pg/ml to 100 ng/ml. The 50% effective dose for LPS was 1 order of magnitude higher than that for activating human macrophages. Macrophages were activated by LPS in the presence of serum or in the presence of albumin demonstrated to be free of LBP. The capacity to react to LPS in the absence of LBP was not due to the acquisition of LBP during a previous culture in serum. It was then established which CD14-specific antibodies block LPS binding to monocytes. Among the CD14-specific antibodies recognizing bovine mononuclear phagocytes (60bca, 3C10, My4, CAM36, VPM65, CMRF31, and TÜK4), the first four blocked the binding of LPS-fluorescein isothiocyanate to bovine monocytes at low concentrations. Anti-CD14 antibodies did not block LPS-mediated activation of bovine bone marrow-derived macrophages, monocyte-derived macrophages, and alveolar macrophages. This was observed in experiments in which anti-CD14 concentrations exceeded the 50% inhibitory dose by >30-fold (3C10 and My4) or >300-fold (60bca), as defined in the binding assay described above. Monocyte-derived macrophages from an animal deficient in β_2 integrins and control macrophages were activated by similar concentrations of LPS, suggesting that β_2 integrins are not important bovine LPS receptors. Thus, in bovine macrophages, LPS recognition pathways which are independent of exogenous LBP, of membrane-expressed CD14, and of β_2 integrins may exist.

Vertebrates respond to minute concentrations of lipopolysaccharide (LPS) from the cell walls of gram-negative bacteria by an array of effector functions, e.g., by the production of inflammatory cytokines, the up-regulation of surface-expressed tissue factor, and, in some species, the high output of nitric oxide (31, 38). These functions provide a nonspecific host defense against LPS-containing organisms, but they also may contribute to the immunopathological sequelae characterizing gram-negative sepsis or a septic shock (39). The major cellular mediators of both host defense and immunopathology are mononuclear phagocytes, although neutrophils and endothelial cells also contribute. A number of cell surface recognition elements were reported to be involved in LPS binding and/or signal transduction (13, 15, 22, 32, 34, 35, 42, 51, 53). Two key constituents in the LPS-mediated activation of mononuclear phagocytes are LPS-binding protein (LBP) and CD14 (21, 49). LBP catalyzes the shuttling of LPS into a complex of LPS and either soluble or cell-bound CD14 (16, 54). Mononuclear phagocytes express CD14 at their surface, which is essential for the binding of LPS and the triggering of LPS-mediated functions, as suggested by studies with blocking anti-CD14 antibodies (10, 40, 47, 53), with cells transfected with CD14 (33), or with animals (18, 19) or humans (9, 44, 46) genetically deficient in cell surface-expressed CD14. Soluble CD14 present in plasma may associate with LPS, and the complex binds to as-yet-unknown receptors on endothelial cells (14, 20, 23). How LPS-cell association results in signal transduction is not known. Incompletely characterized cellular constituents are involved in mediation of a signal by CD14-associated LPS (48), and LPS receptors other than CD14 are expressed by monocytoid cells (13, 35, 42, 51). Their role in mediation of mononuclear phagocyte activation is not yet clear.

Most studies proving the importance of plasma LBP and cellular CD14 in functional activation of phagocytes were performed with human monocytes or monocytoid cell lines. Differentiated macrophages were investigated to a far lesser degree. We reported that during maturation of both human (29) and bovine (30) monocytes to macrophages, the ability to interact with LPS is altered. In particular, human macrophages are not dependent on exogenous LBP for their activation by low concentrations of LPS (27). In the present study, the requirement for exogenous serum factors, including LBP, and for cell surface-expressed CD14 for LPS-induced activation was studied in bovine macrophages from several organs. It was of particular interest to study the role of LBP and CD14 in LPS-induced NO generation, a function which is difficult to measure in human macrophages and which has received little attention in murine studies. Our findings raise the possibility that in addition to the soluble LBP-cell surface CD14 pathway, bovine macrophages have additional high-affinity LPS recognition elements mediating a variety of effector functions.

MATERIALS AND METHODS

Reagents. LPS (*Escherichia coli* O55:B5) was obtained from Sigma (St. Louis, Mo.; catalog no. L 2637). Fluorescein isothiocyanate (FITC)-labeled LPS (*E. coli* O55:B5) was purchased from List Biological Labs, Campbell, Calif. A panel of anti-CD14 antibodies was generously provided by R. Landmann, University Hospital, Basel, Switzerland. The mouse immunoglobulin G (IgG) subclasses and the origins of these antibodies, in parentheses, are as follows: 63D3 (IgG1)

* Corresponding author. Mailing address: Institute of Veterinary Virology, Laenggass-Strasse 122, CH-3012 Berne, Switzerland. Phone: 41 31 631 2502. Fax: 41 31 631 2534. E-mail: jungi@ivv.unibe.ch.

† Present address: Department of Hematology and Oncology, Dana Farber Cancer Institute, Boston, Mass.

(41), 3C10 (IgG2b; S. D. Wright, Rockefeller University, New York, N.Y.), B-a8 (IgG1; Serotec, Oxford, England), RoMo1 (IgG2a; C. Schütt, Greifswald, Germany), FMC32 (IgG1; Silenus, Victoria, Australia), and CMRF31 (IgG2a; D. N. Hart, Christchurch, New Zealand). TÜK4 (IgG2a) was purchased from Dako (Schlieren, Switzerland), Leu-M3 (IgG2b) was from Becton-Dickinson (Basel, Switzerland), MEM-18 (IgG1) was from Sanbio/Monosan (Uden, The Netherlands), and My4 (IgG2b) was from Coulter (Zug, Switzerland). CAM36 (IgG1) was a generous gift from William C. Davis, University of Washington, Pullman, and 60bca (IgG1) was generously provided by Philipp Bochsler, University of Knoxville, Knoxville, Tenn., and by Robert Todd III, University of Michigan, Ann Arbor. VPM65 was the kind gift of Ian McConnell, University of Oxford, Oxford, England. Among these antibodies, 3C10, CMRF31, TÜK4, My4, 60bca, and VPM65 bound to bovine monocytes and macrophages (see Results). The medium was from Seromed, Munich, Germany. Medium additives were from either Seromed or Life Technologies (Paisley, United Kingdom). Fetal calf sera (FCS) of undetectable endotoxin content were either from Life Technologies or from Biological Industries, Kibbutz Beth Haemek, Israel.

Animals. Bone marrow donors were calf fetuses obtained from various abattoirs. Bovine blood was obtained from animals of a Red Holstein herd. A Holstein-Friesian calf with bovine adherence deficiency (BLAD; β_2 integrin deficiency) and an age-matched control calf were also used as blood donors. β_2 integrin deficiency was verified by flow cytometry, restriction fragment length polymorphism, and pedigree analysis (37); all β_2-deficient animals detected to date are descendants of the Holstein sire Osborndale Ivanhoe (45), and the animal used in this study had this progeny both in its paternal and maternal lineage (37).

Cell isolation and culture. Bovine bone marrow cells were procured from tibiae of calf fetuses and cultured in the presence of 20% heated (30 min, 56°C) FCS under nonadherent conditions (2). This led to proliferation of macrophage precursor cells and to their subsequent differentiation into mature nonactivated macrophages; cells of all other hematopoietic lineages were gradually lost during culture. Bone marrow-derived macrophages (BMM) were subcultured in microtiter plates, where they were stimulated with LPS derived from *E. coli* (O55:B5) in the presence or absence of antibodies.

Bovine peripheral blood mononuclear cells (PBMC) isolated by an adapted Ficoll-metrizoate procedure were used either for flow cytometry or for the generation of monocyte-derived macrophages (MDM). For flow cytometry, either fresh PBMC or adherence-purified monocytes were used. The latter were obtained by a 2-h PBMC culture in 75-cm^2 flasks, removal of nonadherent cells, and overnight culture of adherent cells, followed by mechanical dislodgment. For the generation of macrophages, PBMC were sealed in Teflon bags (10 to 20 ml, 4×10^6 PBMC/ml) as described previously (30) and cultured for 6 to 8 days at 37°C in a humidified atmosphere of 5% CO_2. During this time, monocytes had matured to macrophages, which optimally responded to LPS and gram-negative organisms by NO generation. Cells harvested from Teflon bags were subcultured in microtiter plates and stimulated as described for BMM.

Bovine alveolar macrophages (BAM) were procured from fresh cadaver lungs as described previously (28). These cells either were used freshly or were cultured for 3 days under nonadherent conditions as described for monocytes. After being harvested from Teflon bags, they were subcultured in microtiter plates as described above.

Flow cytometry. PBMC were pretreated with different concentrations of antibodies and then stained with anti-murine IgG or IgM $F(ab')_2$ conjugated with phycoerythrin R (Jackson Immunoresearch Laboratories, West Grove, Pa.). Cells were analyzed in a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif.). Monocytes were gated on the basis of their light scatter properties, and the position of the monocyte gate used was confirmed by staining with anti-CD14.

The binding of LPS-FITC to monocytes was determined by flow cytometry. To PBMC (50 μl per Falcon tube [12 by 75 mm]), equal volumes of various dilutions of antibodies in RPMI 1640 containing FCS (10%) were added, and after 10 min at 37°C, LPS-FITC was added in another 50 μl (final concentration, 1 μg/ml). Control tubes containing a 67-fold excess of unlabeled LPS or human serum albumin (HSA; Swiss Red Cross Blood Transfusion Service, Berne, Switzerland; final concentration, 1%) instead of FCS or lacking LPS-FITC were included. PBMC were either 10^6 of freshly isolated bovine mononuclear cells or 0.5×10^6 of adherence-enriched monocytes, which were cultured overnight and then dislodged after 18 h of culture. After incubation for 30 min at 37°C, the cells were washed in phosphate-buffered saline and subjected to flow cytometry. The median fluorescence of cells within the monocyte gate was determined, and inhibition of binding by antibodies was expressed in percent, with 100% representing fluorescence by cells alone, in the absence of LPS-FITC. The LPS-FITC used and nonfluorescent LPS were found to activate bovine MDM at identical concentrations.

Cell stimulation. Bovine macrophage-enriched cell populations harvested from Teflon bag cultures were washed twice with phosphate-buffered saline and subcultured in Iscove's modified Dulbecco's minimal essential medium containing various concentrations of pyrogen-free FCS or in HSA (1%). Neither FCS nor HSA stimulated the macrophage parameters considered in the present study. Cells were dispensed into microtiter plates at concentrations ranging from 3×10^4 to 1×10^5 macrophages/well. Following an adherence phase of 2 h, the cells were exposed to LPS in the presence or absence of antibodies. After 24 h, supernatants were collected for the measurement of nitrite and tumor necrosis factor (TNF) content (see below), and the remaining cells were tested for expression of procoagulant activity (PCA).

Nitrite determination. The amount of NO generated was estimated by determining nitrite, one of the stable end products of NO, by the Griess reaction (1, 3).

TNF measurement. TNF was determined in a colorimetric cytotoxicity assay in which PK(15) cells served as target cells (5) or by a recently described antibody capture enzyme-linked immunosorbent assay (28).

PCA assay. A kinetic turbidimetric recalcification time assay (25) was used to estimate PCA expressed by adherent monolayer cells.

***Limulus* amoebocyte lysate assay.** All reagents to which cells were exposed were tested for endotoxin contamination by a turbidimetric kinetic *Limulus* amoebocyte lysate assay (12, 28). The test had a sensitivity of 2 pg of LPS (*E. coli* O55:B5) per ml. The various agents to which monocytes and macrophages were exposed, including the anti-CD14 antibodies used for inhibition studies, were found to be inactive.

RESULTS

Serum is not required for macrophage activation by LPS. MDM were exposed to LPS (100 pg/ml to 100 ng/ml) in the presence of various FCS concentrations and in medium in which FCS was replaced by 1% HSA. Serum was not required for recognition of LPS by these cells. In the case of NO generation, increasing concentrations of serum reduced the amount of nitrite generated (Fig. 1 and data not shown). In the case of PCA, increasing serum concentrations progressively decreased the sensitivity of macrophages towards low LPS concentrations. TNF induction was optimal at low serum concentrations.

To determine whether macrophages acquired the capacity to recognize LPS in a serum-independent manner during the culture in FCS-containing medium, e.g., by irreversible adsorption of LBP from serum, BAM harvested with serum-free PBS and BAM cultured for 3 days in Teflon bags in FCS-containing medium were assessed for serum requirement for LPS-mediated activation. Cultured (day 3) cells showed a stronger response in all three parameters than fresh (day 0) cells, particularly for nitrite generation (Fig. 2). Teflon bag-cultured BAM behaved like MDM did insofar as increasing serum concentrations dampened the response towards LPS in all three test systems (Fig. 2 and data not shown). A maximal response was observed either in the absence of serum (nitrite generation; TNF production by day 0 macrophages) or with 0.5% serum (PCA; TNF production by day 3 macrophages), but the differences between 0 and 0.5% serum were not significant. The complete omission of serum was associated with a reduction in sensitivity of cultured (day 3) macrophages towards LPS, which was least expressed in NO generation (data not shown). In contrast, fresh (day 0) BAM collected in the absence of serum responded to the lowest stimulatory LPS concentration (1 ng/ml) regardless of whether 1% HSA or 0.5% FCS supplemented the medium (data not shown). Thus, the capacity to react to LPS in the absence of serum does not depend on preculture of macrophages in serum-containing medium.

The apparent lack of serum requirement could be due to incomplete removal of serum constituents after Teflon bag cultivation, i.e., due to incomplete washing of macrophages, or to a contamination of HSA with LBP. These possibilities were tested by measuring the capacity of macrophage supernatants and washing fluids for the presence of LBP activity, by use of a functional assay. LBP in serum is known to support the binding of LPS-FITC to monocytes (21). Therefore, the conditions under which LPS-FITC binds to monocytes were explored (Fig. 3). In MDM activation experiments (see below), LPS-FITC and unconjugated LPS from the same *E. coli* strain showed identical dose-response relationships, suggesting that FITC conjugation did not alter the functional properties of

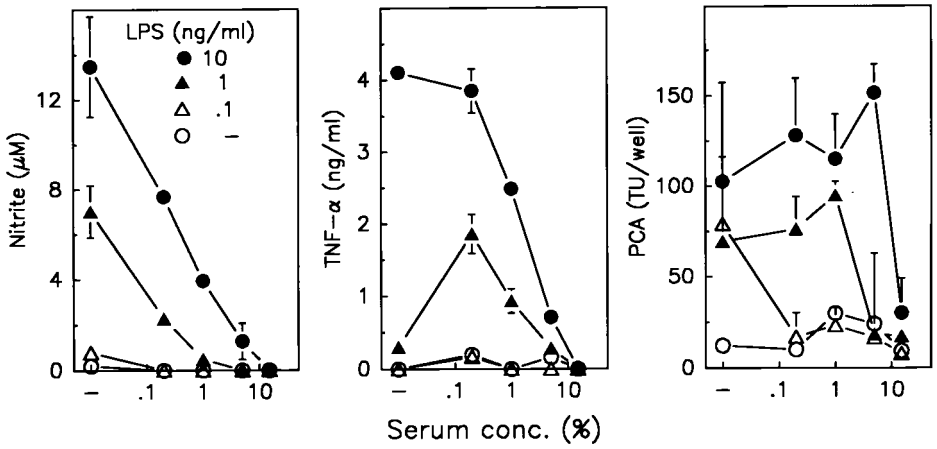

FIG. 1. Activation of bovine MDM by LPS as a function of FCS concentration. Twenty-four hours after LPS stimulation or mock stimulation, supernatants were collected for determination of nitrite content by the Griess reaction and for TNF measurement by enzyme-linked immunosorbent assay. The remaining cell monolayer was used to measure PCA by turbidimetry. Means of triplicates ± standard deviations of a representative experiment are shown. The experiment was repeated four times with essentially similar results. — (*x* axis), serum was replaced by 1% HSA.

LPS (data not shown). LPS-FITC bound well to monocytes in the presence of FCS (Fig. 3A, trace b) but not in the presence of HSA (Fig. 3A, trace c), suggesting that the binding was serum dependent. The binding could be blocked with an excess of unlabeled LPS (Fig. 3, traces a), suggesting that it was specific. MDM were harvested from Teflon bags and repeatedly washed and resuspended in serum-free medium. While the first macrophage supernatant supported the binding of

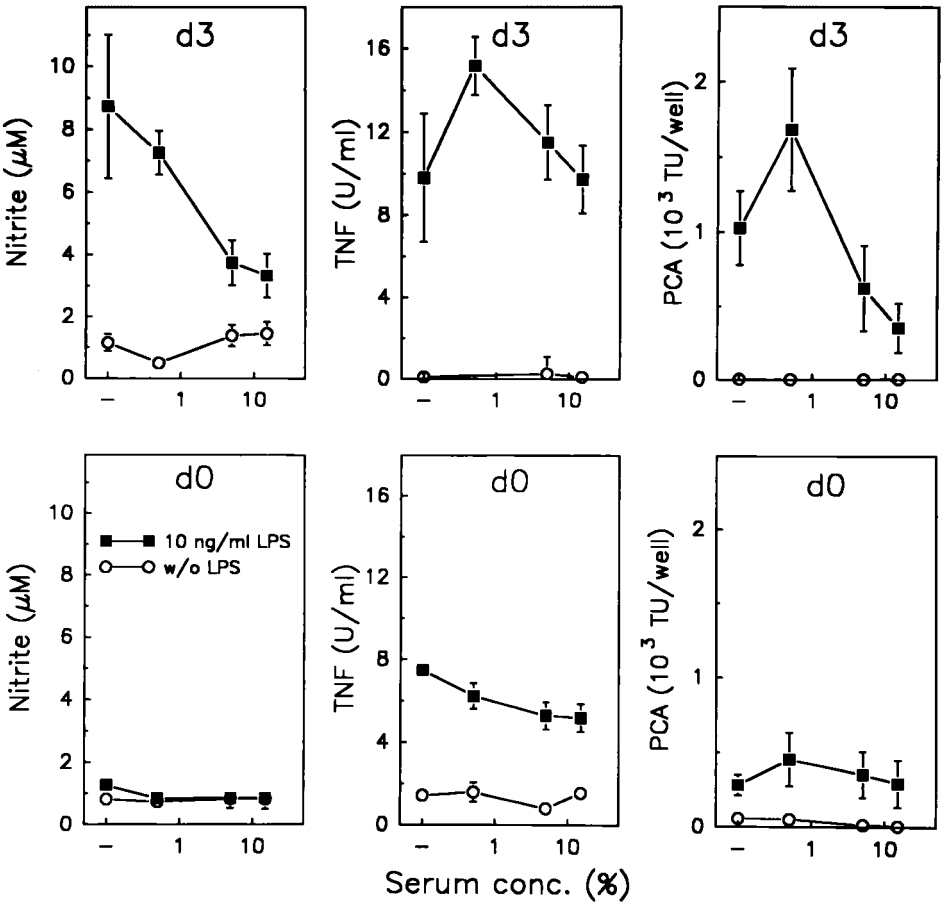

FIG. 2. Activation of BAM by LPS as a function of serum concentration. Cells were subcultured in microtiter plates immediately after collection in serum-free saline (d0) or after 3 days of culture in FCS-containing medium (d3). The indicated effector functions (nitrite generation, TNF secretion, and PCA) were determined 24 h after stimulation by LPS (10 μg/ml) or mock stimulation. — (*x* axis), serum was replaced by 1% HSA. Means ± standard errors of the mean of four independent experiments are shown. Similar results were obtained with 1 and 100 ng of LPS per ml.

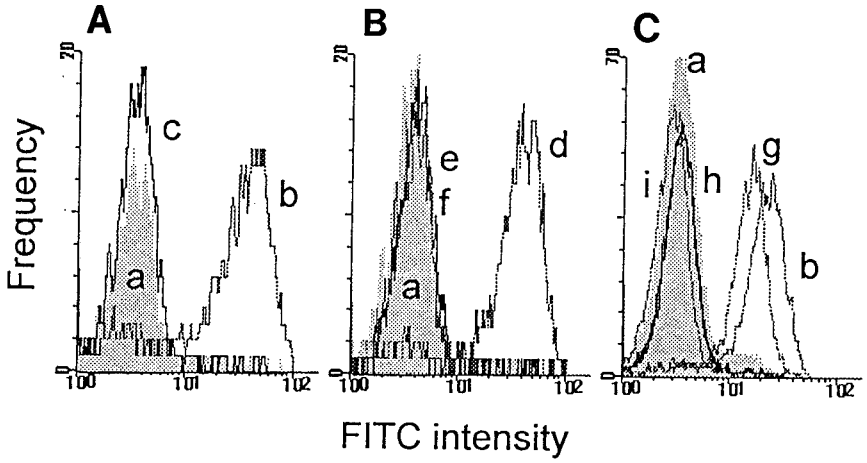

FIG. 3. Binding of LPS-FITC to monocytes cultured overnight, as determined by flow cytometry. Representative histograms of LPS-FITC binding to light scatter-defined monocytes are shown. The binding of LPS-FITC (1 μg/ml) was determined under various conditions. Shaded histograms (a) of all panels represent a 67-fold excess of unlabeled LPS. (A) Binding in the presence of 10% FCS or 1% HSA (b and c, respectively). (B) Demonstration of LBP absence in washed MDM. Monocytes resuspended in 1% HSA were supplemented with MDM culture supernatant (20% FCS) from Teflon bag culture (d), by the first washing fluid from Teflon bag-cultured macrophages (e), or by the second washing fluid from Teflon bag-cultured macrophages (f). (C) Effect of anti-CD14. Binding levels of LPS-FITC in the presence of 9-fold-diluted MEM-18 (g), 9-fold diluted My4 (h), 22-fold diluted CAM36 (superimposed with h), or 9-fold diluted 60bca (i) are shown. Each part of the experiment was repeated at least three times.

LPS-FITC to monocytes, the second and third washing fluids displayed decreased activity in this respect (Fig. 3B, traces d, e, and f), suggesting that with repeated washing, the LBP content of washing fluids decreased. The third washing fluid, which was devoid of LBP activity in the binding assay, was identical to the medium used for cell subculture in the antibody inhibition experiment. Thus, the ability of LPS to activate macrophages cultured in serum-free, albumin-containing serum could not be explained by the presence of functionally active LBP in the medium.

Recognition of bovine CD14 by monoclonal antibodies. We then wished to determine the role of CD14 in the activation of bovine macrophages. This required the identification of antibodies recognizing bovine CD14. A panel of 14 CD14-specific monoclonal antibodies was tested by flow cytometry for their reactivity with bovine mononuclear phagocytes. The human CD14-specific antibodies TÜK4, 3C10, CMRF31, My4, and 60bca reacted with mononuclear phagocytes at high dilutions (Table 1). VPM65 (specific for sheep CD14) and CAM36 (with specificity for bovine monocytes) also reacted with the cells mentioned above. The exposure of monocytes to CAM36 blocked the binding of PE-labeled TÜK4, suggesting that CAM36 is CD14-specific. The human CD14-specific antibodies Leu-M3 and MEM-18 displayed a weak staining at high antibody concentrations, and B-a8, RoMo1, FMC32, 60D3, and Mo2 failed to bind to bovine mononuclear cells (Table 1). The patterns of reactivity were identical for all cell types tested. These included fresh monocytes, adherence-purified monocytes cultured overnight, MDM (day 7), and freshly collected alveolar macrophages (Table 1). Antibodies displaying a low affinity for bovine monocytes and macrophages included antibodies which are known to block LPS binding to human monocytes (e.g., MEM-18) as well as antibodies which have no or a low blocking activity (e.g., anti-Leu-M3).

Blockade of LPS binding to bovine monocytes by CD14-specific antibodies. The effect of the bovine monocyte-specific antibodies on the binding of LPS to monocytes mentioned above was tested by use of LPS-FITC in flow cytometry. LPS-FITC binding to monocytes was shown above to occur in the presence of FCS, but not HSA, and was blocked by excess unlabeled LPS (Fig. 3, traces a to c). LPS-FITC binding to monocytes was blocked by the CD14-specific antibodies 60bca (Fig. 3, trace i), CAM36, and My 4 (Fig. 3, superimposed traces h) but not by MEM-18 (Fig. 3, trace g), which binds poorly to bovine mononuclear phagocytes (Table 1). The relative potencies in binding inhibition of various CD14-specific antibodies are depicted in Fig. 4. Such experiments confirmed that My4, 60bca, CAM36 (Fig. 4), and 3C10 (data not shown) blocked the binding of LPS-FITC to monocytes in a dose-dependent manner. MEM-18 blocked at extremely high concentrations (Fig. 4), and TÜK4 and VPM65 as well as several antibodies which failed to bind to bovine monocytes did not block this interaction (data not shown). Due to a relatively high consti-

TABLE 1. Binding of anti-CD14 to bovine mononuclear phagocytes[a]

Antibody[b]	Mean fluorescence			
	Fresh monocytes (fresh)	Day 1 monocytes	Day 7 MDM	Alveolar macrophages
None	1.0	1.0	1.0	1.0
TÜK4	**39.2**	**20.2**	**124.7**	**46.3**
3C10	**30.1**	ND[c]	ND	ND
CMRF31	**16.6**	**58.3**	**193.2**	**49.4**
My4	**15.0**	**49.4**	**247.5**	**50.7**
60bca	ND	**65.7**	**476.5**	**71.8**
CAM36	ND	**94.2**	**170.6**	**74.0**
VPM65	ND	**50.7**	**114.1**	**23.4**
Leu-M3	3.6	7.3	4.2	4.2
MEM-18	2.2	3.4	5.3	ND
B-a8	1.8	1.8	10.8	4.7
RoMo1	1.3	1.2	5.1	ND
FMC32	1.3	1.0	ND	ND
63D3	1.0	1.6	4.5	3.3
Mo2	1.0	0.35	1.0	1.0

[a] Data were standardized such that the background control (second antibody only) was taken as 1.0. For each cell type, data from two experiments were averaged. Boldface values indicate strong specific staining.
[b] Optimal concentrations of antibodies were used in each case.
[c] ND, not determined.

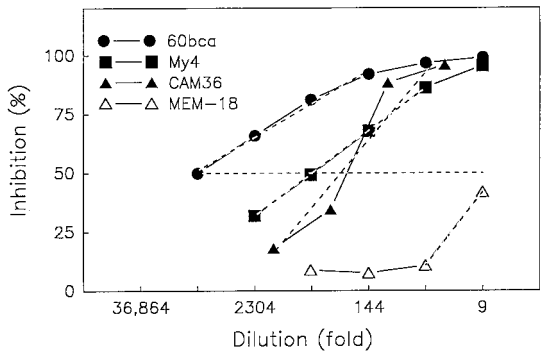

FIG. 4. Dose-dependent inhibition of binding of LPS-FITC to overnight cultured bovine monocytes, as determined by flow cytometry. The *y* axis represents percent inhibition. The *x* axis represents antibody dilution. The calculated ID_{50}s, represented by the broken lines, are 315-fold dilution (CAM36), 0.63 μg/ml (My4), and 0.16 μg/ml (60bca). One of three experiments with similar results is shown.

tutive fluorescence in the absence of added LPS-FITC, the same experiment could not be performed with macrophages. When monocytes were first allowed to react with LPS, then exposed to the antibodies, and finally stained with anti-IgG, fluorescence was not influenced by LPS exposure (data not shown), a result reminiscent of similar findings made with human monocytes (40).

Effect of anti-CD14 on bovine macrophage activation by LPS. The effect of CD14-specific blocking antibodies on activation of bovine macrophages was tested. In these experiments, the lowest LPS concentration evoking significant activation varied between 100 pg/ml and 10 ng/ml, depending on the cell batch and effector function tested, but was usually 1 ng/ml. Table 2 and Fig. 5 show that anti-CD14 failed to block activation of MDM by LPS. The same was observed for BAM (Fig. 5) and BMM (data not shown). Activation of macrophages was not prevented by antibody concentrations far in excess of that required for blocking the binding of 1 μg of LPS per ml to monocytes. In these experiments, antibodies were used at 20 to 27 μg/ml (3C10 and My4; ca. a 30- to 50-fold excess of the 50% inhibitory dose [ID_{50}]) (Fig. 4) or at 40 to 60 μg/ml (60bca; up to 300-fold excess of the ID_{50}). The failure of CD14-specific antibodies to block LPS-mediated activation was not due to their absorption by soluble CD14 in serum, since it was also observed at low serum concentrations (Table 2), or in the absence of serum but the presence of HSA (Fig. 5).

In the previous experiments, parameters of macrophage activation were even higher in the presence of pyrogen-free anti-CD14 than in its absence or in the presence of irrelevant, pyrogen-free antibody preparations (Table 2 and data not shown). This raised the possibility that CD14 cross-linking or CD14-Fc receptor bridging (36) enhances macrophage effector functions. Mimicking this type of cross-linking was attempted by macrophage exposure to anti-CD14. Microtiter plates were coated with My4 (20 μg/ml), and macrophages were added; this was followed by mock stimulation or LPS stimulation 2 h later. After 24 h, supernatants were tested for nitrite and TNF, and cells were assayed for PCA. Antibody coating did not modulate these parameters. Likewise, the addition of anti-CD14-pretreated, washed macrophages to uncoated or anti-mouse IgG-coated wells did not modulate the nitrite, TNF, and PCA responses following either mock stimulation or LPS stimulation (data not shown).

Role of β_2 integrins as LPS receptors of bovine macrophages. In searching for a non-CD14 LPS receptor, the role of β_2 integrins was studied in macrophages from a β_2 integrin-deficient calf. MDM of the deficient animal and a β_2 integrin-expressing, age-matched control animal were stimulated with graded concentrations of LPS, and macrophage effector functions were determined 24 h later. The LPS-induced responses of macrophages from the β_2 integrin-deficient animal were generally higher than those of cells from the control animal (Table 3). The 50% effective doses were similar in both animals and were of the same order of magnitude as that for macrophages from older animals (Table 2). This argues against a major role of β_2 integrins as LPS receptors in bovine macrophages. Notably, LPS responsiveness was not altered when β_2 integrin-deficient macrophages were exposed to LPS in the presence of high concentrations of blocking anti-CD14. This raises the possibility that elements distinct from CD14 and β_2 integrins are involved in recognition of and response to LPS by bovine macrophages.

DISCUSSION

In this study, we investigated the requirements for bovine macrophage activation by LPS. The major findings are that neither serum factors such as LBP nor CD14 are necessary for macrophage activation. This suggests the existence of pathways in macrophages independent of exogenous LBP and cell membrane-expressed CD14. Tests with a genetically β_2 integrin-deficient animal allowed us to rule out CD11b/CD18 as an important LPS receptor as well. Our findings are at variance with the current paradigm of LPS-mediated macrophage activation by showing LPS activation of macrophages being (i) independent of the presence of exogenous serum factors and (ii) independent of surface-expressed CD14. These two issues are discussed separately below.

Several serum-derived constituents were reported to mediate the interaction of LPS with mononuclear phagocytes (6, 14, 43, 52). Among these, the significance and mechanism of action of LBP are characterized best. LBP was earlier thought to participate in the formation of a trimolecular complex formed by LPS, LBP, and CD14 (53). Recent evidence suggests that LBP has a catalytic function and mediates the transfer of LPS monomers from LPS aggregates to membrane-expressed CD14 (mCD14) of monocytes or to soluble CD14 (16, 54). Our finding that serum-derived LBP is not required for LPS-mediated macrophage activation is not without precedence; several earlier reports suggested that human monocytes, monocytoid cell lines, or alveolar macrophages may be activated by LPS in

TABLE 2. Effect of anti-CD14 on activation of bovine MDM[a]

LPS concn	Nitrate produced (μM) with:		
	No antibody	Control antibody (IgG2b)[b]	Anti-CD14 (My4)[b]
0	0.04 ± 0	0.79 ± 0.42	2.69 ± 0.23
0.1	0.29 ± 0.25	0.87 ± 0.14	3.39 ± 0
1	2.69 ± 0.47	2.37 ± 0.14	7.14 ± 0.53
10	5.38 ± 0.47	3.46 ± 0.35	11.05 ± 0.34

[a] Macrophages cultivated for 8 days were exposed to LPS with or without antibody CD14 (My4), and after 24 h, the nitrite generated was determined. Values indicate means ± standard deviations (three determinations per group). The experiment was repeated, and similar results were obtained. Similar results were obtained for TNF production and PCA up-regulation and with antibody 60bca.

[b] Test concentration, 20 μg/ml.

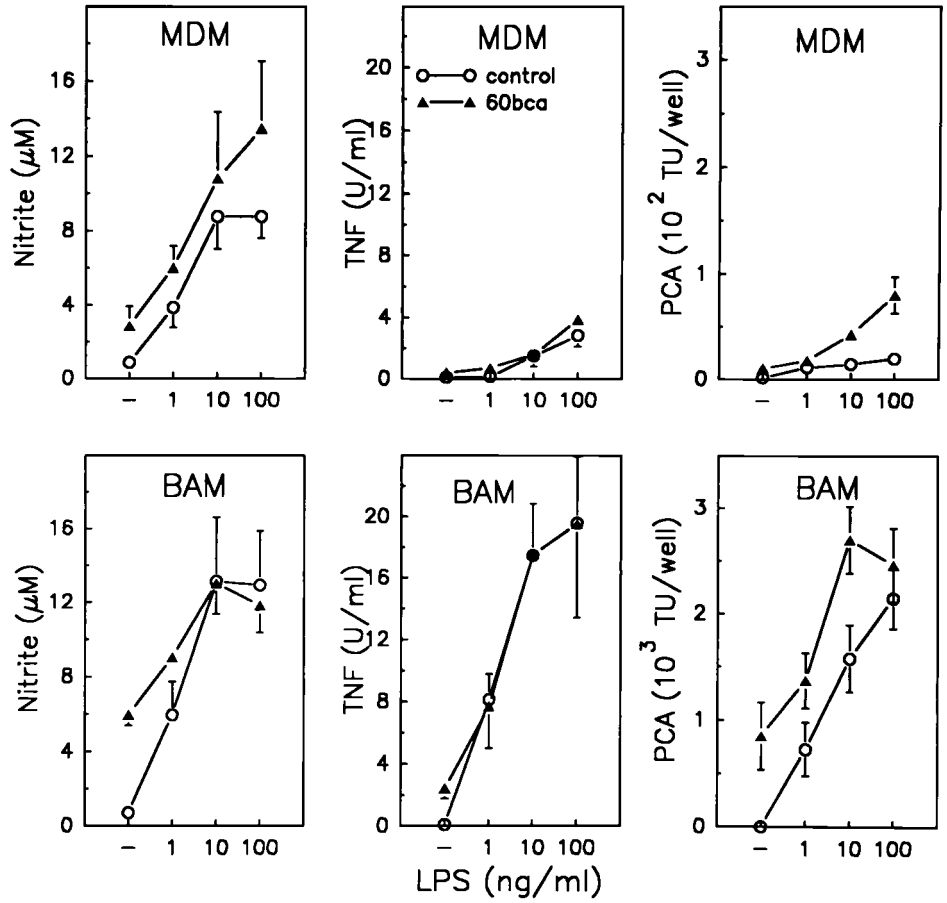

FIG. 5. Effect of anti-CD14 antibodies on activation of bovine MDM and BAM by LPS at various concentrations. Cells were stimulated for 24 h in the presence or absence of 60bca antibody (60 μg/ml), and then nitrite generation, TNF production, and PCA were determined. Data represent means ± standard errors of the means of four (MDM) or five (BAM) independent experiments.

the absence of serum (8, 10, 22, 24). All of these studies differ from the present one either in a significantly lower level of activation in the absence of serum or in a higher LPS concentration required for activation. Two macrophage studies, however, support the present contention. First, a study on nitrite generation by the murine macrophage cell line J774A.1 showed that these cells react to lower concentrations of LPS when tested in the absence of serum than when tested in its presence (50). Second, our laboratory showed that human monocyte-derived macrophages and certain monocytoid cell lines display TNF release and PCA expression to similar extents and with identical dose-response relationships under serum-deficient and serum-sufficient conditions (27). As in this study, a contribution by a small concentration of contaminating LBP could be ruled out.

The discrepancy between our studies and some of the earlier ones demonstrating the importance of serum (21, 32, 53, 55) might be explained in part by suboptimal culture conditions. When serum-free conditions were used, serum was replaced by HSA devoid of LBP activity in our studies (27), since macrophages do not perform in a physiological manner when the absence of serum is not compensated for by the presence of albumin or other proteins. It is our conclusion, therefore, that human macrophages (27), bovine macrophages, and murine

TABLE 3. Effect of anti-CD14 on activation of macrophages from a normal and a β_2 integrin-deficient calf (i.e., with BLAD)[a]

LPS (ng/ml)	My4 (μg/ml)	Nitrite (μM)		TNF (ng/ml)		PCA (TU/well)	
		BLAD	Control	BLAD	Control	BLAD	Control
		0.41 ± 0.2	0 + 0	0 + 0	0 ± 0	22 ± 8.1	1,039 ± 178
0.1		1.43 ± 0	1.04 ± 0.59	0.72 ± 0.2	0 ± 0	120 ± 398	1,757 ± 0
1		20.3 ± 0.57	3.59 ± 0.46	13.6 ± 0.64	0 ± 0	1,607 ± 170	868 ± 146
10		26.4 ± 1.40	6.88 ± 0.67	26.3 ± 1.03	2.79 ± 0.09	6,995 ± 1,061	2,660 ± 549
1	25	19.9 ± 0.78	1.27 ± 0.45	22.5 ± 0.16	0 ± 0	3,359 ± 2,039	1,883 ± 218
10	25	20.7 ± 1.07	6.81 ± 0.38	33.7 ± 0.41	5.47 ± 0.07	4,331 ± 582	4,027 ± 0

[a] MDM from a BLAD calf and age-matched control cells cultivated for 8 days were exposed to LPS with or without anti-CD14 (My4), and after 24 h, nitrite, TNF, and PCA levels were determined. Values indicate means ± standard deviations (three determinations per group). Similar results were obtained in a second experiment.

macrophage cell lines (50) respond to low LPS concentrations regardless of the presence or absence of exogenous serum. It remains to be seen whether this is a differentiation-related trait and how macrophages acquire the capacity to recognize and respond to LPS in the absence of exogenously added serum factors. Moreover, a putative serum factor quenching LPS-induced macrophage effector functions (Fig. 1 and 2) needs to be characterized in further studies.

The importance of mCD14 in mononuclear cell activation by LPS is well documented (10, 18, 19, 33, 40, 46, 53). We now show that LPS activation of bovine macrophages is not blocked by anti-CD14 antibodies. This is in partial contrast to a previous study using BAM and a monoclonal antibody also used here since partial inhibition of LPS-induced tissue factor induction was observed (56). It is also at variance with several studies, including our own, in which CD14-specific antibodies blocked human monocyte or monocytoid cell line activation by LPS (4, 10, 21, 24, 27, 40, 53). The question arises, therefore, whether this variation has a technical or a biological basis.

Technical explanations encompass (i) an insufficient affinity to bovine CD14 of the antibodies used, (ii) fine specificity of anti-CD14 for monocyte rather than macrophage-expressed CD14, (iii) contamination of antibody preparations by endotoxin or other macrophage-activating by-products, and (iv) activation of macrophages due to antibody-mediated cross-linking. The first explanation is highly unlikely since much smaller concentrations of anti-CD14 specifically blocked the binding of higher concentrations of LPS to bovine monocytes. The assumption that the fine specificity of CD14 alters during the maturation of monocytes to macrophages is also unlikely, since the reactivity spectrum of CD14-specific antibodies was exactly the same for bovine monocytes and macrophages. The third assumption is improbable, too, since the antibodies used were devoid of endotoxin activity at the tested concentrations, as judged from a sensitive *Limulus* amoebocyte lysate test. Moreover, it would not explain why macrophage activation was LPS dose-dependent rather than antibody dose-dependent. The fourth possibility was addressed by experiments in which surface CD14 was cross-linked by anti-CD14, followed by a second antibody. Under these conditions, macrophages were not activated. This does not rule out that under the usual test conditions (24 h in the presence of antibodies), cross-linking did result in activation. Nevertheless, the LPS dose dependency needs to be explained. A definitive answer depends on the availability of pyrogen-free CD14-specific F(ab) preparations.

As biological reasons for the discrepancy described above one may consider variations due to (i) distinct effector functions tested, (ii) different macrophage subsets analyzed, and (iii) species differences. Differences due to distinct effector functions tested are improbable, except that LPS binding may not parallel LPS-mediated cell activation. Differences due to distinct cellular subsets might be a contributing reason in that most studies in which an effect of anti-CD14 was demonstrated were performed with monocytes. With regards to macrophages, identical results were obtained with BMM, MDM, and BAM. We favor species variation as a contributing factor. Thus, human and bovine monocyte-derived macrophages were tested under similar conditions (reference 27 and present study), yet the results were completely different.

The putative species differences are also expressed in the sensitivity of macrophages to low LPS concentrations. Smooth LPS from *E. coli* induces half-maximal responses at concentrations of less than 100 pg/ml in human macrophages and certain monocytoid cell lines (12, 27) and at concentrations 1 order of magnitude higher in bovine macrophages expressing mCD14. It is intriguing to note that at 100 ng of LPS per ml, anti-CD14 no longer blocks LPS-mediated activation of human monocytes and macrophages (7, 26). Bovine mCD14 may have a relatively low affinity to LPS in comparison to that of human mCD14. The triggering of bovine macrophages with LPS might therefore reveal an LPS activation pathway that is also present in other species, but that is not seen as clearly, due to redundancy with the high-affinity mCD14 system. It will be of interest to further characterize mCD14-independent LPS activation pathways in bovine macrophages but also in human and murine cells genetically deficient for mCD14. The question arises as to which of the many LPS receptors proposed (11, 13, 15, 17, 22, 34, 35, 42, 51), if any, is operative under the conditions described here. CD11b/CD18 has been put forward as an LPS receptor which becomes important under conditions of low serum concentrations (22, 51). That CD11b/CD18 does not operate as a substitute for CD14 in cattle is supported by our finding that macrophages from animals genetically deficient in all β_2 integrins respond to LPS as well as β_2 integrin-expressing macrophages do. This is even true in the absence of serum and in the presence of blocking concentrations of anti-CD14 (Table 2). This led us to suggest that in addition to the CD11b/CD18 and the mCD14 recognition pathways, additional mechanisms of LPS-mediated macrophage activation may exist.

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