Activation of Murine Macrophages and Lymphocytes by *Ureaplasma diversum*

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ABSTRACT

***Ureaplasma diversum* is a pathogen in the bovine reproductive tract. The objective of the research was to study interactions with macrophages and lymphocytes which might elucidate aspects of pathogenetic mechanisms of this organism. We studied the activation of murine macrophages of C3H/HeN (LPS-responder) and C3H/HeJ (LPS-low-responder) genotype for TNF-α, IL-6, IL-1 and nitric oxide production and blastogenic response of C3H/HeJ splenocytes after *Ureaplasma diversum* stimulation. Live and heat-killed *U. diversum* induced TNF-α, IL-6 and IL-1 in peritoneal macrophage cultures of both C3H/HeN and C3H/HeJ mice in a dose dependent manner. Interferon-gamma modulated the cytokine production, by increasing the production of TNF-α, IL-6 and nitric oxide, but IL-1 secretion was only enhanced in C3H/HeJ macrophages stimulated by live ureaplasmas.**

Supernatant of *U. diversum* sonicate was mitogenic for murine spleen lymphocytes. The blastogenic response was dose dependent, and stimulation with both *U. diversum* and Concanavalin A seemed to have an additive effect. These results suggest that *U. diversum*, similar to other mycoplasmas, activates murine macrophages and lymphoid cells. The studies should be repeated with bovine cells in order to elucidate pathogenetic aspects of inflammation in cattle caused by *U. diversum*.

RÉSUMÉ

***Ureaplasma diversum* est un micro-organisme pathogène du tractus reproducteur des bovins. Dans le but d'élucider certains aspects des mécanismes de sa pathogénie, une étude sur les interactions entre les macrophages et les lymphocytes et cette bactérie a été effectuée. L'étude a porté sur l'activation de macrophages murins provenant des souris de lignée C3H/HeN et C3H/HeJ pour la production de TNF-α, IL-6, IL-1 et oxide nitrique, ainsi que sur la réponse blastogénique de splénocytes de souris C3H/HeJ après stimulation par *U. diversum*. Des cellules d'*U. diversum* viables et tuées par la chaleur ont induit une production, dose-dépendante, de TNF-α, d'IL-6 et d'IL-1 par des macrophages péritonéaux en culture provenant de souris C3H/HeN et C3H/HeJ. L'interféron gamma a modulé la production de cytokines en augmentant la production de TNF-α, d'IL-6 et oxide nitrique, mais la sécrétion d'IL-1 était augmentée seulement chez les macrophages de souris C3H/HeJ stimulés par des uréaplasmes vivants.**

Le surnageant de cellules d'*U. diversum* soniquées s'est avéré mitogénique pour les lymphocytes spléniques murins. La réponse était dose dépendente et une stimulation simultanée avec *U. diversum* et de la concanavaline A a semblé avoir un effet additif. Ces résultats suggèrent qu'*U. diversum*, tout comme d'autres mycoplasmes, a une action stimulante sur les cellules lymphoïdes et les macrophages murins. Ces études devraient être répétées avec des cellules d'origine bovine afin d'élucider la pathogénie de l'inflammation observée lors d'infection à *U. diversum* chez les bovins. (*Traduit par Dr Serge Messier*)

INTRODUCTION

Ureaplasmas are wall-less prokaryotes in the family of *Mycoplasmataceae* in the class of *Mollicutes*. They are distinct from other genera in possessing urease (1). *Ureaplasma diversum* inhabits the respiratory and reproductive tracts of cattle. Although frequently isolated from the genital tract of cows and bulls without clinical signs, the organism may, under certain circumstances, cause herd outbreaks of granular vulvitis, abortion and infertility (2–5).

Experimentally, *U. diversum* can cause granular vulvitis, endometritis, salpingitis, seminal vesiculitis, infertility, embryonic death, and abortion (6–9). Histopathological changes, during natural or experimental infections, are often characterized by moderate or severe neutrophil and mononuclear cell infiltration (6), suggesting stimulation of cellular responses and inflammatory mediators during the course of infection. Although adherence and urease activity are recognized as factors of virulence (10,11), pathogenetic mechanisms in general remain unresolved.

Many species of the genera *Mycoplasma* and *Acholeplasma* have been shown to be mitogenic for murine lymphoid cells and induce blastogenesis and cytokine production (12–16). This phenomenon may contribute to pathogenesis of mycoplasma disease, although the evidence of such nonspecific

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Submitted October 27, 1993.

interactions during natural infection is scarce (17).

We decided to determine if *U. diversum* induces a cytokine response and is mitogenic for murine macrophages and spleen lymphocytes, because such information is not available and may encourage further research on pathogenetic mechanisms during ureaplasmosis in cattle.

Our data showed that *U. diversum* stimulates murine macrophages to release IL-1, IL-6, TNF-α and nitric oxide (NO), and that it is mitogenic.

MATERIALS AND METHODS

PREPARATION OF *UREAPLASMA DIVERSUM SAMPLES*

The virulent strain 2312 of *Ureaplasma diversum* (8th in vitro passage) was cultured in 1 L of buffered U 4 broth medium for 16 h at 37°C under normal atmospheric conditions (18). The culture was centrifuged (15,000 × *g* for 30 min) and the pellet was washed three times in sterile phosphate buffered saline, pH 7.2, (PBS) and finally suspended in 5 mL of PBS and stored in 1 mL aliquots at −70°C until used. The numbers of colony forming units (CFU) and color changing units (CCU) in the culture before centrifugation were: 2.4×10^7 CFU/mL, and 10^7 CCU/mL, respectively. The viability of the ureaplasmas was also determined after thawing one of the aliquots and found to be: 2.5×10^7 CFU/mL, and 10^7 CCU/mL. The concentration of the ureaplasmas by centrifugation was not reflected in increased numbers of CFU/mL or CCU/mL after freezing and thawing, probably due to the fragility of the organisms.

Suspensions of live ureaplasmas (L), and ureaplasmas killed by heat (H) at 56°C for 1 h were used for cell stimulation. In addition, the supernatant of a sonicated *U. diversum* suspension was prepared as follows: 2 mL containing 2 mg protein/mL (19) were sonicated on an ice-bath, by ten 1 min bursts, separated by 1 min intervals, at 35% output through a microprobe from an ultrasonic disintegrator (Virsonic Cell Disrupter, Model 16-850, Virtis Comp., Gardiner, New York). The suspension was centrifuged at 15,000 × *g* for 30 min and the supernatant was filtered through a 0.22 μm pore-size low protein binding membrane filter (Nuclepore Canada, Toronto, Ontario). The number of CCU in the suspension before sonication, in the resuspended pellet after centrifugation, and in the supernatant was 10^7, 10^4 and 10^3, respectively. *Ureaplasma diversum* colonies grew on agar medium from the suspension before sonication and from the resuspended pellet after centrifugation, but not from the supernatant of the sonicate. Thus, the low CCU titer determined in the supernatant of the sonicate was not due to viable ureaplasmas, but was residual urease activity. Protein concentration in the supernatant of sonicate was 1.2 mg/mL, determined according to Lowry *et al* (19).

MACROPHAGES

Six eight-week-old female mice of C3H/HeN (LPS-responder) and C3H/HeJ (LPS-low-responder) genotypes were purchased from Charles River Laboratories (St. Constant, Quebec) and Jackson Laboratory (Bar Harbor, Maine), respectively. They were housed according to guidelines of the Canadian Council on Animal Care.

Peritoneal exudate macrophages were harvested in sterile PBS with antibiotics, four to five days following intraperitoneal injection of 1.5 mL of sterile thioglycolate medium. Cells were washed and suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10mM HEPES, 2mM glutamine, and antibiotics (penicillin 10 U/mL and streptomycin 10 μg/mL), adjusted to a concentration of 1.5×10^6 cells/mL, dispensed in 100 μL volumes in a 96-well flat bottom plate (Nunclon Microwell Plates, Roskilde, Denmark). The medium with nonadherent cells was replaced after 2-3 h incubation at 37°C in normal atmosphere with 5% CO_2. Incubation was continued and the medium was changed the next day with medium without FCS, but containing live or heat-killed *U. diversum* at the following final concentrations: 25, 10, 1.0 and 0.1 μg/mL, and *E.coli* LPS (Sigma Chemical Co., St.Louis, Missouri) at a concentration of 0.1 μg/mL for C3H/HeN and 25 μg/mL for C3H/HeJ macrophages. U 4 broth medium diluted to a final concentration of 25 μg protein/mL, was used as a negative control.

In order to examine the possible modulatory effect of interferon-gamma (IFN-γ) on response of macrophages stimulated with *U. diversum*, macrophages were treated with 15 U/mL of mouse recombinant IFN-γ (Genzyme, Cambridge, Massachusetts) 2 h before activation with the appropriate stimulators.

Each sample was tested in triplicate. After 24 h of incubation, supernatants were removed and stored at −20°C for IL-6, NO, TNF-α and −70°C for IL-1 assays.

SPLEEN LYMPHOCYTES

Spleen lymphocytes of C3H/HeJ mice were obtained according to Cole *et al* (13). Cells were suspended in RPMI-1640 medium (Gibco, Grand Island, New York), supplemented with 10% FCS, 5×10^{-5} M of β-mercaptoethanol, 1% (v/v) of nonessential amino acids (Gibco), 1% (v/v) Na-pyruvate (Gibco), 2 mM glutamine and antibiotics. Flat bottom plates (96-wells) were seeded with 200 μL of 1.25×10^6 cells per well. Cells were stimulated by concanavalin A (Con A) (Sigma) in the following concentrations: 1.0, 0.5 and 0.25 μg/mL, and LPS of *E. coli* (Sigma) at 10 μg/mL, and filtered supernatant of *U. diversum* sonicate at 10, 5, 1 and 0.1 μg protein /mL. Plates were incubated at 37°C for 72 h in normal atmosphere with 5% CO_2. After 48 h, 20 μL of ^{3}H-labelled thymidine (20 μCi/mL) (ICN, St-Laurent, Quebec) was added. At the end of incubation, the plates were frozen at −20°C until assayed. Cells from thawed plates were harvested on glass-fiber filters with an automated cell harvester (Pharmacia LKB Biotechnology, Quebec). Thymidine incorporation was determined by liquid scintillation counting with an LKB flatbed scintillation counter. Results were expressed as the means of six replicates ± SD.

CYTOKINE AND NITRITE ASSAYS

TNF-α was determined in a cytotoxicity assay using BALB/c CL7 cells (American Type Culture Collection, Rockville, Maryland) as target. Cells at concentration of 4×10^5/mL were seeded in microtiter plates and left overnight to form monolayers. Serial threefold dilutions of test macrophage supernatants, in triplicate were applied to the plate, after which actinomycin D to a final concentration of 2 μg/mL

TABLE I. TNF-α production by IFN-γ (15 U/mL) primed or unprimed peritoneal macrophages of C3H/HeN and C3H/HeJ mice, stimulated with various doses of live (L) and heat-killed (H) *Ureaplasma diversum*, *E. coli* LPS, and *U. diversum* broth medium. The results are means ± SD of reciprocals of dilutions giving 50% cytotoxicity (U) in Cl 7 cells

Dose of *U. diversum* μg/mL	TNFα (U/mL) produced by C3H/HeN macrophages		TNFα (U/mL) produced by C3H/HeJ macrophages	
		IFNγ[a]		IFNγ[a]
L 25	29853 ± 1396	97238 ± 29490	25413 ± 2813	13827 ± 1495
L 10	9663 ± 6057	42304 ± 6586	10101 ± 1029	12859 ± 485
L 1	1046 ± 24	36782 ± 13449	4081 ± 447	11956 ± 488
L 0.1	2785 ± 68	4352 ± 739	7147 ± 1643	5879 ± 1429
H 25	13646 ± 6672	40700 ± 10628	5879 ± 1605	17215 ± 1075
H 10	4959 ± 662	33790 ± 6908	1865 ± 262	11001 ± 1838
H 1	722 ± 67	10592 ± 923	4520 ± 276	9485 ± 965
H 0.1	1135 ± 776	3872 ± 494	1107 ± 548	7229 ± 2394
LPS 0.1	97205 ± 27691	52999 ± 15355	ND	ND
LPS 25	ND	ND	28 ± 7	54 ± 3
Medium 25 μg protein/mL	0	0	0	0

[a] The release of TNFα was statistically significantly enhanced by IFNγ except in the cases of C3H/HeJ macrophages stimulated with live ureaplasmas and C3H/HeN macrophages stimulated with LPS
ND: not done

was added. Plates were incubated at 37°C for 18 h in normal atmosphere with 5% CO_2. Supernatants were discarded and the remaining cells in the wells were fixed in a solution of 5% buffered formalin, washed with tap water and stained with 1% aqueous solution of crystal violet. The dye was dissolved with 33% glacial acetic acid and absorbency of solutions was measured with a Microplate Autoreader (Bio-Tek Instruments, Winooski, Vermont) at 550 nm wave length.

Calculations of TNF-α were performed by using a logit transformation computer program developed by Yoram Hassidim (The Department of General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem). The TNF-α titer was expressed in U/mL ± SD as a reciprocal dilution of test supernatants giving 50% cytotoxicity in CL7 cells as compared with controls without addition of test supernatants. r-TNF-α (Genzyme) was used as internal standard for the assay. The presence of TNF-α was confirmed by ELISA on random samples using a mouse Tumor Necrosis Factor Elisa Test kit (Genzyme), according to the manufacturer's instructions.

A commercial ELISA kit (Genzyme) was used to determine mouse IL-1α in macrophage culture supernatants.

The release of IL-6 was measured with the IL-6 dependent B-9 cells as indicators (21). B-9 cells were cultured at 37°C in atmosphere with 5% CO_2 in complete RPMI-1640 medium, as described above for spleen lymphocytes, supplemented with fibroblast conditioned medium (FCM) as a source of IL-6. The cells were washed twice in complete RPMI-1640 medium without FCM and incubated overnight and washed one more time before they were added to the samples. Twofold serial dilutions in volumes of 100 μL of each sample prediluted 1:200 in RPMI-1640 medium without FCM were made in 96-well flat bottom plates. One hundred μL of B9 cell suspension, adjusted to contain 5×10^4 cells/mL were added to each well. Proliferation was measured after 72 h by reduction of MTT (3-(4,5)-dimethylthiazol-2,5-diphenylotetrazolium bromide) (Sigma) to formazan (22). Six hours before the end of incubation 20 μL of MTT dye, at a concentration of 5 μg/mL was added. After completed incubation the formazan crystals were dissolved in 20% sodium dodecyl sulfate (SDS) in 50% N'N dimethylformamide solution. The OD was measured the next day in the microplate autoreader at 550 nm. Calculations of IL-6 concentration were performed by using the logit transformation computer program. The IL-6 titer was expressed as a reciprocal dilution of test supernatant giving 50% of maximal proliferation and compared with controls stimulated by IL-6 (Genzyme) at a concentration of 500 pg/mL. The amount of IL-6 in test samples was expressed as pg/mL ± SD.

The nitric oxide release from stimulated macrophages was measured as nitrite (20). Fifty μL aliquots of macrophage supernatant were incubated with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthylene and 2% H_3PO_4) for 5 min at room temperature. The absorbency of samples was measured at 550 nm wave length with the microplate autoreader. The concentration of nitrite in nM ± SD of three replicates was calculated by reference to a standard serial dilution of sodium nitrite ranging from 0.5 to 10 nM.

STATISTICAL ANALYSES

The effect of IFNγ on cytokine release was evaluated for each preparation of ureaplasmas at multiple doses using 2-way analysis of variance and the SAS statistical program. The NO-response (nitrite concentration) was evaluated by multiple Student's *t* tests with an overall p value of <0.05.

RESULTS

CYTOKINES

Ureaplasma diversum induced TNF-α, IL-1 and IL-6 in peritoneal macrophage cultures of both C3H/HeN and C3H/HeJ mice. Both live (L) and heat-killed (H) *U. diversum* induced production of TNF-α by C3H/HeN and C3H/HeJ macrophages in a dose related manner (Table I). Recombinant IFN-γ clearly enhanced TNF-α production in the C3H/HeN macrophages stimulated with live and heat-killed ureaplasmas ($p<0.0001$), but not LPS ($p>0.05$). There was negligible TNFα response to LPS in the C3H/HeJ macrophages (<100 units are generally regarded as negative), and clear effect of IFN-γ in cultures stimu-

TABLE II. TNF-α concentration in ng/mL determined with a commercial ELISA kit in supernatants of C3H/HeN and C3H/HeJ macrophage cultures stimulated with live (L), heat-killed (H) *U.diversum* (25 μg/mL) and LPS in the presence or absence of IFN-γ (15 U/mL)

Source of macrophages	C3H/HeN		C3H/HeJ	
IFN-γ	−	+	−	+
U.diversum (L)	7.5	>32	15.6	17.4
U.diversum (H)	10.32	>32	9.0	10.4
LPS 0.1 μg/mL	30.3	>32	ND	ND
25 μg/mL	ND	ND	0.26	0.52
Medium (25 μg prot/mL)	0.17	0.25	0	0

ND: not done

TABLE III. Concentration of IL-6 in pg/mL ± SD in culture supernatants of peritoneal macrophages stimulated with live (L) or heat-killed (H) *U.diversum*; LPS and *U.diversum* culture medium with or without IFN-γ (15 U/mL). The amount of IL-6 was calculated as follows: titer of supernatant giving 50% of maximal proliferation was compared with the concentration of an internal standard of recombinant murine IL-6 giving 50% proliferation

Dose of *U.diversum* and LPS	C3H/HeN	C3H/HeN IFN-γ[a]	C3H/HeJ	C3H/HeJ IFN-γ[a]
L 25 μg/mL	105.7 ± 0.7	156.8 ± 8.4	32.1 ± 5.8	70.1 ± 17.1
L 10	43.7 ± 17.6	88.24 ± 11.8	23.8 ± 8.2	37.6 ± 4.0
L 1	25.95 ± 8.0	56.8 ± 10.6	5.2 ± 1.3	22.3 ± 7.8
L 0.1	3.45 ± 3.6	5.3 ± 1.8	0.6 ± 0	0.6 ± 0.4
H 25	33.95 ± 20.4	168.4 ± 33.1	0.6 ± 0.1	48.3 ± 7.9
H 10	96.8 ± 4.0	97.7 ± 10.5	0.1 ± 0.1	28.7 ± 6.8
H 1	16.5 ± 5.2	55.8 ± 8.6	0.63 ± 0.6	26.7 ± 1.3
H 0.1	1.9 ± 1.7	2.4 ± 1.2	0	0.3 ± 0.3
LPS O.1	>500	96.0 ± 8.0	ND	ND
LPS 25	ND	ND	0	0
Medium (25 prot μg/mL)	0	0	0	0

[a] IL-6 release was statistically significantly enhanced by IFNγ, except in macrophages stimulated with LPS
ND: not done

TABLE IV. IL-1 concentration (pg/mL) determined by a commercial IL-1-α ELISA kit in supernatants of C3H/HeN and C3H/HeJ macrophage cultures stimulated with various doses of live(L) or heat-killed (H) *U.diversum*, LPS and *U.diversum* broth medium with or without costimulation with IFN-γ (15 U/mL)

Dose of *U.diversum* and LPS	C3H/HeN	C3H/HeN IFN-γ	C3H/HeJ	C3H/HeJ IFN-γ
L 25 μg/mL	204	111	36	55
L 10	107	82	29	60
L 1	143	64	23	53
L 0.1	41	28	15	21
H 25	148	67	114	91
H 10	133	39	89	72
H 1	124	32	60	44
H 0.1	48	22	23	17
LPS 0.1	>405	50	ND	ND
LPS 25 μg/mL	ND	ND	<15	<15
Medium (25 prot μg/mL)	19	<15	<15	<15

ND: not done

lated with heat-killed ureaplasmas ($p<0.0001$), but not live ureaplasmas ($p>0.05$) (Table I). Analysis of samples of supernatants of macrophages stimulated with 25 μg/mL of live or heat-killed ureaplasmas using an ELISA test confirmed the presence of TNFα and the response of C3H/HeJ macrophages to ureaplasmas, and the low response to LPS. The augmenting effect of IFN-γ was seen in C3H/HeN macrophages (Table II). Small amounts of TNF-α in C3H/HeN macrophages stimulated with U4 medium were detected with the ELISA assay but not the cytotoxicity assay, possibly indicating traces of LPS contamination.

IL-6 was produced in response to ureaplasma material in a dose related manner, enhanced by IFN-γ ($p<0.0001$) (Table III). However, there was no clear response of C3H/HeJ macrophages to heat-inactivated *U. diversum*, except when the macrophages were primed with IFN-γ. Rat monoclonal antibody against mouse IL-6 (Genzyme) neutralized 60% or more of the B-9 stimulatory activity of selected test supernatants (data not shown).

IL-1 was detected in supernatants of both C3H/HeN and C3H/HeJ macrophages stimulated with live and heat-killed ureaplasmas (Table IV). There was a clear trend towards dose related release of IL-1, although a concentration of ureaplasma below the response level was not tested. The IL-1 production was increased by IFN-γ in the C3H/HeJ cultures in response to all dose levels of live ureaplasmas.

NITRIC OXIDE PRODUCTION

Both C3H/HeN and C3H/HeJ macrophages produced NO in response to ureaplasmas. Only C3H/HeN macrophages challenged with 25 μg live ureaplasmas or 0.1 μg LPS showed a significant response ($p<0.05$) without IFN-γ pretreatment, in all other cases the release of NO was dependent upon IFN-γ

TABLE V. Nitric oxide measured as nitrate in nM/mL ± SD secreted by peritoneal macrophages of C3H/HeN and C3H/HeJ mice, stimulated with different doses of live (L), heat-killed (H) *Ureaplasma diversum*, and LPS with or without IFN-γ (15 U/mL)

Dose of *U.diversum* and LPS	C3H/HeN	C3H/HeN IFN-γ	C3H/HeJ	C3H/HeJ IFN-γ
L 25 μg/mL	0.96 ± 0.08*	2.4 ± 0.1*	0.25 ± 0.05	1.82 ± 0.08*
L 10	0.58 ± 0.09	2.3 ± 0.2*	0.2 ± 0.04	1.30 ± 0.08*
L 1	0.58 ± 0.07	2.4 ± 0.3*	0.19 ± 0.07	1.20 ± 0.06*
L 0.1	0.50 ± 0.06	1.7 ± 0.09*	0.4 ± 0.34	0.77 ± 0.06*
H 25	0.58 ± 0.08	2.5 ± 0.25*	0.17 ± 0.01	2.04 ± 0.2*
H 10	0.55 ± 0.1	2.5 ± 0.05*	0.17 ± 0.08	1.57 ± 0.1*
H 1	0.49 ± 0.08	2.2 ± 0.14*	0.2 ± 0.1	1.4 ± 0.2*
H 0.1	0.46 ± 0.07	1.7 ± 0.08*	0.2 ± 0.1	1.17 ± 0.03*
LPS 0.1	1.16 ± 0.13*	2.2 ± 0.16*	ND	ND
LPS 25	ND	ND	0.2 ± 0.018	0.3 ± 0.02
Medium (25/μg/mL)	0.46 ± 0.13	0.19 ± 0.06	0.26 ± 0.04	0

* Concentrations of nitrate significantly different from medium control ($p<0.05$)(Student's *t* test)
ND: not done

TABLE VI. Thymidine uptake of C3H/HeJ mouse spleen lymphocytes in response to supernatant of *Ureaplasma diversum* sonicate, Con A or LPS. The data shown are the mean of six replicates ± SD and represent one of three experiments

Mitogen μg/mL	^{3}H thymidine uptake ccpm ± SD
Con A 1.0	100433 ± 13197
" 0.5	38630 ± 6007
" 0.25	8186 ± 931
LPS 0.01	8401 ± 573
Con A 0.25 + LPS 0.01	16972 ± 2029
U.diversum 10	47161 ± 3839
" 5	32687 ± 4516
" 1	17166 ± 999
" 0.1	7304 ± 720
U.diversum 10 + LPS 0.01	47435 ± 2419
Con A 0.25 + *U.diversum* 10	56608 ± 3876
" 0.25 " 5	46571 ± 4601
" 0.25 " 1	27033 ± 3105
" 0.25 " 0.1	12650 ± 2315
Medium (25 prot μg/mL)	3134 ± 150

(Table V). The NO production was highest in the macrophages stimulated with 25 μg/mL and less in macrophages stimulated with 0.1 μg/mL indicating dose related response, but the ureaplasmas were not titrated to a concentration below the response level.

MITOGENICITY

Supernatant of *U. diversum* sonicate was mitogenic for C3H/HeJ spleen lymphocytes (Table VI). ^{3}H-thymidine uptake by splenocytes in response to *U. diversum* was dose dependent, as was the response to Con A. *Ureaplasma diversum* and Con A costimulation had an additive effect on the mitogenic response.

DISCUSSION

The data indicate that *U. diversum*, is able to stimulate murine macrophages and lymphocytes similar to other members of the *Mycoplasmataceae* family (17,23,24,25). We did not attempt to determine the endpoint of activity, nor did we attempt to compare specific activity with activity of bacterial LPS, but rather included LPS as positive control for response of C3H/HeN macrophages and as control for the low response of the C3H/HeJ macrophages. We used the cytotoxicity assay to determine relative amounts of TNFα, and we confirmed the presence of this cytokine using a commercial ELISA specific for murine TNFα, because cytotoxicity may not be specific for TNFα. We also showed that IFN-γ augmented the production of TNF-α, IL-6 and NO, but only IL-1 in C3H/HeJ macrophages stimulated by live ureaplasmas. IFNγ enhanced TNFα in C3H/HeN macrophages stimulated with live and killed ureaplasmas, but only C3H/HeJ macrophages stimulated with killed ureaplasmas was enhanced, but for reasons unknown to us this pattern seemed reversed in respect to IL-1. An augmenting effect of IFN-γ has also been found for *Mycoplasma fermentans*, where IFN-γ increased IL-6 production by CBA/J and C3H/HeJ peritoneal macrophages stimulated with a high molecular weight fraction (15). TNF-α secretion was augmented in the presence of IFN-γ in C3H/HeJ peritoneal macrophages induced by the large colony type of *Mycoplasma mycoides* subsp *mycoides* (23). The augmenting effect of IFN-γ on cytokine secretion by macrophages may be important during mycoplasma infection because these organisms may also induce lymphocytes to produce interferons (26,27) and therefore may amplify the inflammatory process.

Although, mycoplasmas are frequently observed to stimulate lymphoid cells, the nature of the biochemical component with this activity is not known. Poorly characterized extracts labelled "high-molecular-weight material" from *M. fermentans* induce cytokine release, and a protein of *M. arthritidis* obtained from the culture supernatant has superantigen characteristics and cytokine inducing capacity (14,28). The lipoglycans of mycoplasmas have been shown to share some of the biological activities of bacterial endotoxin and may induce tumor cell killing possibly mediated by TNFα and NO (29). We have only shown that the activity of *U. diversum* is heat-stable and recognize the need for further biochemical studies.

Ureaplasma diversum stimulated macrophages from both LPS responder and low-responder mice. Thus, these organisms activate macrophages in a manner different from LPS. Similar to cytokine inducing factors from other mycoplasmas, the *U. diversum* factor seems to be heat resistant (23,30). With the exception of the decreased IL-6 response of C3H/HeJ macrophages stimulated with ureaplasma material only, we found that activity was retained in ureaplasmas heated at 56°C for 1 h.

Various mycoplasma species have the capacity to cause antigen independent blast transformation of lymphocytes (13). Both T and B lymphocytes are transformable but it seems that

B cells are stimulated more readily. We stimulated the whole population of splenocytes and did not attempt to differentiate between subsets of transformed lymphocytes. However, the additive effect of *U. diversum* and Con A costimulation might suggest that either the target cells or the receptors for the two stimuli are different.

The C3H/HeJ mouse strain, has a defect in a somatic autosomal gene that limits responsiveness of B lymphocytes to LPS (31). It is likely that, the mitogenic effect of *U. diversum* is different from the effect of LPS, similar to what has been observed for other mycoplasmas (30).

In conclusion, we found direct stimulation of murine macrophages and lymphocytes by *U. diversum* leading to cytokine production and blastogenesis. This may be responsible for recruitment of lymphoid cells to inflammatory sites during acute and chronic forms of ureaplasmosis in cattle. It remains to be shown if *U. diversum* stimulates bovine mononuclear cells.

ACKNOWLEDGMENTS

B-9 cells and IL-6 standard were kindly provided by Dr. Carl Richards, Department of Pathology, McMaster University, Hamilton, Ontario. Dr. M. Shoukri, Department of Population Medicine, Ontario Veterinary College, gave valuable assistance with the statistical analyses. Support from the Natural Sciences and Engineering Research Council of Canada, and the Ontario Ministry of Agriculture and Food, is acknowledged.

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