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Involvement of Interleukin-1 (IL-1), IL-6, IL-2, and IL-4 in Generation of Cytolytic T Cells from Thymocytes Stimulated by a Mycoplasma fermentans-Derived Product

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The capacity of Mycoplasma fermentans-derived high-molecular-weight material (MDHM) to generate cytolytic T cells from mitogen-stimulated murine thymocytes was studied in detail. The role of MDHM and the involvement of monokines and lymphokines resulting from the addition of MDHM to thymocyte cultures were examined in complete and adherent cell-depleted culture systems by the addition of neutralizing monoclonal antibodies against interleukin-2 (IL-2), IL-4, and IL-6 and in reconstitution experiments with recombinant mediators. The data presented here suggest that MDHM is crucial only in the first phase of a reaction sequence beginning with the stimulation of adherent accessory cells and resulting in the synthesis of IL-1 and IL-6. The lymphokines IL-2 and, primarily, IL-4 are required in a second step which, once these lymphokines are formed, can proceed in the absence of MDHM and accessory cells and lead to the formation of cytolytic T cells. The elucidation of the MDHM-induced reaction sequence may be of relevance in view of the hypothetical role of mycoplasmas in rheumatic disease in humans. M. fermentans is an organism capable of infecting humans and in an early report has been discussed as a causative agent for rheumatoid arthritis.

Several mycoplasmal products derived from different strains have been proposed to modulate immune cells in a number of ways. MAS, a product from Mycoplasma arthritidis, stimulates lymphocyte proliferation (1) and gamma interferon production (12). Acholeplasma spp. (25) and membranes from Spiroplasma spp. (23) stimulate tumor necrosis factor alpha synthesis, and membranes from M. arginini or M. arthritidis induce granulocyte-macrophage colony-stimulating factor-dependent macrophage proliferation (24).

In a previous publication, we described high-molecular-weight material from M. fermentans (MDHM) that induces the in vitro release of interleukin-6 (IL-6) from human monocytes and murine peritoneal macrophages (20). In concanavalin A (ConA)-stimulated thymocyte cultures, MDHM causes the formation of cytolytic T lymphocytes (CTLs). Since neutralizing monoclonal antibodies (MAbs) to IL-6 abolish this effect, we ascribed the MDHM activity in this system to its potential to generate IL-6 (20).

In this study, we explored the CTL-inducing capacity of MDHM in more detail. The question as to which mediators are required to allow CTL formation, although studied in many laboratories under different experimental conditions, is still not unequivocally answered. Of the known lymphokines, IL-1 (6, 21), IL-2 (5, 6), IL-4 (19, 31), IL-5 (27), IL-6 (18, 21, 26), IL-7 (3), tumor necrosis factor (30), and gamma interferon (6, 26) have been implicated at one time or another, either singly or in combination. We wished to identify the relevant mediators involved and clarify the sequence of events which follow the MDHM-mediated formation of CTLs in ConA-stimulated murine thymocyte cultures. Our data suggest that MDHM has a primary function in stimulating the synthesis of IL-1 and IL-6 by adherent accessory cells and that IL-2 and, primarily, IL-4 are required for CTL formation in a second step which, once these lymphokines are formed, can proceed in the absence of MDHM and accessory cells.

MATERIALS AND METHODS

Cultivation of M. fermentans. M. fermentans D15-86 was originally isolated from a contaminated human cell line (20). Mycoplasmas were grown in GBF-1 medium for 3 days at 37°C in an atmosphere containing 5% CO2. GBF-1 medium consists of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1% (vol/vol) cell sonicate. To prepare the sonicate, we suspended P815 mastocytoma cells at 107/ml in phosphate-buffered saline (PBS) and sonicated them in an ice bath by two 2-min bursts in a Branson Sonifier equipped with a conical tip. Cell debris was removed by 45 min of centrifugation at 21,000 × g, and the nonsedimented sonicate was passed through a 0.22-μm-pore-size sterile filter. GBF-1 medium was, in contrast to conventional mycoplasma media, nontoxic for thymocytes. It was also free of contaminating endotoxin (<10 pg/ml, as determined by the Limulus assay) and showed no activity in the lectin-dependent killing assay.

Preparation of MDHM. Two different preparations were used in this study. MDHM-A was prepared from culture media of HL60 cells infected with M. fermentans D15-86 by ammonium sulfate precipitation (90% saturation), dialysis of the redissolved precipitate against PBS, removal of insoluble material by centrifugation for 1 h at 21,000 × g, and filter sterilization as described previously (20). MDHM-M was prepared from M. fermentans grown in 120 ml of GBF-1 medium and collected by sedimentation at 21,000 × g. Mycoplasmas were washed in PBS, resuspended in 7 ml of RPMI 1640 medium with 5% FCS, and sonicated for 30 s at 60 W in a Branson Sonifier equipped with a conical tip. The

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preparation contained about 200 μg of mycoplasma protein per ml, as determined by the Lowry method from the sediment of a parallel sample without FCS. MDHM preparations were kept frozen at −20°C until use. MDHM-M has the advantage of being free of contaminating host cell constituents.

Mice. Female CBA/J mice were purchased from Bomholtgaard (Ry, Denmark) or Charles River (Sulzburg, Germany) and used at the age of 6 to 8 weeks.

Antibodies and mediators. Rat MAbS against murine IL-2 (immunoglobulin G2a [IgG2a]) from clone S4B6.1 (13) and against IL-4 (IgG1) from clone 11B11 (17) were generous gifts of T. Mosmann and W. Paul, respectively, to E. Schmitt. They were used as ascites fluids at dilutions of 1:800 and 1:5,000, respectively. Hybridoma clone 6B4, which produces anti-murine IL-6 IgG1 (29), was kindly provided by J. van Snick. The anti-murine IL-6 MAb was used as ascites fluid at a 1:20 dilution. Natural human IL-2 (8 × 10^3 U/μg) was produced and purified in our institute as described previously (7). IL-2 units are defined by the supplier. Human recombinant IL-2 (rIL-2) (1.25 × 10^5 U/μg) was obtained from Genzyme. Activity units are defined as described previously (8). Human rIL-6 (10^3 U/μg) was obtained from Boehringer, Mannheim, Germany. Activity units are defined as described previously (28).

Mito- mycin-poisoned spleen accessory cells. Single-cell suspensions were prepared by teasing minced spleen tissue in Hanks balanced salt solution buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4) (BSS) through a nylon sieve. Cells were adjusted to 5 × 10^6/ml in RPMI 1640 medium with 5% FCS and 25 μg of mitomycin per ml. Mitomycin was removed after 30 min of incubation at 37°C by washing the cells three times in medium.

Cell culturing, proliferation, and lectin-dependent killing assay. Thymocytes from mice at the age of 5 to 7 weeks were prepared in BSS by gentle teasing of thymus tissue through a nylon sieve. For removal of adherent accessory cells, single cells were adjusted to 10^6/ml in BSS–5% FCS and passed over a 20-ml Sephadex G10 column containing this buffer. Depleted or untreated thymocytes were washed and suspended in RPMI 1640 medium containing 5% FCS, 2 mM glutamine, and 2.5 × 10^{-5} M 2-mercaptoethanol. Cell culturing was performed in this medium at a final cell concentration of 1.5 × 10^6/ml or 2 × 10^6/ml in the presence of 4 μg of ConA per ml, and accessory cells, MDHM, mediators, or MAbs were added. Thymocytes were cultured either in 1-ml volumes in Falcon tubes or in 0.2-ml volumes in round-bottom microtiter plate wells. After 3 days of culturing at 37°C in a moist atmosphere containing 7.5% CO₂, lectin-dependent killing was measured in the microtiter cultures by removing 100 μl of culture supernatant and replacing it with 100 μl of medium with 10^6 phytohemagglutinin-coated (5 μg/ml) ^{51}Cr-labelled P815 mastocytoma cells. ^{51}Cr release was determined after 4 h of incubation, and specific lysis was calculated as follows: [(cpm_{experimental} - cpm_{spontaneous}) / (cpm_{100%} - cpm_{spontaneous})] × 100. Viable effector cells from the 1-ml cultures (done in triplicate) were counted with trypsin blue and adjusted to 10^6/ml in medium. For proliferation assays, 200-μl portions were removed and cultured in flat-bottom microtiter plate wells in the presence of 1 μCi of [3H]thymidine, which was adjusted with unlabelled thymidine to a final concentration of 20 μM. Thymidine incorporation was determined after 6 h with an automatic cell harvester (Scatron). For the determination of cytolytic activity at different effector-to-target (E/T) ratios, 100-μl serial dilutions of effector cells were added to 10^6 ^{51}Cr-labelled, phytohemagglutinin-coated P815 target cells in round-bottom microtiter plate wells and specific lysis was determined as described above.

Bioassay of IL-6. IL-6 was determined with hybridoma cell line 7D1 as described previously (28).

Immunofluorescence and flow cytometry analysis. Thymocytes were cultured for 3 days under the same conditions as those described for CTL generation. Thirty minutes before harvesting of the cells for immunophenotyping, methyl-α-D-mannopyranoside (Sigma) was added to a final concentration of 11 mM. The immunophenotypes were determined by a double staining procedure. Cells were labelled in a first step with CD4 (rat IgG; anti-L3T4; clone GK1.5) and CD8 (mouse IgG; anti-Lyt-2.1; NEN, Dreieich, Germany) MAbs. Binding of MAbs was assessed with fluorescein-conjugated rabbit anti-rat IgG antiserum (Medac, Hamburg, Germany) in a second staining step and phycoerythrin-conjugated goat anti-mouse IgG antiserum (Southern Biotechnology, Birmingham, Ala.) in a third staining step. The expression of Thy-1.2 was determined with the appropriate MAb (rat IgG; Beeton Dickinson, Heidelberg, Germany) and fluorescein-conjugated rabbit anti-rat IgG antiserum. The distribution of antigens was analyzed by flow cytometry (FACScan; Beeton Dickinson). The addition of propidium iodide (10 μg/ml) to cells prior to analysis allowed gating out of dead cells.

RESULTS

MDHM induces CTLs and an increase in CD8^+ cells in ConA-stimulated thymocyte cultures. The assay system used here was originally developed in our laboratory to detect lymphokines that would induce the in vitro differentiation of CTL precursors (CTL-P) to CTLs. Conditions for ConA-stimulated thymocyte cultures were chosen such that only

![FIG. 1. Concentration dependency of MDHM-mediated CTL generation. Thymocytes (4 × 10^7) were cultured in 0.2-ml volumes with 0.8 μg of ConA and the indicated concentrations of MDHM-M. Cytolytic activity was determined after 3 days by phytohemagglutinin-dependent lysis of ^{51}Cr-labelled P815 mastocytoma cells. Data represent means ± standard deviations for triplicate determinations.](image-url)
marginal CTL development occurred when no lymphokines were added. There was increased CTL activity after 3 days when lymphokine-containing material was added from the onset of culturing. MDHM-M, when introduced into this assay system, caused a dose-dependent increase in CTL activity, which reached a plateau at 2.5% (vol/vol) MDHM-M, corresponding to about 5 µg of mycoplasm protein per ml (Fig. 1). Flow cytometry analysis showed that there was a relative increase in the quantity of Thy-1+ CD4- CD8+ cells after 3 days of culturing with MDHM-A (12% CD8+ cells) over that in ConA control cultures (7% CD8+ cells). Omitting ConA from the assay abolished all CTL formation, regardless of whether it was MDHM or lymphokine mediated.

Depletion of adherent cells results in nonresponsiveness to MDHM. To clarify whether MDHM acted directly on CTL-P or whether other cells were the primary targets, we depleted thymocytes of adherent cells by passing them over Sephadex G10. Such adherent cell-depleted thymocyte cultures contained CTL-P, as they still responded to IL-2 or IL-4 by CTL development (Fig. 2) but were unable to respond to MDHM (Fig. 3).

Response of adherent cell-depleted thymocytes to MDHM can be reconstituted by mitomycin-treated spleen cells or peritoneal cells. Nonresponsiveness to MDHM of adherent cell-depleted thymocytes could possibly be due to a lack of helper T cells producing lymphokines, a lack of adherent accessory cells, such as macrophages, as a source of monokines, or both. In a first test of these hypotheses, mitomycin-poisoned spleen cells were added as a potential source of lymphokines and monokines. Four percent of these cells was sufficient to reconstitute the CTL response to MDHM but had little effect on their own, other than inducing some proliferation (Fig. 3). Next, resident peritoneal cells, rich in macrophages and almost devoid (we measured 1% Thy-1+ cells) of lymphokine-producing T cells, were added in graded amounts to adherent cell-depleted thymocytes to provide a source of monokines. Only 0.4% of these cells sufficed to reconstitute the response to MDHM (25% specific lysis versus 2% in controls), whereas 1% of these cells led to an increase in CTL activity even in the absence of MDHM. Since freshly isolated peritoneal macrophages stimulated by

FIG. 2. CTL generation in adherent cell-depleted thymocyte cultures by IL-2 and rIL-4. Thymocytes (4 x 10⁶) depleted of adherent accessory cells by passage over Sephadex G10 were cultured in 0.2-ml volumes with 0.8 µg of ConA and the indicated concentrations of lymphokines. Cytolytic activity was determined as described in the legend to Fig. 1.

FIG. 3. Reconstitution of the MDHM-mediated cytolytic response in adherent cell-depleted thymocyte cultures by spleen cells. Adherent cell-depleted thymocytes (2 x 10⁶) were cultured in 1-ml volumes in the presence of 4 µg of ConA. Where indicated, 8 x 10⁴ mitomycin-treated spleen cells (SCm), 1.5% (vol/vol) MDHM-A, or 0.02% (vol/vol) anti-IL-4 MAb-containing ascites were added. Cells were harvested after 3 days, and numbers of viable cells were determined. From one portion of the cells thymidine incorporation (Inc.) was determined over a 7-h period with 2 x 10⁵ cells. Incorporation data are means ± standard deviations for triplicate measurements. From the other portion of the cells cytolytic activity at various E/T ratios was determined as described in the legend to Fig. 1. Data are means ± ranges for duplicate determinations at an E/T ratio of 10.
FIG. 4. Effects of neutralizing anti-IL-4 and anti-IL-6 MAb's on MDHM-mediated CTL generation. Thymocytes were cultured in 1-ml volumes with ConA and, where indicated, with 2.5% (vol/vol) MDHM-M, 0.02% (vol/vol) anti-IL-4 MAb-containing ascites, 5% (vol/vol) anti-IL-6 MAb-containing ascites, or 1,000 U of murine rIL-4 per ml. Proliferative and cytolytic activities were determined as described in the legend to Fig. 3. Data are means ± ranges for duplicate determinations at an E/T ratio of 10.

the adhesion process are a source of monokines, these results suggested that monokines might be a limiting component in the adherent cell-depleted thymocyte system. MDHM might thus act in untreated thymocyte cultures by stimulating monokine production of a few adherent cells or in reconstituted thymocyte cultures by stimulating monokine production of macrophages added with spleen or peritoneal cells to the adherent cell-depleted cultures.

**Response to MDHM involves monokines.** If our assumption is correct that the effects of MDHM on CTL formation from thymocytes are indirect and caused by monokines released from adherent accessory cells, MDHM action should be inhibitable by neutralizing MAb's against such monokines. MAb's against murine IL-6 did indeed abolish MDHM action, as previously published (20) and shown in Fig. 4. In further support of this assumption, the addition of a combination of rIL-1 and rIL-6 to adherent cell-depleted thymocytes led to extensive CTL formation, whereas the addition of each monokine alone was less efficient (Fig. 5). Lower concentrations of rIL-1 (20 U/ml) only gave rise to a proliferative response (data not shown). Assays for rIL-6 in the supernatants of MDHM-containing, ConA-stimulated thymocyte cultures repeatedly revealed titers of up to 160 U/ml, whereas in control cultures without MDHM, IL-6 activities of between 8 and 32 U/ml were found.

**MDHM and monokine effects on thymocytes involve IL-2 and IL-4.** Since both IL-2 and IL-4 could lead to CTL formation in our adherent cell-depleted, ConA-stimulated thymocyte cultures (Fig. 2), the question arose as to whether IL-1 and IL-6 had an indirect effect on CTL formation by acting primarily on helper T cells and giving rise to IL-2, IL-4, or both, which are then required for CTL-P to proliferate and differentiate into CTL effector cells. The addition of a neutralizing MAb against IL-4 to either MDHM- or monokine-stimulated thymocytes inhibited most CTL formation without having much effect on the proliferative activity of the cultures (Fig. 3 and 5, respectively). In the experiment shown in Fig. 4, there was consistent inhibition of CTL formation by anti-IL-4 MAb's but an increase in thymidine uptake. It is possible that proliferation, which is normally maximal before the third day of culturing, was delayed in this case and was therefore comparatively higher than in the uninhibited control. These data suggest a primary role for IL-4 in the formation of CTLs under these conditions. However, IL-2 also appears to be required, as neutralizing anti-IL-2 MAb's abolished MDHM-driven CTL formation equally well (Fig. 6). Surprisingly, we were unable to detect IL-2 or IL-4 in the culture supernatants of MDHM-containing, ConA-stimulated thymocytes, although many workers, including ourselves (4), have shown that thymocytes can liberate IL-2, albeit under different conditions. However, the frequency of IL-4-producing cells, as determined in an ELISPOT assay (22), increased from 1 in 12,000 to 1 in 6,000 upon the addition of IL-6 to the cultures (21a), suggesting a role for this monokine in IL-4 production.

**Addition of IL-2 or IL-4 can overcome the inhibition of the effects of MDHM by anti-IL-6 MAb's.** The experiment described in the preceding section suggests a dual requirement for IL-2 and IL-4, but it does not exclude IL-6 as an additional prerequisit for CTL development, in particular since anti-IL-6 MAb's inhibited CTL formation in our assay (Fig. 4). If IL-6, in conjunction with lymphokines, were directly required for CTL development rather than for the generation of these lymphokines by helper T cells, then one should not be able to overcome the MAb-mediated inhibition of IL-6 by the addition of lymphokines. We had previously demonstrated that such inhibition could be overcome by IL-2 (20) and found in this study (Fig. 4) that it could be counteracted equally well by rIL-4. Similarly, rIL-4 was able to abolish the inhibitory action of neutralizing anti-IL-2 MAb's (Fig. 6). This result suggests that both IL-4 and IL-2 are limiting factors for the development of CTLs in this system.
DISCUSSION

MDHM caused a concentration-dependent increase in CTL activity in ConA-stimulated thymocyte cultures. This increase was accompanied by a moderate increase in CD8\(^+\) cells after 3 days of culturing. Surprisingly, MDHM showed no significant proliferative effects. Diverging cytolytic and proliferative activities in thymocyte cultures have been previously reported (3).

Analysis of the MDHM-mediated CTL response of ConA-stimulated thymocytes is made difficult by the complexity of the system. It consists of two different subpopulations of helper T cells (14), CTL-P at different maturation stages and an unknown number of ill-defined accessory cells. Our experimental conditions were chosen such that only a marginal CTL response occurred in the absence of any additional components. The system appears not to be limited by the number of CTL-P-expressing receptors for IL-2 and IL-4 (Fig. 2), but rather by the capacity of limited numbers of cells to synthesize monokines and lymphokines upon stimulation. For the purpose of discussion, we differentiate among (i) factors that stimulate the synthesis of mediators from accessory cells, (ii) accessory cell-derived mediators required for lymphokine synthesis by helper T cells, and finally (iii) lymphokines that presumably act directly on CTL-P.

Thymocytes depleted of adherent cells lost responsiveness to MDHM but still responded to IL-2 or IL-4 by CTL formation (Fig. 2). The response to MDHM could be reconstituted by the addition of mitomycin-poisoned spleen cells (Fig. 3) or peritoneal macrophages. This result suggested that MDHM acts on adherent accessory cells, presumably but not necessarily macrophages (16), reacting to MDHM by monokine synthesis. Indeed, increased IL-6 was measured in MDHM-stimulated thymocyte cultures, and MDHM-mediated CTL formation was reduced to control levels by neutralizing anti-IL-6 MAbs (Fig. 4) (20). In keeping with this hypothesis are the findings that rIL-1, rIL-6 and, in particular, a combination of both monokines were capable of causing a CTL response in adherent cell-depleted thymocyte cultures (Fig. 5) (30) and that these monokines were synthesized in MDHM-stimulated macrophage cultures (14a, 20).

We regard these monokines as early mediators in this system which are primarily required for the formation of lymphokines but not for CTL differentiation or generation of lymphokine receptors on CTL-P, because the inhibitory action of anti-IL-6 MAbs could be overcome by IL-2 (20) as well as by rIL-4 (Fig. 4) and both IL-2 and rIL-4 could lead to CTLs in the absence of adherent accessory cells (Fig. 2). In agreement with a key role for IL-4 is the finding that neutralizing anti-IL-4 MAbs inhibited the effects of MDHM (Fig. 3 and 6) as well as those of the monokines (Fig. 5) on CTL formation. However, IL-2 was found to be equally limiting, as anti-IL-2 MAbs also inhibited the effects of MDHM (Fig. 6). According to a recent paper, IL-2 is required for IL-4 production (2). In agreement with these findings, inhibition of CTL formation by anti-IL-2 MAbs could be overcome by rIL-4 (Fig. 6).

A recent publication reported that IL-7 can support the generation of CTLs from thymocytes (3). Two points argue against the involvement of IL-7 in MDHM-mediated CTL formation. (i) Neither macrophages nor spleen cells are known producers of IL-7, yet both were particularly potent in restoring the MDHM response in adherent cell-depleted thymocyte cultures. (ii) Anti-IL-4 MAbs inhibited the effects of MDHM but not those of IL-7 on CTL formation (3).

Our data are compatible with the following sequence of events resulting from MDHM stimulation. MDHM, added to thymocyte cultures, leads to the synthesis of IL-1 and IL-6 by adherent accessory cells. These monokines are early mediators required for ensuing IL-2 and IL-4 synthesis. Our experiments do not clearly distinguish between a direct requirement of IL-2 for CTL differentiation and an indirect effect of IL-2 on helper T cells resulting in IL-4 synthesis.
They suggest, however, that IL-4 may be responsible for the final step in MDHM-mediated CTL differentiation.

The elucidation of the MDHM-induced reaction sequence leading to CTLs as a result of monokine liberation may be of relevance in view of the hypothetical role of mycoplasmas in rheumatic disease. *M. fermentans* is an organism capable of infecting humans and, in an earlier report, was discussed as a causative agent for rheumatoid arthritis (32). Activated CD8+ T cells have in fact been detected in synovial fluid in cases of reactive arthritis (15), and increased levels of IL-1 (10) and IL-6 (9, 11) have been measured in synovial fluid in rheumatoid arthritis patients.

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