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Roles of Interferon and Cellular Adhesion Molecules in Bacterial Activation of Human Natural Killer Cells

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Interaction of lipopolysaccharide (LPS) from enteric and oral bacteria with natural killer (NK) cells enhanced cytotoxicity against NK-sensitive and NK-resistant targets. This activation occurred without expansion of the NK cell population or without changes in the leukocyte function-associated antigen family of cellular adhesion molecule (CAM) expression on NK cells. Significant interferon (IFN) titers were measured in LPS-lymphocyte supernatants, and antibody to IFN-α blocked LPS activation. LPS-induced NK cytotoxicity was inhibited by antibodies to individual alpha chains of CAM and, more profoundly, by antibody to the beta chain of CAM. However, LPS, when preincubated with NK cells, did not compete with subsequent anti-CAM antibody binding as detected by flow cytometry. Anti-CAM antibodies had no effect on NK activation by IFN, but antibodies to either CD11a or CD11c abrogated IFN production induced by LPS. These findings suggest that LPS binds NK cells at non-CAM sites, resulting in the release of IFN. IFN then acts in an autocrine manner independent of CAM to enhance NK cytotoxicity. Interaction of anti-CAM antibodies with CAM may provide a negative signal in regulating LPS-induced IFN production.

Culture of human peripheral blood lymphocytes (PBL) with pathogenic bacteria causes a rapid increase in the cytotoxicity of natural killer (NK) cells measured against bacteria-infected cells (4) and tumor targets (7, 12, 13). This cytotoxicity can be comparable to interleukin-2 (IL-2)-induced cytotoxicity when measured early in culture (5). However, bacteria-induced cytotoxicity peaks after approximately 24 h despite continued presence of bacterial antigens. This activation phenomenon also occurs without significant lymphocyte proliferation over a 1-week culture period (5). Modest concentrations of IL-2 were measured in bacteria-lymphocyte cultures, and anti-IL-2 antiserum did not block all cytotoxicity induced (5). Activation was found to be independent of the IL-2 (Tac, CD25), T-cell (CD3), and E-rosette (CD2) receptors (5).

Whole gram-negative bacteria were shown to activate NK cells via surface lipopolysaccharide (LPS), as treatment with polymyxin B abrogated cytotoxicity (5). When LPS was isolated from oral and enteric bacteria and tested, the NK activation kinetics were similar to those induced by whole bacteria. LPS, a major outer membrane component of gram-negative bacteria, is a potent immune mediator, especially affecting B-cell proliferation and monocyte-macrophage activation. The effects of LPS on NK cells have not been as extensively studied.

When purified LPS from *Actinobacillus actinomycetemcomitans* Y4 was tested, significantly higher concentrations of LPS (1 μg/ml) were necessary to activate NK cytotoxicity (R. A. Lindemann and F. Eilber, Arch. Oral Biol., in press) than to activate monocytes (1 ng/ml, as measured by production of IL-1 and tumor necrosis factor) (6). If LPS was preincubated with lymphocytes and monocytes, subsequent activation of NK cells by IL-2 was inhibited, demonstrating that LPS preferentially activated monocytes. This high sensitivity of monocytes to LPS is presumably due to specific receptors for LPS. Recently, it was suggested that LPS binds cellular adhesion molecules (CAM) on human macrophages (16). Therefore, it was important to determine whether LPS activation of NK cells occurred via similar receptors. The study reported here examined the mechanism of LPS interaction with NK CAM and subsequent cytotoxic enhancement utilizing LPS derived from enteric and oral organisms.

MATERIALS AND METHODS

Isolation of lymphocytes. PBL from healthy volunteers were obtained by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation. Adherent cells were depleted by adherence to plastic. PBL were suspended in RPMI 1640 medium with 10% human AB serum. The percentage of monocytes remaining, determined with a universal rosetting reagent (3), was 1 to 3%. Large granular lymphocytes, rich in NK cells, were separated by a discontinuous Percoll (Pharmacia) gradient (15).

Antibodies. The monoclonal antibody OKM-1 (anti-CD11b), directed against the alpha chain of CR3, was purchased from Ortho Pharmaceuticals (Raritan, N.J.). Leu-M5 (anti-CD11c), directed against the alpha chain of P150,95, was purchased from Becton Dickinson Inc. (Mountain View, Calif.). NKH-1-RD1 (phycoerythrin conjugate) was purchased from Coulter Immunology (Hialeah, Fla.). Antibodies against the human leukocyte function-associated antigens (LFA) LFA-1 (TS1/22) (CD11a) and LFA-β (TS1/18) (CD18) were kindly provided by T. Springer (Dana-Farber Cancer Institute, Boston, Mass.). All antibodies were utilized at a 1:100 dilution for antibody-blocking experiments and flow cytometric analyses.

LPS. LPS from *A. actinomycetemcomitans* Y4 was kindly provided by A. Nowotny and F. Sanavi (University of Pennsylvania). This highly purified LPS was free of contaminating protein and contained approximately 0.5% bound amino acids. LPS from *Escherichia coli* 026:B6 (L-3755) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Antibody blocking of cytotoxicity. Lymphocyte-LPS cultures (1×10^6 lymphocytes per ml, 1 μg of LPS per ml) were incubated for 24 h in a humidified incubator at 37°C with 5%

CO_2, and then the lymphocytes were tested in a 4-h cytotoxicity assay (described below). Antibodies against CAM were added for the entire 24-h culture period or only during the final 4-h cytotoxicity assay. Antibodies were used singly and in combination.

Competition assay. Lymphocytes (1×10^6 in 200 μl of 1× phosphate-buffered saline with 2.5% fetal calf serum and 0.1% sodium azide) were placed in test tubes. LPS were added at 10 μg/ml for 45 min at room temperature. Antibodies to CD11a, CD11b, CD11c, and CD18 were added individually at 1:100 concentrations for 20 min on ice. The lymphocytes were washed twice, and a secondary antibody, goat anti-mouse immunoglobulin conjugated to fluorescein (Coulter), was incubated on ice for 20 min. Lymphocytes were washed twice, and anti-NKH-1-RD1 antibody (phycoerythrin conjugate) was added for 20 min on ice. Lymphocytes were washed twice and suspended in the original solution prior to dual-color flow cytometric analysis (described below).

Effects of LPS on NK surface markers. LPS were added to lymphocyte cultures as described above. After 24 h, cells were double stained with antibodies to CD11a, CD11b, CD11c, or CD18 and NKH-1-RD1 (described above) and compared with untreated cells by flow cytometry (described below).

Flow cytometry. Cells were analyzed for two-color fluorescence on a Coulter Epics C or Epics V flow cytometer equipped with a single argon laser (Coherent, Palo Alto, Calif.). Ten thousand cells were analyzed in each sample. The results are presented as density plots with logarithmic intensity scaling (see Table 3). Percentages of stained and nonstained cells were calculated with the MDADS programs (Coulter). These calculations were made by using density plots of control samples which involved substitution of diluent alone or isotypic controls for one or both antibodies.

IFN. Lymphocyte-LPS cultures were prepared as described above. During the 24-h culture period, a rabbit polyclonal antibody to human alpha interferon (IFN-α) (Interferon Sciences, Inc., New Brunswick, N.J.) with a neutralizing titer of 1×10^5 neutralizing units per ml was added at a concentration of 1,000 neutralizing units per culture. Cytotoxicity of lymphocytes was determined by the standard ^{51}Cr assay and compared with that of lymphocytes incubated with LPS alone. IFN-α (500 U/ml; Amgen, Thousand Oaks, Calif.) was tested as a positive control for activation in some cultures, and the effectiveness of antibody blocking was determined by adding the anti-IFN-α antibody.

IFN assay. IFN titers were measured by a modification (14) of the micro-dye uptake method described elsewhere (1). Large plaque-purified encephalomyocarditis virus, propagated in CCL-L cells and stored at −70°C, was used as a challenge virus in the IFN assays. Confluent WISH cells (2) were incubated overnight in microdilution plates containing 0.1 ml of maintenance medium (Eagle basal metabolic medium, 2% fetal bovine serum, 1% glutamine) per well and twofold dilutions of sample supernatants. Media were removed 18 to 24 h later, and cultures were infected with sufficient encephalomyocarditis virus to produce a 100% cytopathic effect in 24 h. After incubation for 24 h at 37°C in a 5% CO_2 atmosphere, cultures were fixed in methanol, stained with 1% aqueous crystal violet, and examined under a 10× microscope. All samples were run in duplicate. IFN titers were defined as the reciprocal of the dilution giving approximately 50% protection of the cultures from destruction by encephalomyocarditis virus. An international standard leukocyte IFN from the National Institutes of Health was assayed simultaneously in each assay. All titers are expressed in international units, and the lowest detectable titer was 10 IU.

TABLE 1. Effect of IFN-α antibody on LPS-activated cytotoxicity of PBL against K562 targets

Condition(s)	Lytic units for expt no.:		
	1	2	3
Untreated	2.2	4.1	8.1
Untreated plus anti-IFN[a]	2.0	4.2	7.7
E. coli[b]	5.5	12.5	33.0
E. coli plus anti-IFN	2.7	4.9	14.3
A. actinomycetemcomitans Y4[b]	4.2	12.0	37.5
A. actinomycetemcomitans Y4 plus anti-IFN	1.6	3.9	10.5
IFN-α[c]	6.2	6.0	18.8
IFN-α plus anti-IFN	1.7	2.2	11.3

[a] Anti-IFN-α antibody added at 1,000 neutralizing units per culture for 24 h.
[b] LPS added at 1 μg/ml for 24 h.
[c] IFN-α added at 500 U/ml for 24 h.

Target cells. The NK-sensitive human erythroleukemia cell line K562 and the NK-resistant melanoma cell line UCLA SO-M14 (M14) (17) were used as targets in the cytotoxicity assays. Target cells (5×10^6 in 1 ml of RPMI 1640 with 10% fetal calf serum) were labeled with 250 μCi of ^{51}Cr for 1 h at 37°C.

Cytotoxicity assay. K562 or M14 target cells (5×10^3) were mixed with effector cells (at 50:1, 25:1, and 12.5:1 ratios) in round-bottom microdilution plates containing 200 μl of RPMI 1640 with 10% AB serum. The plates were centrifuged at 65 × *g* for 4 min to initiate cell-to-cell contact and then incubated for 4 h at 37°C in a humidified incubator with 5% CO_2. At the end of the assay, plates were centrifuged at 150 × *g* for 8 min, and 100 μl of supernatant was removed from each well and counted for ^{51}Cr released from target cells. Each assay was performed in quadruplicate. Cytotoxicity was defined as percent specific ^{51}Cr released, calculated as [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. Experimental release was defined as the counts of ^{51}Cr released from target cells caused by effector cells, measured in counts per minute; maximal release, as the ^{51}Cr released from target cells induced by 2% Nonidet P-40 detergent; and spontaneous release, as the counts of ^{51}Cr from target cells incubated alone.

Statistical analysis. Significance of the results of cytotoxicity assays was determined by first converting the three effector-to-target ratio values to lytic units (10), defined as the number of cells required to cause a specified amount of target lysis (in this case, 30%) and usually expressed as lytic units per 10^6 cells. This method allows a more accurate comparison between lymphocyte donors. The paired *t* test (two tailed) was then applied to determine the significance of 30% lysis values.

RESULTS

Effect of antibodies to IFN on LPS-induced cytotoxicity. To determine the role of IFN in the activation phenomenon, antibody to IFN-α was incubated in culture during the 24-h LPS activation period. Presence of IFN-α antibody abolished cytotoxicity of LPS-activated cultures (Table 1). Cytotoxicity of the positive control, IFN-α (500 U/ml)-stimulated lymphocytes, was also blocked by the antibody.

TABLE 2. IFN production from 24-h culture supernatants of large granular lymphocytes stimulated by 10 μg of LPS per ml

Subject no.	IFN (IU) from cultures of:					
	A. actinomycetemcomitans Y4			*E. coli*		
	Untreated[a]	CD11a[b]	CD11c[b]	Untreated[a]	CD11a[b]	CD11c[b]
1	240	40	15	240	60	60
2	120	40	20	120	20	15
3	120	ND[c]	40	120	ND	30
4	160	60	30	180	80	60

[a] Supernatants from untreated cultures contained 10 IU of IFN.
[b] Antibodies to CD11a and CD11c were added at a 1:100 dilution.
[c] ND, Not done.

TABLE 3. Effects of LPS on CAM expression in NK and PBL cells

Antibody	NKH-1$^+$				PBL			
	Untreated		LPS[a]		Untreated		LPS	
	% Positive	MPC[b]	% Positive	MPC	% Positive	MPC	% Positive	MPC
NKH-1					7	6	6	6
CD11a	6	15	7	16	44	13	49	14
CD18	8	21	8	23	83	16	88	16
CD11b	7	31	6	31	21	21	20	23

[a] LPS from *E. coli*, added at 1 μg/ml for 24 h. Cytotoxicity values were consistent with activation by LPS (data not shown).
[b] MPC, Mean peak channel fluorescence.

Induction of IFN by LPS. IFN titers were measured in culture supernatants after 24 h of incubation of large granular lymphocytes with LPS or with LPS and specific antibody. Cultures of large granular lymphocytes activated by LPS from both oral and enteric bacteria released significant amounts of IFN (Table 2). However, when either anti-CD11a or anti-CD11c antibody was present during LPS activation, IFN production was significantly inhibited.

LPS and antibody competition for lymphocyte-binding sites. To determine if LPS bound CAM, LPS was preincubated with PBL. This procedure was performed to see if bound LPS would block subsequent antibody binding to CAM. Labeled antibodies to CAM were added, and percent antibody binding was determined by dual-color flow cytometry. Preincubation with either LPS tested had no effect on subsequent antibody binding to any CAM of NKH-1$^+$ cells tested. The percentages of positive cells in the untreated population were 5.6, 8.8, and 6.4% for CD11a, CD18, and CD11b, respectively. The percentages for the LPS-pretreated population were 4.1, 8.2, and 7.3% for CD11a, CD18, and CD11b, respectively. The density of receptor-bound antibody was not altered for any subject tested. Mean peak channel fluorescences for the untreated population were 60, 60, and 73 for CD11a, CD18, and CD11b, respectively. Peak fluorescences for the LPS-treated population were 57, 56, and 73 for CD11a, CD18, and CD11b, respectively.

Effect of LPS on NK surface markers. The experiments described above determined LPS-CAM binding competition. The experiments described here were designed to determine modulation or induction of CAM markers. Flow cytometry was used to analyze changes in CAM after LPS activation. Results for both LPS tested were equivalent; data for *E. coli* LPS are presented. The percentage of cells bearing an NK marker (NKH-1) did not change in 24-h cultures (Table 3). The effects of LPS on the NKH-1$^+$ CAM were analyzed with dual-color staining. The number of CD11a-, CD11b-, CD11c-, or CD18-positive NK (NKH-1$^+$) cells did not change, and the density of expression of CAM on NKH-1$^+$ cells was not altered (Table 3). In addition, when the entire lymphocyte population was analyzed for CAM, LPS had no effect on the percentage of positive cells or on the density of antigen (Table 3).

Effect of antibodies to CAM on LPS-induced cytotoxicity. Antibodies to CAM were incubated in culture with lymphocytes and LPS to determine if the activating effect of LPS occurred via interaction with these molecules. Antibodies were also added to unstimulated lymphocytes to measure any general effects related to target binding that would be independent of LPS activation. Unstimulated lymphocytes were not significantly affected by 24 h of culture with the antibodies to CAM (Table 4). In LPS cultures, all four antibodies to CAM significantly inhibited cytotoxic activation measured by target cell lysis when incubated for 24 h against K562 targets (Table 4). M14 targets were equally inhibited (data not shown). In control experiments in which antibodies were added after the 24-h LPS activation period and present during the whole 4-h cytotoxicity assay, only anti-CD11a had a slight but significant inhibitory effect on both LPS-activated and unstimulated lymphocyte cytotoxicity measured against M14 targets (data not shown).

To determine if the cytotoxic inhibition was general for any activation stimulus and not strictly related to LPS, antibodies to CAM were added to lymphocyte cultures containing IFN-α (500 U/ml). Cytotoxic activation by IFN was not blocked by these antibodies (Table 4).

DISCUSSION

NK cells display the leukocyte function-associated antigen family of structurally related CAM CD11a, CD11b, and CD11c on their cell surfaces (for a review, see reference 8). These receptors are antigenically distinct with respect to their alpha chains, but they share a common beta chain (CD18). It has been suggested that these same receptors recognize and bind LPS on human macrophages (16). The study reported here sought to determine the relation between LPS and NK CAM, as LPS has been shown to activate NK cytotoxicity (5).

First, however, the role of IFN in the LPS-NK activation process was assessed. Klimpel et al. (4) demonstrated that high levels of IFN were contained in bacteria-activated lymphocyte supernatants. Both *Shigella flexneri* and *Salmonella typhimurium* stimulated release of IFN-α and IFN-γ.

TABLE 4. Cytotoxicity of PBL against K562 targets measured 24 h after culture

Antibody[a]	Lytic units[b] for:			
	Untreated cultures	*A. actinomycetemcomitans* Y4	*E. coli*[c]	IFN-α (500 U/ml)
None	3.8 ± 2.4	13.0 ± 4.9	14.2 ± 5.3	18.8 ± 7.4
CD11a	3.0 ± 1.5	3.7 ± 2.0*	6.9 ± 3.1*	13.1 ± 6.1
CD18	4.1 ± 3.5	2.7 ± 1.6*	3.1 ± 2.9*	17.7 ± 7.2
CD11b	3.5 ± 2.9	5.6 ± 3.8*	6.6 ± 3.5*	17.8 ± 8.5
CD11c	2.0 ± 1.1	3.1 ± 3.3*	4.5 ± 5.3*	16.4 ± 9.0

[a] Antibodies were added at a 1:100 dilution.
[b] Means ± standard deviations for four subjects. Values with asterisks are significantly different from control ($P < 0.05$).
[c] LPS were added at 1 μg/ml.

Tarkkanen et al. (13) also demonstrated that activation of NK-rich PBL fractions by *Salmonella* bacteria involved interferon production. Cloned human NK cells were shown to produce IFN-α, IFN-γ, or both (9), but in vivo IFN sources may include both NK cells and other mononuclear cells. In this study, when antibody to IFN-α was incubated in LPS-PBL cultures for 24 h, PBL cytotoxicity against NK targets was significantly inhibited (Table 1). This finding suggested that IFN was responsible for NK activation. In support of these results, high titers were detected when IFN was measured in LPS-lymphocyte culture supernatants. However, if anti-CAM antibodies CD11a and CD11c were added during LPS-lymphocyte cultures, production of IFN was blocked (Table 2), which initially suggested that LPS may interact with NK CAM to produce IFN.

To test the hypothesis that LPS binds CAM, a competition assay was conducted. LPS, when preincubated with lymphocytes, failed to compete with subsequent binding of specific antibody to alpha or beta chains of CAM as detected by flow cytometry. In addition, LPS affected neither the percentage of CAM^+ PBL or NK cells nor the expression of CAM on these cells (Table 3). Therefore, the possibility that antibodies against CAM did not block LPS binding but instead blocked autoactivation by IFN was investigated. However, antibodies to alpha or beta chains of CAM had no effect on IFN activation of NK cells (Table 4).

The finding that anti-CAM antibodies blocked cytotoxicity induced by LPS but not IFN suggested that these antibodies have an indirect effect on NK activation by down-regulating LPS-induced IFN production. If this hypothesis were true, one might predict that anti-CAM antibodies would also inhibit NK cytotoxicity because data suggested that IFN-α activated NK cells. In support of this hypothesis, it was found that if antibodies to CAM were added to 24-h LPS-PBL cultures, NK killing was inhibited (Table 4). NK cytotoxic inhibition by anti-CAM antibodies required the presence of antibody during an 18- to 24-h lymphocyte-LPS culture incubation. When antibodies to alpha or beta chains were incubated only during the 4-h cytotoxicity assay, cytotoxicity was rarely diminished. Anti-CD11a had a slight inhibitory effect, which was attributed to interference with target binding. That antibody has been shown to have a weak blocking effect on killing of K562 targets by an NK clone (11). However, its inhibitory effect on LPS induction of cytotoxicity was more profound than the inhibitory effect on unstimulated lymphocytes incubated for 24 h, demonstrating that it also interfered with activation, not only binding.

LPS did not compete with anti-CAM antibodies, which suggested two possible explanations for this finding. First, that LPS bound CAM but the affinity of specific antibody was higher than that of LPS and therefore bound LPS was displaced. Second, that LPS did not bind CAM, but activation via IFN was regulated by anti-CAM antibodies. The current data support the second possibility. When a single anti-CAM antibody (CD11a or CD11c) was added to LPS-NK cultures, IFN production was inhibited (Table 2). An antibody to a single alpha chain should not have prevented LPS from binding other CAM sites and inducing activation if this pathway were active. This contradiction supported the LPS-CAM competition data and suggested that LPS does not exclusively bind CAM but binds other sites on NK cells to induce cytotoxic activation via IFN release. Antibodies to CD11a and CD11c may trigger an IFN inhibitory pathway rather than block LPS-CAM binding. Therefore, LPS-induced IFN release would be initiated by a non-CAM biochemical pathway. This pathway appears subject to down-regulation via anti-CAM antibodies. Because antibodies to CAM did not block exogenously added IFN, it was concluded that the subsequent activation effects of IFN were also mediated independently of CAM. These findings suggest that a novel IFN regulatory pathway for NK cells exists via certain CAM.

LPS released in vivo from gram-negative organisms during infection may trigger NK activation by autocrine regulation via IFN. This may be a component of the inflammatory response against such infections. Down-regulation of this NK activation through cell surface CAM could be a normal homeostatic mechanism for limiting inflammation. However, the in vivo counterparts of the monoclonal antibodies used in these experiments have not been identified. Because they are adhesion molecules, there may be many sources of ligands able to bind to them and thus regulate this system.

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