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Comparison of Mitogens from *Mycoplasma pulmonis* and *Mycoplasma neurolyticum*

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Studies on *Mycoplasma pulmonis* and *Mycoplasma neurolyticum* mitogenesis demonstrated that macrophages are not essential for the interactions of *M. neurolyticum* with B lymphocytes, whereas *M. pulmonis* that stimulates B and T lymphocytes depends on macrophages to fully exert its mitogenic effects.

Membranes of both species carry the mitogenic as well as pathogenic potentials. However, *M. neurolyticum* major mitogens are biochemically distinct from those of *M. pulmonis*. Furthermore, *M. pulmonis* mitogenesis is directly correlated to the mycoplasmal-induced pathogenic effects, while no such correlation could be established with *M. neurolyticum*. It was, therefore, concluded that *M. pulmonis* and *M. neurolyticum* differ in the biochemical and biological natures of their mitogens.

INTRODUCTION

Mycoplasma pulmonis and *Mycoplasma neurolyticum* are both pathogens in their natural hosts. *M. pulmonis* causes respiratory diseases in rats and mice whereas *M. neurolyticum* is the etiological agent of conjunctivitis and rolling diseases in mice [1].

In addition to their pathogenic potentials, these two mycoplasma species have been demonstrated to share the capacity to non-specifically stimulate rat and mouse lymphocyte cultures [2].

To understand the physiological significance of mycoplasma mitogenesis, several aspects of this biologic activity needed clarification. We have studied the lymphocyte cell population responsive to *M. pulmonis* and *M. neurolyticum*. It has been shown that *M. neurolyticum* stimulates only the B-lymphocyte populations of both rat and mouse [3,4]. On the other hand, *M. pulmonis,* which stimulates mostly B cells, also activates a portion of the T cells in cultures of rat lymphocytes [5].

Further experiments on *M. pulmonis* mitogenesis revealed that this activity is exhibited by the membranes isolated from the organism. The major membrane mitogens of *M. pulmonis* were identified as heat-labile outer-surface proteins [6]. In addition, our data indicated that membrane carbohydrates might also be involved in *M. pulmonis* mitogenesis, although to a lesser extent than the proteins [6,7]. Additional studies showed that membranes isolated from *M. pulmonis* exhibit not only mitogenic capacity but are also capable of inducing tracheitis and interstitial pneumonia in rats [8]. Reduction in membrane mitogenic effects resulted in a parallel decrease in the membrane's pathogenic potential. It was further demonstrated that administration of the T-cell mitogen, concanavalin A, produced

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pneumonia in rats [8]. These results suggested that the mitogenic activity of *M. pulmonis* toward T lymphocytes plays a role in the pneumonia induced by this organism.

M. neurolyticum differs from *M. pulmonis* in the ability to activate T lymphocytes as well as in the diseases it produces. We were motivated, therefore, to compare the mitogens of these two mycoplasma species.

In the studies reported here we have tested the dependence of *M. pulmonis* and *M. neurolyticum* mitogenic activities on the presence of macrophages in cell cultures. The biochemical natures of mycoplasmal mitogens were compared as well as the relation of mitogens to the mycoplasmas' pathogenicity.

MATERIALS AND METHODS

Animals

Inbred Lewis rats and Balb/C mice 8–12 weeks of age were purchased from the Weizman Institute of Science, Rehovot, Israel, where they had been reared under specific pathogen-free conditions.

Mycoplasmas

The *Mycoplasma pulmonis* strain used was the one isolated by Ginsburg and Nicolet [10]. *M. neurolyticum* strain Type A was kindly provided by Dr. J.G. Tully from the NIAID-NIH stock, Bethesda, Maryland. Mycoplasmas were grown as previously described [2,3]. Both species have been through 11–15 broth passages in our laboratory. To label mycoplasma lipids, growth medium was supplemented with methyl-^{3}H-palmitic acid 50 mCi per liter medium (Sp. Act. 33.5 Ci/mmole).

Cell Cultures and Lymphocyte Transformation

Rat and mouse lymph node cells were cultured at 2×10^{6}/ml in culture media, as previously described in detail [2,3]. Lymphocyte transformation was assayed by ^{3}H or C^{14} labeled thymidine uptake as previously described [2,3]. The values presented are the means of four individual experiments, each performed in triplicate. The standard deviations did not exceed 5 percent in all cases. To remove macrophages from lymphocyte suspensions, cultures were incubated in 100 mm tissue culture plastic petri dishes for one hour at 37°C. The non-adherent cells were collected gently and incubated again in petri dishes for an additional hour at 37°C. Non-adherent populations contained 81 percent of the original cells with less than 1 percent macrophages.

Mycoplasma Membrane Preparations

Non-viable lysed mycoplasma cells and purified membranes were prepared as previously described [6,9]. Membrane preparations (500 μg protein/ml) were exposed to 60°C for one hour, to 10 μg/ml or 100 μg/ml of protease for one hour at 37°C or to 2.5×10^{-3}M (final concentration) of sodium-m-periodate for one hour at 37°C. Control membranes were incubated in parallel but without enzyme or periodate. Washings and collection of membrane residues were performed as described in the past [6]. Membrane lipids from mycoplasma membranes prelabeled with ^{3}H-palmitic acid were extracted twice, using chloroform-methanol and washed with potassium chloride [11,12]. These procedures yielded 100 percent of *M. pulmonis*-labeled lipids and 96.4 percent of *M. neurolyticum* lipids in the lipid fraction. The procedures described by Marchesi and Andrews [13] as modified by

Kahane and Brunner [14] were employed to isolate membrane glycoproteins from *M. neurolyticum* with lithium diiodosalicylate and 50 percent phenol in water. No radioactive lipids could be demonstrated in the purified fractions. Proteins were determined according to Lowry et al. [15], using bovine serum albumin as standard. Carbohydrates were determined by the Anthron reaction [16], using D-galactose as standard. It was our experience that heat, protease, or exposure to sodium-m-periodate did not affect the amount of ^{3}H-labeled lipids in membrane residues as compared to control membranes.

Antisera to Mycoplasma Membranes

Antisera to *M. pulmonis* and *M. neurolyticum* membranes were produced in rabbits as described by others [17].

RESULTS

Effect of Macrophages on the Lymphoproliferative Responses to M. pulmonis *and* M. neurolyticum

Recently Cole et al. reported that mitogenic stimulation of mouse T lymphocytes exerted by *M. arthritidis* requires the presence of macrophages [18]. To test whether macrophages are essential for the interaction of *M. pulmonis* and *M. neurolyticum* mitogens with lymphocytes, mitogenesis of mycoplasmal membranes was tested in rat lymph node cell cultures from which macrophages were depleted. During removal of macrophages from lymph node cells 19 percent of cells were depleted. We, therefore, suspended the non-adherent population at the original volume of unfractionated cells and thus obtained a culture of non-adherent cells containing 81 percent of cells as compared to unfractionated cultures. In addition, cultures containing equal cell concentrations of fractionated and unfractionated cells were prepared. Table 1 compares the results of lymphocyte transformation induced in

TABLE 1
Effect of Macrophage Depletion on Lymphocyte Transformation Induced by *M. pulmonis* and *M. neurolyticum*

Mitogen	^{3}H-Thymidine Uptake (CPM) and Stimulation Index[a]		
	2×10^6/ml LN Cells	2×10^6/ml Non-Adherent LN Cells	1.62×10^6/ml Non-Adherent LN Cells
M. neurolyticum membranes	68,626 (66.5)	96,914 (78.7)	72,130 (75.2)
M. pulmonis membranes	173,241 (168.0)	140,868 (114.5)	107,695 (112.2)
Concanavalin A	194,198 (188.3)	81,563 (66.3)	60,401 (62.9)
–	1,031 (1)	1,230 (1)	959 (1)

Lewis rat lymph node (LN) cells or macrophage-depleted, non-adherent lymph node cells at the specified concentrations were cultured in the presence of 20 μg/ml of various mitogens. Thymidine incorporation was assayed by addition of 1 μci ^{3}H-thymidine at 48 hours and harvesting of cultures 24 hours later.

Depletion of adherent cells was achieved by two successive incubations of one hour at 37°C in plastic petri dishes. 19 percent of the cells were removed during these procedures. The remaining non-adherent populations contained < 1 percent macrophages.

[a] Numbers in parentheses represent stimulation indices which were calculated by dividing the mean cpm of mitogen-stimulated cultures by the mean cpm of control cultures.

these cultures by *M. neurolyticum* and by *M. pulmonis* membranes, as well as by Concanavalin A (Con A). The blastogenic responses induced by both *M. pulmonis* and Con A were reduced upon removal of macrophages from cell populations. However, comparison of the stimulation indices revealed a reduction of 65–67 percent in Con A stimulation and 32–33 percent reduction in the stimulation of lymphocytes by *M. pulmonis*. Stimulation of lymphocytes induced by *M. neurolyticum* was not reduced upon depletion of macrophages from cell cultures but was rather slightly enhanced. The results indicate that *M. neurolyticum*, previously reported to activate only B lymphocytes [3,4], is able to interact with these cells without the involvement of macrophages in this process. On the other hand, *M. pulmonis*, mostly a B-cell mitogen and, to a lesser degree, a T-cell mitogen [5] requires the presence of macrophages for induction of lymphocyte blastogenesis to its full expression.

Biochemical Comparison of M. pulmonis *and* M. neurolyticum *Membrane Mitogens*

Having established differences between *M. pulmonis* and *M. neurolyticum* mitogenic activities toward T lymphocytes and their different dependence on macrophages, we were motivated to compare the biochemical nature of their mitogens.

In previous studies on the biochemical nature of *M. pulmonis* mitogens, we have demonstrated that the organism's membranes carry this mitogenic capacity [6]. To identify the mitogens of *M. neurolyticum*, mycoplasmal membranes were isolated and their mitogenic capacity was compared to that of non-viable lysed cells. It was revealed (data not shown) that isolated membranes induce a greater lymphoproliferative response in mouse and rat lymphocytes than equal protein doses of unfractionated lysed cells. In subsequent studies we have, therefore, examined that nature of *M. neurolyticum* membrane mitogens by subjecting these membranes to different physical, chemical, and enzymatic treatments. In each experiment we have tested the mitogenic stimulation of Balb/C lymphocytes exerted by pretreated, *M. neurolyticum* membranes in comparison with the stimulation induced by control membranes exposed to buffers without enzymes or chemicals.

Table 2 summarizes our previous data [6,7] on the biochemical nature of *M. pulmonis* membrane constituents stimulating rat lymphocytes, together with additional data on both *M. pulmonis* and *M. neurolyticum* mitogens. Our results demonstrate that the mitogenic activity of membranes from both organisms is reduced upon heating for 60 minutes at 60°C. However, most of *M. pulmonis* mitogens are heat-labile, whereas 18 percent of *M. neurolyticum* mitogenesis resisted heating, even when membranes were heated for 60 minutes at 100°C. These differences motivated us to compare the sensitivity of membrane mitogens of both species to proteolytic digestion. Following mild proteolysis, *M. pulmonis* membranes lost 90 percent of their mitogenic capacity when 57 percent of the membrane proteins were digested. Similar proteolytic treatment of *M. neurolyticum* membranes removed only 20 percent of the proteins and resulted in a 20 percent decrease of the mitogenic activity. Exposure of mycoplasmal membranes to higher concentrations of protease almost abolished the mitogenesis of *M. pulmonis*, whereas the mitogenic activity of *M. neurolyticum* decreased by 30 percent even when 57 percent of membrane proteins were digested. It should be mentioned that the additional digestion of proteins was also accompanied by a slight decrease in membrane carbohydrate concentrations. We could, therefore, conclude that when only membrane proteins are affected the residual mitogenesis of *M. neurolyticum* is 80 percent as

TABLE 2
Biochemical Comparison of *M. pulmonis* and *M. neurolyticum* Membrane Mitogens

Mitogen in Culture	*M. pulmonis*			*M. neurolyticum*		
	% of Control Membranes			% of Control Membranes		
	Protein concentration	Carbohydrate concentration	Residual mitogenesis	Protein concentration	Carbohydrate concentration	Residual mitogenesis
Membranes	100	100	100	100	100	100
Membranes heated at 60°C for 60 minutes	100	98.4	1.5	100	98.1	18.4
Membranes digested with 10 μg/ml protease	43.3	99.6	10.3	80	101	79.9
Membranes digested with 100 μg/ml protease	15.8	83.3	5.2	43	86	70.0
Membranes exposed to sodium-m-periodate	99.3	98.0	73.4	97	102	15.4
Membrane lipids	3.2	ND	0	0.2	16.4	18.6
Membrane glycoproteins	ND	ND	ND	1.5	8	35.4
Membranes + 50 mM N-acetyl glucosamine	–	–	19.1	–	–	72.3
Membranes + 1 percent rabbit anti *M. pulmonis*	–	–	16.7	–	–	95.7
Membranes + 1 percent rabbit anti *M. neurolyticum*	–	–	98.5	–	–	9.3

The mitogenicity of *M. pulmonis* toward Lewis rat lymphocytes was compared to the mitogenicity of *M. neurolyticum* toward Balb/C mice lymphocytes. Lymph node cells were cultured at 2×10^6 cells/ml and exposed to the specified mycoplasmal preparations with or without addition of sugars or antisera. ^{3}H-thymidine (1 μci/ml) was added to the cultures after 48 hours for a pulse period of 24 hours.

compared to 10 percent residual activity of *M. pulmonis*. In contrast to proteolysis, *M. neurolyticum* mitogens were found to be very susceptible to oxidation with sodium-m-periodate. Treatment of membranes, using low concentrations of periodate to avoid oxidation of proteins [7,19], significantly (85 percent) reduced *M. neurolyticum* mitogenesis. In comparison, *M. pulmonis* mitogenesis decreased by 27 percent upon exposure to low concentrations of periodate. These results suggested that carbohydrates within *M. neurolyticum* membranes play a major role in the mitogenic activity of the organism. On the other hand, carbohydrates are minor constituents in *M. pulmonis* membrane mitogens.

Further experiments showed that, unlike the lipids isolated from *M. pulmonis* which were not mitogenic, lipids isolated from *M. neurolyticum* exhibited 18 percent of the membrane mitogenesis. Interestingly, 18.4 percent and 15.4 percent of *M. neurolyticum* mitogenic activity were retained after heating and exposure to periodate, respectively. It seems that the mitogenesis exhibited by membrane lipids is unaffected by the later treatments. Studies repored by Kahane and Tully [20] indicated that glycoproteins might be present in the *M. neurolyticum* membranes. We have extracted a fraction from *M. neurolyticum* which contained proteins and carbohydrates and did not contain any residual lipids prelabeled with ^{3}H-palmitic acid. This fraction exhibited mitogenic activity which was abolished by either protease or oxidation with periodate. These data suggest that glycoproteins present in our preparation are responsible for at least 35 percent of the mitogenesis of *M. neurolyticum* membranes. Furthermore, we have found that N-acetylglucosamine, when present in lymphocyte cultures, inhibits 28 percent of *M. neurolyticum* mitogenic activity and 80 percent of the activity of *M. pulmonis*. Other sugars tested—glucose, galactose, N-acetyl-galactosamine and α-methylmannoside—did not significantly affect the mycoplasmal mitogenesis and the results were therefore omitted from Table 2.

That *M. pulmonis* mitogens differ from *M. neurolyticum* mitogens was further demonstrated using antisera against mycoplasmal membranes. Antibodies to *M. pulmonis* significantly blocked the expression of *M. pulmonis* mitogens and had almost no effect on lymphoproliferative response to *M. neurolyticum*. The same phenomenon was true with antisera to *M. neurolyticum* membranes that inhibited *M. neurolyticum* but not *M. pulmonis* mitogenic activity.

The Relationships Between Mycoplasmal Mitogenic and Pathogenic Effects

We have previously demonstrated that mitogenesis of *M. pulmonis* plays a major role in the pneumonia induced by the organism in rats [8]. In this study we were interested in clarifying the relationships between *M. neurolyticum* mitogenic and pathogenic components. In agreement with previous reports from the laboratories of Tully and Thomas [21–24] we have found that broth cultures (from which mycoplasma cells were removed by centrifugations) when administered intravenously, produced rolling disease followed by death of mice. Control groups of mice which received equal volumes of sterile broth or cell-free *M. pulmonis* cultivation broth did not develop any apparent effect. Further experiments revealed that the toxigenic broth medium from *M. neurolyticum* had no mitogenic effects on mouse lymphocytes. Additional series of experiments revealed that non-viable lysed cells as well as membranes isolated from *M. neurolyticum* produced rolling disease and subsequent death of mice. The toxicity of lysed cells and membranes was dose-dependent. Protein doses equivalent to 4×10^{9} colony-forming units produced

disease in all animals. Equal protein doses and volumes of either *M. pulmonis* lysed cells or bovine serum albumin were not toxigenic. Surprisingly, and in contrast to the suggested heat lability of cell-free toxin and whole cell toxicity [21–24], we have found that lysed cells heated at 60°C or 100°C for 60 minutes retained their toxicity. Of 10 animals inoculated, nine developed rolling syndrome and died within five minutes.

On the other hand, these heated preparations lost about 80 percent of the original mitogenic potential displayed by non-heated lysed cells. These data, together with the observation that cell-free *M. neurolyticum* cultivation broth is toxigenic but not mitogenic, suggest that the pathogenicity of *M. neurolyticum* is not related to the organism's mitogenic effects. Moreover, our data demonstrate that *M. neurolyticum* membranes produce rolling disease in mice.

DISCUSSION

The studies reported here revealed distinct differences between *M. pulmonis* and *M. neurolyticum* mitogens in their ability to activate T lymphocytes, in their dependence on macrophage functions, and in the biochemical nature of the mycoplasmal mitogenic moieties, as well as in the relations between the mycoplasmal mitogenic and pathogenic activities.

Our studies with macrophage-depleted lymphocyte cultures revealed that macrophages are not essential for the interactions between *M. neurolyticum* and the B lymphocytes that respond to this mitogenic mycoplasma [3,4]. On the other hand, *M. pulmonis*, exerting its mitogenic effects mostly on B cells and partially on T cells [5], needed macrophages to display maximal activity. Recently Cole et al. [18] demonstrated that a mitogen derived from *M. arthritidis* that stimulates mouse T cells depends on radio-resistant adherent cells to exert its mitogenic effects. In view of these data, together with ours, it seems that macrophages are required during the process of T-lymphocyte stimulation whereas macrophages are not essential for the activation of B lymphocytes by mycoplasmal mitogens. In view of these differences between *M. neurolyticum* and *M. pulmonis*, we were interested in comparing the biochemical natures of their mitogenic constituents. The data reported here and previously [6,7] clearly demonstrate that the major mitogens of *M. pulmonis* are heat-labile, protease-sensitive membrane proteins. In addition, our results strongly suggest that membrane substances which contain carbohydrates are involved to a certain extent (between 20 percent to 30 percent) in the lymphoproliferative responses induced by *M. pulmonis* membranes. We are currently attempting to identify these mitogenic, carbohydrate-containing components from *M. pulmonis*. Moreover, assuming that these latter constituents induce only partial mitogenesis, as compared to the response displayed by membranes, further experiments will be needed to explain the profound inhibition exerted by N-acetylglucosamine on the mitogenic response to *M. pulmonis* membranes.

As in *M. pulmonis*, the mitogens from *M. neurolyticum* are present in the organism's membranes and most of their activity is reduced upon heating. However, unlike *M. pulmonis* mitogens, the membrane mitogens from *M. neurolyticum* are highly susceptible to oxidation with sodium periodate while being less affected by proteolytic enzymes. Further, lipids isolated from *M. neurolyticum* membranes display a certain degree of mitogenic activity, whereas lipids from *M. pulmonis* are non-mitogenic. Studies on the effects of antisera on the mycoplasmal mitogenesis showed that antibodies produced against membranes from one species inhibited the

mitogenic response to the organism's membranes but had no effect on the mitogenic activity of the other mycoplasma species. Considered together, the above data reveal that *M. neurolyticum* mitogens differ from those of *M. pulmonis* and are composed of various biochemical moieties. Although membrane proteins and lipids exhibit a certain degree of activity, the mitogenic activity of *M. neurolyticum* is mostly displayed by substances containing carbohydrates.

It is also possible that the mitogenic activity of isolated lipids is in fact exerted by the organism's glycolipids [25]. Our observation that the lipid preparation was serologically active indicated that glycolipids were present [25]. However, only isolation of *M. neurolyticum* glycolipids will clarify whether these compounds display mitogenic activity and are therefore the active components within the lipid fraction. Using the procedures described by others for isolation of glycoproteins [13,14], we have isolated from *M. neurolyticum* membranes components containing proteins and carbohydrates which were more potent as mitogens than proteins and lipids. Kahane and Tully [20] have already suggested that N-acetylglucosamine linked to proteins is present in *M. neurolyticum* membranes. Since the mitogenic activity of the isolated components was sensitive to both proteolysis and periodate it is plausible to assume that we have isolated from *M. neurolyticum* glycoproteins and that the inhibitory effect exerted by N-acetylglucosamine on *M. neurolyticum* mitogenesis indicates that this sugar is an integral part of the mitogenic glycoproteins. Although preliminary experiments to isolate lipoglycans from *M. neurolyticum* [26] did not yield any mitogenic fractions, these compounds should also be considered in further studies on the mitogenic carbohydrate-containing subtances from *M. neurolyticum*.

In a further series of experiments, we have examined the relations between *M. neurolyticum* mitogens and the components responsible for the induction of rolling syndrome in mice. It was found that toxigenic cell-free *M. neurolyticum* broth cultures are not mitogenic. Moreover, non-viable lysed cells as well as purified mycoplasmal membranes were demonstrated to be highly toxigenic. These preparations retained their toxicity even upon 80 percent reduction in their mitogenic capacity. It was therefore concluded that, unlike *M. pulmonis,* the mitogens from *M. neurolyticum* are not directly responsible for the mycoplasmal toxic effects. Moreover, our ability to induce rolling disease and death in mice administered *M. neurolyticum* membranes augments the doubts recently raised by Tully [24] on the concept of *M. neurolyticum* exotoxin.

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