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Regulation of Major Histocompatibility Complex Class II Expression by *Pasteurella haemolytica* Leukotoxin†

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Many properties have been associated with *Pasteurella haemolytica* leukotoxin and other repeat-in-toxin toxins, including their cytotoxic activity on various cells of the lymphoid and nonlymphoid systems as well as their ability to modulate the immunological activity of lymphocytes and monocytes. In this study, we assessed the ability of *P. haemolytica* leukotoxin to affect the expression major histocompatibility complex (MHC) class II molecules on bovine peripheral monocytes. Peripheral blood mononuclear cells were isolated from *P. haemolytica* leukotoxin-seronegative calves and incubated with various concentrations of authentic leukotoxin as well as the recombinant *lktA* gene product (LktA). Expression of MHC class II antigen on cells was evaluated by flow cytometric methods. The results indicated that both a crude, authentic leukotoxin preparation and LktA were able to affect MHC class II expression by inducing a marked downregulation of MHC class II expression on bovine monocytes. However, when cells were activated with gamma interferon (IFN-γ), LktA and Lkt had little or no detectable effect. By using a cell line which expresses MHC class II only after activation by IFN-γ, we were able to confirm the observation that LktA had no effect on the expression of MHC class II after IFN-γ treatment. Leukotoxin affected the functional capacity of monocytes to present antigen, as demonstrated by the ability of LktA or authentic leukotoxin to totally inhibit a mixed lymphocyte culture from MHC-mismatched calves. Thus, leukotoxin was able to downregulate constitutive expression of MHC class II expression, and we propose that this is a novel way in which this molecule can affect the immune function of monocytes, playing an important role in bacterial pathogenesis and survival of organisms at the infection site.

Pasteurella haemolytica (serotype type 1, biotype A) is a bacterium commonly found in the upper respiratory tract of cattle. Under appropriate conditions, the organism can colonize the lower respiratory tract and cause acute fibrinous pneumonia. The acute pulmonary lesions are characterized by pulmonary edema, the onset of which is characterized by microvascular injury. Several potential *P. haemolytica* virulence factors have been identified, including lipopolysaccharide (LPS) (23) and leukotoxin (Lkt) (1, 2).

P. haemolytica leukotoxin is cytotoxic for polymorphonuclear cells (PMN) (7) and macrophages (21). Originally, it was thought that this property was responsible for enhancing the virulence of invading organisms by killing pulmonary macrophages and neutrophils (6). However, it is now apparent that the pathogenesis of pasteurellosis is not due to the cytotoxic activity of Lkt alone, as indirect tissue damage at sites of infection may occur through the release of neutrophil (19, 25) and macrophage (18) products that can mediate endothelial cell damage. In support of this, animals depleted of neutrophils did not develop any gross pneumonic lesions after exposure to *P. haemolytica* (26). However, PMN may also have a protective effect, particularly in the presence of immune serum (6).

Leukotoxin also modulates lymphocyte function. In a series of studies, Majury and Shewen (19, 20) indicated that a partially purified Lkt preparation was able to inhibit the mitogen or antigen responsiveness of bovine peripheral blood monocytes. The inhibition was present even when the Lkt was heat inactivated and could be abrogated by the addition of anti-Lkt antibody (19). Therefore, some effect other than cytotoxicity must have been occurring. The inhibition was not restricted to bovine species; lymphocyte proliferation could also be inhibited in human and dog lymphocyte cultures. In a further series of experiments, the mechanism of inhibition of proliferation was investigated by adding back cytokines and other immune modulators. To some extent, the addition of exogenous interleukin-2 (IL-2) or of the glycolipid monosialoganglioside was able to overcome the inhibition. The addition of exogenous IL-1, however, was only marginally effective. It was suggested that the inhibition of lymphocyte proliferation may be the result of a failure of IL-2 production or altered IL-2 receptor expression (20).

The leukotoxin genes have been cloned (17) and expressed in *Escherichia coli*, and the *lktA* gene product is accepted as being highly immunogenic, yet it has no cytolytic activity (8, 12). The production of these molecules has allowed the in-depth study of the relationship between LktA and host cells without induction of any of the harmful cytolytic mechanisms. Recently, we have assessed antibody and cellular responses both in vivo and in vitro using both recombinant LktA and a chimeric molecule derived from a gene fusion between the *lktA* and the bovine IL-2 genes (14). We have since produced another fusion protein consisting of LktA and bovine gamma interferon (IFN-γ) and have assessed the immunomodulatory capacity of these molecules. Our results indicate that LktA can modulate the expression of major histocompatibility complex (MHC) class II molecules, and we propose that this novel activity may be another method by which *P. haemolytica* Lkt can affect the immune status of the host by regulating the early stages of antigen recognition and lymphoproliferation. These findings are discussed in the context of the pathogenesis of *P. haemolytica* infection.

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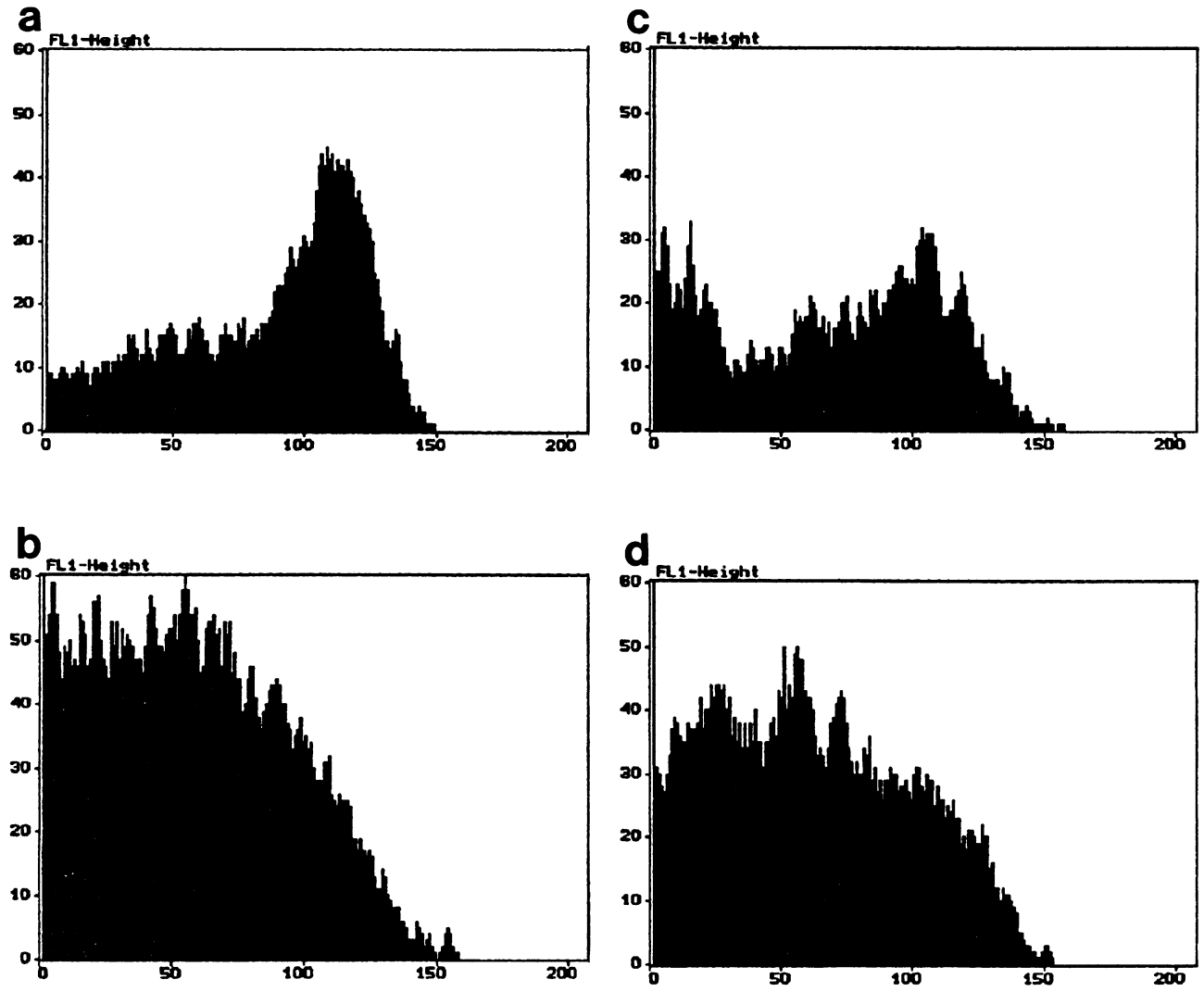

FIG. 1. Flow cytometric profiles of peripheral blood monocytes from two animals, stained with anti-MHC class II. Two-parameter analysis of forward angle versus 90° light scatter was used to gate the population for fluorescence analysis. Typically, 80 to 90% of those cells that were gated stained positive for the monocyte-granulocyte marker DH59B. (a and c) Untreated monocytes stained for MHC class II (TH14B). The peak fluorescence occurs at channel 118. (b and d) Monocytes from the same animal, treated with LktA and stained with TH14B. Peak fluorescence occurs at channel 60. This experiment has been repeated on at least 25 blood samples with similar results.

MATERIALS AND METHODS

Animals and cell lines. Hereford beef calves, 7 to 8 months old, that were seronegative for antibodies against *P. haemolytica* biotype A, serotype 1, were used throughout the study. Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (Rockville, Md.) and cultured by serial passage in Dulbecco's modified minimal essential medium (GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum (Hyclone, Logan, Utah). All reagents were kept free of bovine viral diarrhea virus, endotoxin, and *Mycoplasma* spp.

Leukotoxin and cytokines. Recombinant LktA was prepared from *E. coli* as inclusion bodies as described previously by Harland et al. (12).

Authentic leukotoxin (Lkt) was obtained from *P. haemolytica* in log-phase growth. *P. haemolytica* serotype 1 strain B122 was grown in brain heart infusion broth medium at 37°C with vigorous agitation until the A_{660} was 1.1. Cells were removed by centrifugation, and the supernatant was filter sterilized. The amount of leukotoxin in test samples was measured by reaction with monospecific rabbit serum against recombinant LktA. Briefly, 96-well plates (Nunc Immulon II) were coated with twofold dilutions of a reference leukotoxin solution and the test sample by incubation at 4°C overnight in

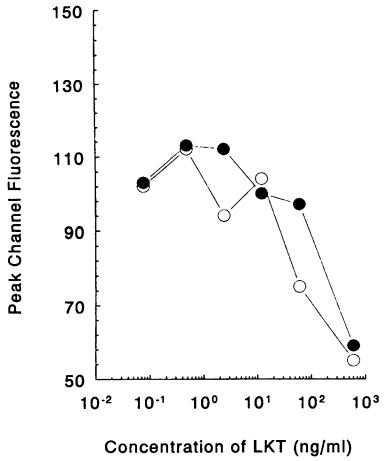

FIG. 2. Effects of LktA on peak channel fluorescence. Peak channel fluorescence was assessed after flow cytometry of cells stained with TH14B and plotted against the concentration of LktA. There was a dose-dependent relationship between the concentration of LktA used to treat cells and the diminished level of MHC class II expression. Results for two animals (○, ●) are shown and are representative of at least 25 replicates.

carbonate buffer. The plates were washed with double-distilled water, and rabbit antileukotoxin was added in Tris-buffered saline plus 1.5% bovine serum albumin at a 1:5,000 dilution. After 1 h at room temperature, the plates were washed eight times with water, and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; heavy and light chains) was added at a 1:10,000 dilution (50 ng/ml). After 1 h at room temperature, the plates were developed with a commercially available substrate-amplification kit (Bethesda Research Laboratories). The amount of leukotoxin was confirmed by running twofold dilutions of leukotoxin on a 7.5% polyacrylamide gel and comparing the intensity of the Coomassie blue-stained bands with that of a bovine serum albumin control.

Recombinant bovine IFN-γ (rBoIFN-γ; CIBA-GEIGY) had

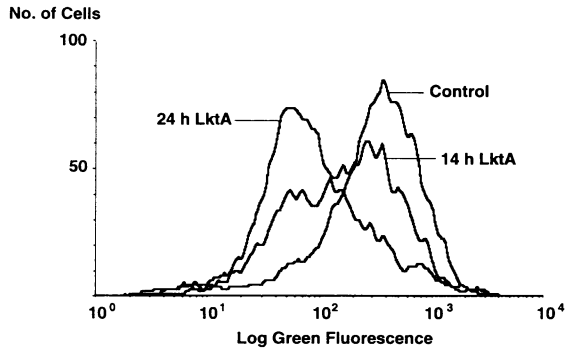

FIG. 3. Effects of Lkt on MHC class II induction over time. PBMC were incubated in the presence of LktA for between 10 and 48 h. By 14 h after incubation, there was a noticeable decrease in the percentage of cells expressing MHC class II. At 24 h, only 31% were fluorescing at or above channel 133 (high responders). There was no difference between the results at 24 and 48 h of incubation. Only profiles from three time points (0, 14, and 24 h) are shown for simplicity, and cells incubated in medium alone (control) for different times showed no change in MHC class II expression. This experiment was repeated on three separate occasions with similar results.

TABLE 1. Kinetics of LktA-induced inhibition of MHC class II[a]

Time (h)	% of monocytes fluorescing		Peak channel
	High	Low	
0	83	17	378
10	80	20	392
12	82	18	352
14	69	31	305
16	63	37	264
18	58	42	254
20	54	46	177
22	49	51	70
24	31	69	52

[a] High, monocytes fluorescing at or above channel 133; low, monocytes fluorescing at or below channel 133. The peak channel is the channel at which the highest frequency of cells fluoresced.

a specific activity of approximately 3×10^6 U/mg of protein and was supplied free of detectable endotoxin. IFN-γ purity was >95%, and it was provided at a typical protein concentration of 0.54 mg/ml. All reagents were stored at −20°C; cytokines were stored at 4°C. Recombinant leukotoxin was assessed for LPS contamination by the *Limulus* amebocyte lysate assay (Whittaker Bioproducts Inc., Walkersville, Md.).

Cell culture and flow cytometry. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by using Ficoll-Hypaque (14, 15) and incubated in triplicate wells of 24-well cluster trays with serial dilutions of Lkt, LktA, or cytokine in Dulbecco's minimal essential medium (GIBCO) containing 10% fetal bovine serum (Hyclone) and supplemented with glutamine and antibiotics (GIBCO). After a 24-h incubation, adherent cells were isolated, washed, and prepared for flow cytometry as described before (4). The adherent cell population was consistently >90% monocytes, as assessed by flow cytometry. Contamination by PMN was <1%. Cells from triplicate wells were pooled, and the cells were incubated with the following lineage-specific monoclonal antibodies: BoLa class II (nonpolymorphic determinant; TH14B) (VMRD, Inc., Pullman, Wash.), monocyte-macrophage-granulocyte (DH59B) (VMRD), and an irrelevant antibody control (1D7; anticoronavirus). After being washed, the cells were further incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Becton Dickinson, Mountain View, Calif.), washed, fixed in 2% formaldehyde, and stored at 4°C until analyzed. The fluorescent intensity of cells was evaluated with a Becton Dickinson FACScan, and data were collected from 10,000 cells. Two-parameter analysis of forward angle versus 90° light scatter was used to gate the population for fluorescence analysis. The percent positive cells and the peak channel for each were determined, and the percentage of PBMC stained with irrelevant monoclonal antibody was subtracted to obtain the net percent positive cells. The peak channel fluorescence for any given population was used to assess the relative intensity of staining with a particular monoclonal antibody. Thus, after incubation, a shift towards higher peak channel numbers (>0.5 log fluorescence units) indicated an increased number of receptors, whereas a shift to a lower peak channel (>0.5 log fluorescence intensity) indicated a reduced number. Controls were cells incubated in medium alone.

MLC. For the mixed lymphocyte culture (MLC) experiments, stimulator cell populations were prepared from gamma-irradiated (1,500 rads) PBMC isolated from venous blood of calves and then plated at a concentration of 5×10^4 cells per well in triplicate wells of a 96-well culture tray; responder

cells from genetically unrelated animals were added at 2×10^5 per well. Negative-control wells contained stimulator or responder cells cultured in medium alone, and positive-control wells contained responder cells stimulated with 5 μg of concanavalin A (Sigma, St. Louis, Mo.) per ml.

Initial studies were carried out with a large number of animals, from which six animals were selected for their high proliferative response in the MLC compared with the concanavalin A-induced proliferation of responder cells. In order to assess the effects that LktA or anti-MHC class II antibodies had on the ability of responder cells to proliferate, different concentrations of LktA or a 1:100 dilution of TH14B were added to MLC. This concentration of TH14B was previously determined as being optimal for fluorescence-activated cell sorting (FACS) analysis and was therefore used to inhibit BoLa class II recognition in the MLC. After 4 days of culture, 0.4 μCi of [*methyl*-^{3}H]thymidine (Amersham Canada Ltd., Oakville, Ontario, Canada) was added to all wells. Incorporation of radiolabel into cell DNA was assessed after cells were harvested onto glass fiber filter mats (Skatron, Sterling, Va.) and counted with a Beckman 1701 scintillation counter (Beckman, Richmond, British Columbia, Canada). Results are expressed as the increase in counts per minute over that in control cultures (15).

RESULTS

Effects of leukotoxin on MHC class II induction in peripheral blood cells. When LktA was cultured with macrophages, there was a sharp decline in the ability of these cells to constitutively express MHC class II molecules (Fig. 1). The reduction in class II was dependent on the dose of LktA; at a dose of 600 ng/ml, the level of MHC class II expression on peripheral monocytes was negligible (Fig. 2). The *Limulus* amebocyte lysis assay for endotoxin indicated that these preparations had between 0.05 and 0.1 EU of contaminating endotoxin per ml. Equivalent doses of LPS had no effect on MHC class II expression in macrophages (data not shown). The reduction in MHC class II expression occurred 14 h after treatment and increased with time until, at 24 h, there was almost a 10-fold decrease in MHC class II expression by monocytes. There was no decrease in MHC class II expression compared with the time zero level in medium-treated cultures. Concurrent with this, there was a 52% drop in the number of monocytes expressing high levels of MHC class II (Fig. 3, Table 1).

In order to assess whether this reduction in MHC class II expression could be prevented by activating monocytes, cells were treated with LktA and/or IFN-γ and then assessed for class II expression. When peripheral blood monocytes were treated with IFN-γ alone, there was a dose-dependent increase in the amount of MHC class II that could be detected by flow cytometry (Fig. 4a). Furthermore, when cells were incubated with a constant concentration of IFN-γ (20 ng/ml) and cocultured with different concentrations of LktA, there was minimal reduction in MHC class II expression (Fig. 4b).

In order to investigate this further, MDBK cells were assessed after treatment with IFN-γ and/or leukotoxin. This cell line does not constitutively express MHC class II; expression of MHC class II occurs only after activation with cytokines, such as IFN-γ. Thus, when cells were analyzed for the presence of MHC class II without activation, none could be detected (Fig. 5a). However, after cells were cultured in the presence of IFN-γ, MHC class II was expressed (Fig. 5b). Expression of MHC class II could be inhibited by the addition of antibodies against MHC class II (1:100 TH14B) to the medium (Fig. 5c), but LktA was unable to inhibit IFN-γ induction of MHC class II expression in this cell line at any of the doses tested (600 to 0.06 ng/ml) (Fig. 5d).

In a limited number of studies, authentic leukotoxin (Lkt) was assessed for its ability to reduce MHC class II expression. Because of the high toxicity of authentic leukotoxin, this experiment could only be conducted within a narrow leukotoxin dose range which had been determined previously by assessing its ability to bind and lyse peripheral blood PMN and macrophage (data not shown). However, when authentic leukotoxin was compared with recombinant leukotoxin, it was apparent that authentic leukotoxin could inhibit MHC class II expression similarly to the nontoxic LktA (Fig. 6).

The ability of LktA to compete with TH14B for binding to

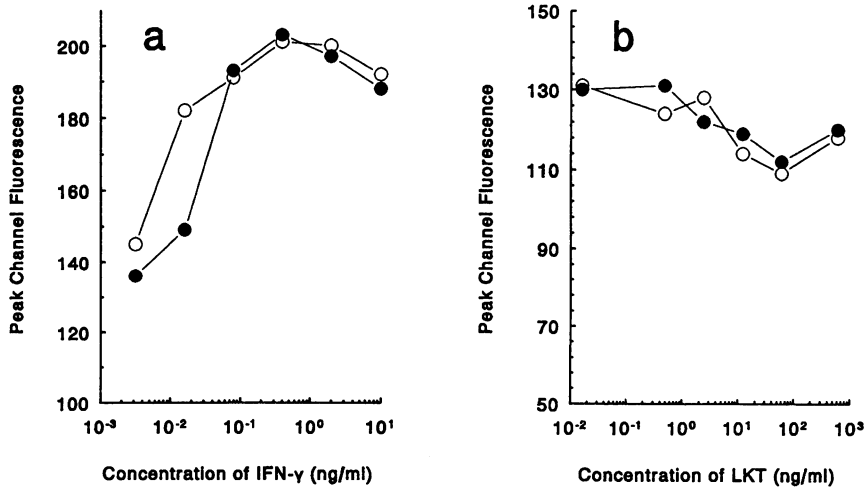

FIG. 4. Effects of IFN-γ on MHC class II induction. (a) Monocytes were assessed for MHC class II expression by flow cytometry after incubation with different concentrations of IFN-γ for 24 h. After treatment, there was a dose-dependent elevation in MHC class II expression, which reached a maximum at 20 ng/ml. (b) Peripheral blood monocytes were isolated and cocultured with IFN-γ (20 ng/ml) and different concentrations of LktA for 24 h. Cells were stained with monoclonai antibodies against MHC class II and then analyzed by fluorescence analysis. (a and b) Results for two animals (○, ●) are shown and are representative of four separate experiments.

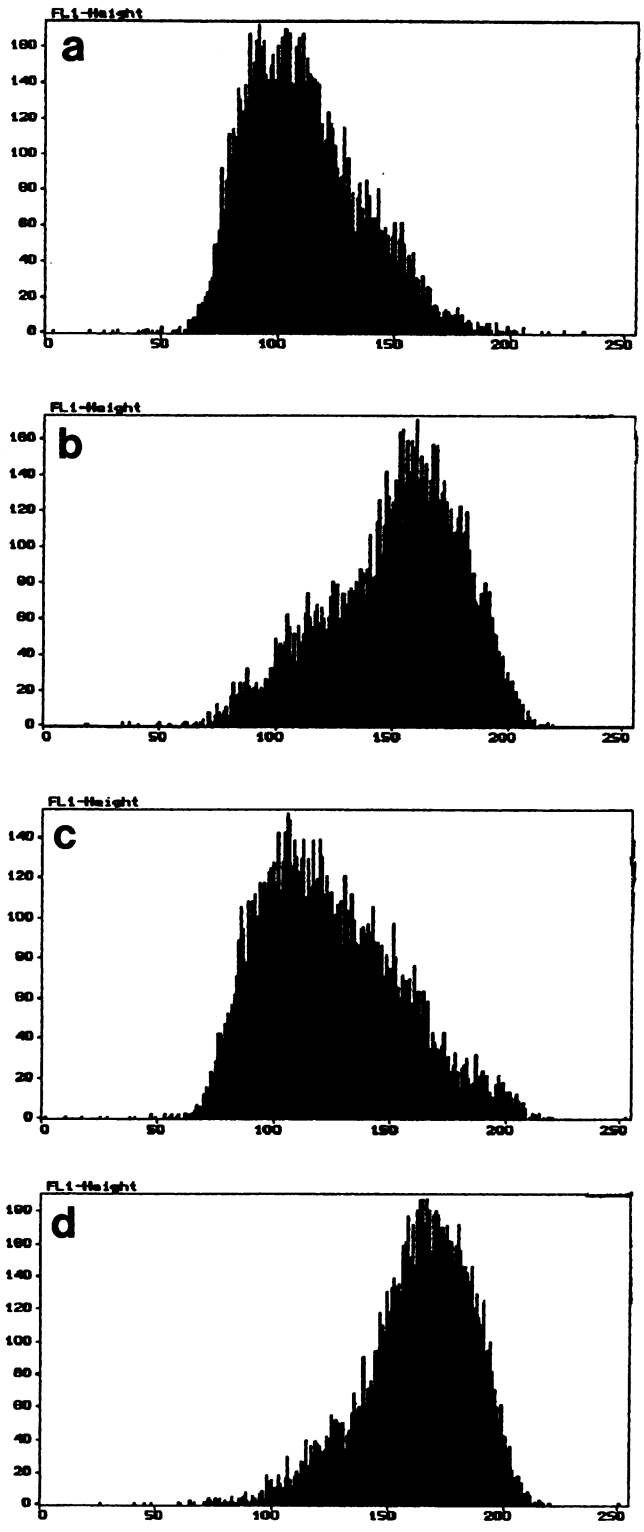

FIG. 5. Flow cytometric profiles of MHC class II expression on MDBK cells after treatment with (a) medium alone, (b) IFN-γ, (c) IFN-γ in the presence of antibodies against MHC class II, and (d) IFN-γ and LktA. Expression of MHC class II was analyzed by using monoclonal antibody TH14B and flow cytometry.

MHC class II was examined. Different molar ratios of LktA and TH14B from 20:1 to 1:100 were added to fixed cells. There was no significant difference in the binding of TH14B to MHC class II in the presence of LktA, as assessed by flow cytometry (data not shown).

MLC studies. In order to correlate the effects that leukotoxin had on MHC class II induction with the functional ability of monocytes, LktA was added to mixed-lymphocyte reaction assays. When PBMC (responder cells) were incubated in the presence of concanavalin A, they underwent rapid mitogenesis, resulting in the uptake of [^{3}H]thymidine, which was readily assessed by β spectroscopy (Fig. 7). Similarly, when responder cells were incubated with irradiated stimulator cells from a non-MHC-compatible recipient, a similar degree of proliferation took place (Fig. 7). When TH14B was added to cultures at an optimal dilution (1:100), there was a marked reduction in the ability of responder cells to proliferate (Fig. 7). Similarly, when LktA was added to MLCs, it was able to inhibit proliferation in a dose-dependent manner as effectively as anti-class II antibody (Fig. 7).

DISCUSSION

P. haemolytica produces a ruminant leukocyte-specific cytotoxin (leukotoxin; Lkt) which is thought to play a pivotal role in the pathogenesis of pneumonic pasteurellosis (6). Alveolar macrophages, peripheral blood macrophages, lymphocytes, and neutrophils are all susceptible to the cytolytic effects of leukotoxin. Leukotoxin can also affect the function of immune effector cells; it can impair bacterial uptake by bovine macrophages and inhibit the chemiluminescent response of neutrophils (5, 21).

More recently, it has been found that sublethal (i.e., noncytolytic) doses of leukotoxin can inhibit lymphocyte proliferation in a dose-dependent fashion in vitro (19). In a further series of experiments, IL-1 and IL-2 were shown to restore lymphocyte function after Lkt treatment (20). It was suggested that leukotoxin was responsible for a direct effect on macrophages and that IL-1 was able to counteract this effect through its lymphocyte-activating function. It was proposed that addition of exogenous IL-2 could restore a deficiency in either IL-2 production or receptor expression brought about by the direct effects of leukotoxin on lymphocytes. Furthermore, the glycolipid monosialoganglioside (GM-1) was also able to reverse the anti-proliferative effects of leukotoxin, and this reversal was particularly marked when it was added in the presence of IL-2. In this case, it was suggested that GM-1 may directly or indirectly interfere with leukotoxin-cell association (20).

Interference with antigen presentation by means of direct inhibition of MHC molecules has been proposed as a common strategy by which viruses may evade immune surveillance. It has been proposed that this mechanism would be advantageous to viruses because it would interfere with antigen presentation and it would block the MHC-inducible expression associated with IFN and other antiviral cytokines. Furthermore, the downregulation of MHC has been related directly to the pathogenicity of myxoma virus and malignant rabbit fibroma virus (3). Cytotoxic T lymphocytes play a crucial role in immunity against human cytomegalovirus infection, and this virus can also reduce MHC class I expression. However, as the critical level of MHC class I expression required to provide an adequate target for cytotoxic T-lymphocyte recognition is unclear, it may be premature to extrapolate MHC class I reduction to a functional in vivo effect (27). It is generally recognized that interference with MHC class I expression may delay or even interfere with the cellular response to lymphocytic choriomeningitis virus (11),

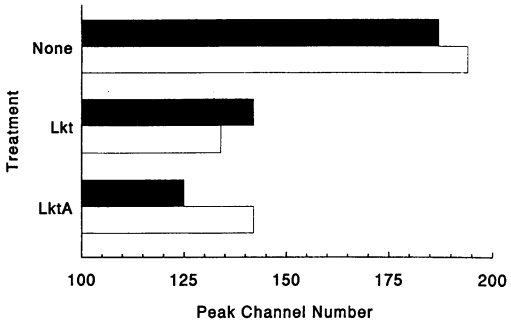

FIG. 6. Effects of different forms of leukotoxin on MHC class II expression. PBMC were isolated and treated for 24 h with either recombinant LktA (600 ng/ml) or approximately equimolar concentrations of authentic Lkt. Cells were then stained with monoclonal antibody against MHC class II and analyzed by flow cytometry. Results for two animals are shown and are expressed as peak channel fluorescence.

herpes simplex virus (16), human immunodeficiency virus (24), myxoma virus (3), and adenovirus (22). Our studies have indicated the LktA can interfere with class II expression to a greater extent than has been reported for viral interference with class I and that this interference can block expansion of alloreactive T lymphocytes.

In this study, we have shown that a bacterial exotoxin (Lkt) from *P. haemolytica* is able to directly affect the expression of MHC class II molecules on bovine macrophages and proliferation during a mixed-lymphocyte reaction. This effect was not due to LPS contamination, as the levels of LPS that were present (0.05 to 0.1 ED/ml) had no effect on MHC class II expression. It was also not due to a simple allosteric effect of leukotoxin binding to the MHC class II molecule directly, as we were unable to demonstrate any competitive binding between it and a monoclonal antibody directed to a nonpolymorphic determinant (TH14B). The toxic (authentic) leukotoxin and the nontoxic LktA appeared to have similar effects at all

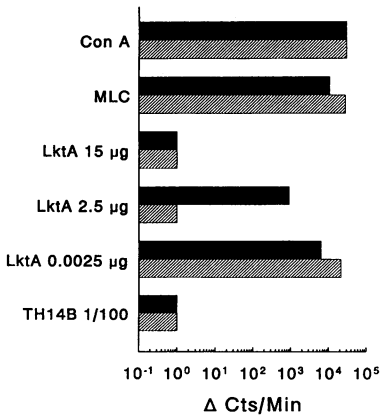

FIG. 7. Effects of LktA on MLC. Results for two animals are shown. Responder cells were incubated with 5 µg of concanavalin A (Con A) per ml or with stimulator cells (MLC). Responder and stimulator cells were incubated with anti-MHC class II antibody (TH14B, 1:100 dilution) or different concentrations of LktA. Proliferation was assessed after incubation with [^{3}H]thymidine, and results are expressed as the increase in counts per minute, with cells incubated in medium alone used as a control. Representative results from four separate experiments are shown.

doses tested. This indicates that while the whole Lkt molecule is required for the cytotoxic effect on macrophages and PMN (7, 14), LktA alone is able to affect at least this immune function.

MHC class II molecules play a critical role in the recognition of nonself in the immune system. T helper cells will only react to conventional antigens when they are presented on cell surfaces in conjunction with autologous MHC class II antigens and other signals, and this first signal then triggers a number of events which give rise to T-cell activation. Alloreactivity and tissue rejection after transplantation are direct consequences of the recognition of foreign MHC molecules from a mismatched individual (10). Therefore, in order to assess the functional effect of LktA on MHC class II, we chose to use an MLC, which is recognized as being able to detect alloreactivity between MHC class II-mismatched individuals. Furthermore, by irradiating stimulator cells sufficiently (1,500 rads), we were able to abrogate antigen expression by B lymphocytes, ensuring that we were examining the effects of Lkt on antigen presentation by macrophages. We demonstrated that monoclonal antibodies against MHC class II were able to inhibit the MLC, further demonstrating that in this case, the reaction was dependent on the recognition of allogeneic MHC class II antigen peptides by macrophage antigen-presenting cells. When leukotoxin was added to cultures, the same degree of inhibition occurred as was afforded by anti-class II antibody. This indicated that leukotoxin was able to downregulate MHC class II expression on (macrophage) antigen-presenting cells to the same extent as antibody was able to block it. Preliminary evidence indicated that this downregulation of MHC class II occurs only on macrophages; B cells are not affected (data not shown).

When macrophages are activated, they undergo a number of functional and phenotypic changes. They exhibit an enhanced level of oxygen and nitrogen metabolism, and this has been correlated with a functional increase in the ability of these cells to kill intracellular pathogens and tumor cells (9, 13). Fc receptor status and MHC class II expression are also enhanced, resulting in opsonization and enhanced killing and antigen presentation. In order to assess whether LktA could affect activation, macrophages were treated with combinations of IFN-γ and LktA. Leukotoxin did not affect the expression of MHC class II in the presence of IFN, and it also did not affect the de novo induction of MHC class II in MDBK cells, which do not constitutively express MHC class II.

These data clearly indicate that LktA and a crude preparation containing similar concentrations of authentic leukotoxin from *P. haemolytica* are both able to affect the constitutive expression of MHC class II molecules but not their expression mediated by IFN-γ activation of macrophages. While other factors may also modulate MHC molecules, it appears that LktA is most effective at downregulating MHC class II expression. The implications of this for the pathogenesis of pasteurellosis cannot be overlooked. Leukotoxin can directly affect a number of cells of the immune system and indirectly affect their function. The decreased expression of MHC class II, which essentially resulted in an anergic state in an MLC, may be at least partially responsible for the suppression of proliferative responses in vitro (14) as well as any unreactive state that occurs after infection. Surprisingly, though, higher concentrations of LktA were able to induce antigen-specific lymphoproliferative responses in LktA-immune calves (14), indicating that this effect is limited to naive animals and that it is not the result of any cytopathic or cytotoxic effect. We propose that this mechanism, while not inducing a state of immunosuppression, may be able directly to affect or redirect

antigen presentation so that the bacterium is able to colonize more efficiently the site of infection. Therefore, it may be anticipated that vaccines incorporating Lkt would not only neutralize the toxic effects of this molecule, but also reduce the ability of this molecule to inhibit immune responsiveness. This effect may be of particular relevance in a multivalent vaccine, when a defect in macrophage presentation may significantly affect responses to third-party antigens.

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