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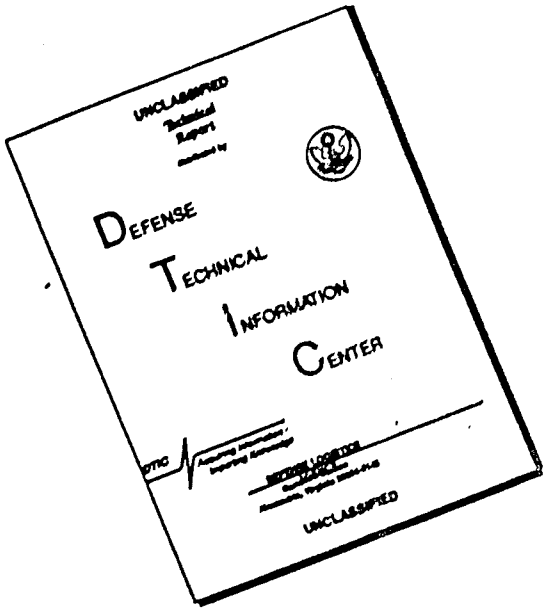

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THE EFFECT OF MYCOPLASMA INFECTION ON THE REPRODUCTION OF SOME RNA VIRUSES

R. Ya. Kagan, F. I. Ershov, I. V. Rakovskaya, A. A. Tsareva, V. M. Zhdanov

Summary

Depending upon the reaction of cells to the presence of mycoplasma, the latter may be divided into 2 groups: (1) mycoplasma producing the cytopathic effect and (2) conditionally pathogenic mycoplasma.

Members of the two groups were found to exert a selective inhibitory effect upon reproduction of three RNA-containing viruses (EEE, VEE and NDV).

Activation of interferon production after pre-treatment of cells with mycoplasma of the first and second groups and subsequent infection with EEE virus was demonstrated.

The character of associative relationships between mycoplasma and viruses and possible causes of the inhibitory effect of mycoplasma infection upon virus reproduction are discussed.

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THE EFFECT OF MYCOPLASM INFECTION ON THE REPRODUCTION OF CERTAIN RNA-CONTAINING VIRUSES

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Associative interrelationships between the Mycoplasmataceae family and viruses in mixed infection form one of the least studied aspects of contemporary bacteriology and virology. Furthermore, the necessity has now arisen to investigate the nature of the relationship between these groups, to determine whether mutual interaction or interference between them is possible, and to discover the underlying causes of these phenomena. The separate facts presently available indicate that associative interrelationships between mycoplasms and viruses may be expressed in completely different ways. In some cases they are manifested in an increase in pathogenic effect, in others in a weakening. Thus, associations between certain species of mycoplasms and viruses are known to promote more active reproduction of the former with an increase in their pathogenic effect. Examples of such interrelationships may be found in increased reproduction and virulence for Swiss mice of Nelson's catarrhal-type mycoplasm under the influence of mouse hepatitis virus [12], and the increased virulence of certain species of mouse mycoplasms under the influence of ectromelia virus [11]. Investigators have also noted more active pathogenicity for white mice of certain species of human-origin mycoplasms under the influence of ectromelia, vaccinia, neurolapina and myxomatosis viruses [8], selective activation of mycoplasm infections of human and avian origin for white mice under the influence of ectromelia virus and the virus of ascites sarcoma 80 [7].

Associative interrelationships between mycoplasms and viruses may also have another character. Thus, Powelson [13], Rouse and his

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co-workers [14], and Butler and Leach [4] established the inhibitory effect of mycoplasms on adenoviruses and certain mouse viruses. Somerson and Cook [16] recently reported on the inhibitory effect of M. orale on the virus of Rouse's sarcoma and a virus associated with Rouse's sarcoma.

The mechanism of the inhibitory effect of mycoplasms on viruses has not been explained; this process may be based on competitive relationships associated with the need for amino acids, nucleotides, growth factors etc., the production of macromolecular toxic substances such as Kraemer's lytic factor [9] and, finally, the effect of mycoplasm infection on tissue metabolism. It may also be assumed that one possible mechanism of inhibitory action in mixed mycoplasm-virus infection is stimulation of the production of interferon, which in turn suppresses virus reproduction.

We studied the interrelationships between certain species of mycoplasms and RNA-containing viruses in tissue culture, and also demonstrated the possibility of interferon synthesis in mixed mycoplasm-virus infection of tissue.

Materials and Methods

The following species of mycoplasms were investigated: M. laidlawii and M. agalactiae[1], M. hominis 1 and M. salivarium[2], mycoplasm contaminants of HeLa, NEr-2, SOTs, KV and A-1 tissue cultures isolated from the corresponding tissues by I. V. Rakovskaya [1, 2].

All the mycoplasm strains investigated were cultured in an agar medium (0.3%) prepared in tryptic digestion of beef heart and containing 10% normal horse serum and penicillin (100 units/ml). After culturing for 4-5 days, the growth zone was drawn off with a Pasteur pipette and transferred to physiological solution (5 ml); the mixture was then carefully blended. Infection was carried out with a 1-milliard mycoplasm suspension (in accordance with the optical standard of the L. A. Tarasevich Control Research Institute of Medical Biological Samples) freed from the agar by centrifugation at 1,500 RPM for 30 minutes. This suspension contained 10^6-10^7 colony-forming units of mycoplasm per one ml of physiological solution.

The number of mycoplasms was determined by preparing serial tenfold dilutions and isolating test samples from each dilution onto the medium described above.

RNA-containing viruses of Eastern (EEE) and Venezuelan (VEE)

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[1]Obtained from Prof. G. Keller's laboratory (GDR).
[2]Obtained from Prof. D. Edwards' laboratory (England).

equine encephalitis were used, along with Newcastle disease virus (NDV). The viruses were obtained from the collection of the D. I. Ivanovskiy Institute of Virology of the USSR Academy of Medical Sciences and maintained on chick embryo fibroblasts. Up to the time of the experiments the EEE viruses had undergone six passages, the VEE viruses 42 passages, and the NDV viruses 9 passages. Culture fluid collected 24 hours after infection of the cells served as the virus-containing material. The infectious virus titer was 10^8-10^9 PFP/ml.

A chick fibroblast culture prepared through trypsinization of 10-day-old embryos was used in the majority of experiments. The cells were suspended in lactalbumin hydrolyzate with 10% warmed bovine serum and antibiotics added. Each milliliter of cell suspension contained 1-1.5 million cells. The suspension was poured into Carrel dishes in 10 ml portions. After a cell layer was formed (in 24-48 hours) and negatively screened for the presence of mycoplasms, mycoplasms were introduced into the dishes in quantities of 0.5 ml of one-milliard suspension; the cells were then infected with viruses after 1, 2 and 3 days (depending on the experiment). The culture fluids were poured off 24 hours after infection and their virus titers were determined under agar by the plaque method. In another series of experiments mycoplasm-infected cells were infected with strictly determined amounts of viruses (100-150 PFP), and after adsorption of the viruses the dishes were covered with an agar overlay. Thus, in the latter case the effect of the mycoplasm on the plaque-forming effectiveness was determined.

The overlay consisted of 0.8 ml of Hay's A solution, 0.2 ml of Hay's B solution, 0.2 ml of lactalbumin hydrolyzate, 0.3 ml of bovine serum and 0.2 ml of chick embryo extract. The pH of the medium was increased to 7.4 with sodium bicarbonate, and 0.3 ml of tris buffer and 2 ml of 2% Difko's agar were added. The dishes were incubated in a thermostat for two days at 37°, after which the cells were stained with neutral red.

The possibility of interferon production was examined by infecting chick embryo fibroblasts with mycoplasm cultures, with the corresponding virus strains, and also with a combination of mycoplasm and virus. Interferon was obtained by collecting the culture fluids 1, 2 and 3 days after the cells had been infected with mycoplasms, viruses and combined mycoplasms-viruses, and heating them at 65° for 30 minutes. It was determined in special experiments that this manner of treatment fully inactivates both the virus and the mycoplasm while not reducing interferon titers.

The activity of the interferons was tested with respect to EEE virus. For this purpose double dilutions of interferon were placed in Carrel dishes for 24 hours, then the cells were washed with Hanks' solution and infected with 100-150 PFP of EEE virus per dish. The

virus was left in contact with the cells for one hour and then drawn off; the culture was washed with Hanks' solution and covered with an agar overlay.

Results

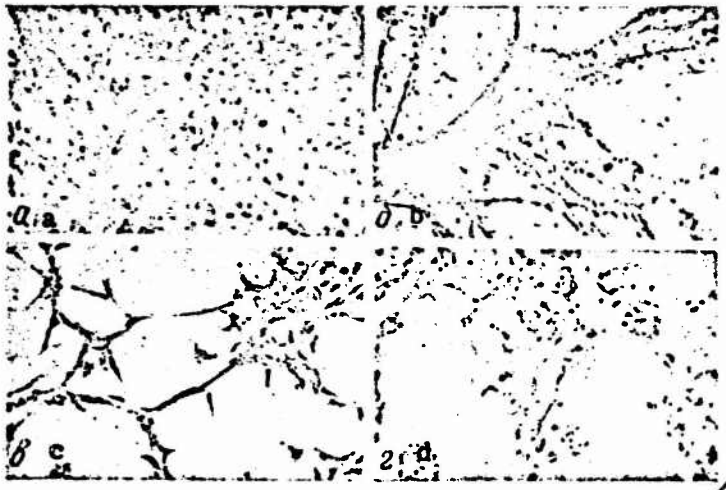
The first step in our investigation was to study the behaviour of mycoplasms in tissue culture, which was necessary for the subsequent analysis of their interrelationships with viruses. The results of our work, as well as the data obtained by other investigators regarding the manner in which mycoplasms behave in tissue cultures, indicate that mycoplasms may be divided into two groups.

The first group consists of mycoplasms which exhibit cytopathic activity, produce destruction of cell plasts and changes in cell metabolism, and actively reproduce in tissue. To this group belong Nelson's catarrhal-type mycoplasm [12], Shepard-type M. hominis [18], M. Gallisepticum [4] and M. laidlawii. The last was isolated by Laidlaw and Elford from sewer water and identified as a saprophyte. According to our data [3], this view should be revised, since M. laidlawii exhibits a marked cytopathic effect with respect to chick embryo fibroblasts and actively reproduces in that culture. A comparison of the nature of the cytopathic effect of mycoplasm species such as M. laidlawii and M. agalactiae with that of certain viruses reveals the similarity of the cytodestructive changes which they produce (see illustration). They all disrupt the integrity of the monolayer and produce vacuolization and granularity in the cellular cytoplasm and pycnosis of the nucleus. The existence of a definite similarity in the picture of the cytopathic lesions produced by mycoplasms and viruses may naturally be a source of error in analyzing the results of virological study. For this reason identification of virus-containing material only by its cytopathic effect is a quite risky procedure, particularly in view of the high seminability of tissues by mycoplasms.

The second group includes mycoplasms not exhibiting a visible cytopathic effect. The results of our experiments indicate that to this group may be assigned mycoplasm contaminants of NEr-2, SOTs and KV tissue cultures, which are able to reproduce in chick embryo fibroblasts and in the tissues of pig embryo kidney, cow embryo, and rhesus monkey kidney; i.e., they stimulate latent mycoplasm infections of tissue cultures. Other species of mycoplasms can also be assigned to this group, such as M. pneumoniae, which reproduces without cytopathic effect in the bronchial epithelium of chick embryos and in guniea pig, monkey and human kidney tissues which have first been trypsinized [5, 6]. We observed active reproduction of M. hominis 1 and M. salivarium in chick embryo fibroblasts and in cow embryo kidney tissue without marked cytopathic effect. These species also belong to the second group of mycoplasms.

In studying the effect of representatives of the two mycoplasm

groups on the reproduction of VEE and EEE arboviruses and NDV paramyxovirus (table 1), certain species of mycoplasms were found to exercise a selective inhibitory effect. Thus, M. laidlawii (first mycoplasm group) exhibited a sharply marked inhibitory effect with respect to all the viruses tested. M. agalactiae, which exhibited a less marked cytopathic effect but actively reproduced in tissue, did not differ from M. laidlawii in terms of its inhibitory effect on virus reproduction.



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Cytopathic effect of mycoplasms and VEE virus after infection of chick embryo fibroblasts

a -- uninfected cells after two days of culturing (X 106); b -- 36 hours after infection with M. laidlawii; cell destruction, plast discharge, X 106; c -- 72 hours after infection with M. agalactiae; plast discharge, appearance of "windows" in the monolayer (X 106); d -- 24 hours after infection with VEE virus; total cell destruction (X 280). Stained with hematoxylin-eosin.

Mycoplasms of the second group had various effects on the viruses tested. Thus, M. salivarium, which did not have a cytopathic effect but reproduced in tissue, did not significantly suppress reproduction of any of the three viruses (VEE, EEE and NDV). M. hominis 1 exhibited a marked inhibitory effect with respect to the VEE and NDV viruses and did not suppress reproduction of EEE virus. Mycoplasms contaminating SOTs and NEr-2 tissues had a weak inhibitory effect on the reproduction of VEE, EEE and NDV viruses, while mycoplasm contaminants of HeLa and A-1 tissues possessed no inhibitory capability with respect to the same viruses.

Thus, the results of these experiments established the selective inhibitory effect of various species of mycoplasms on the reproduction of certain RNA-containing viruses.

Table 1

Effect of Mycoplasms on Virus Reproduction and Plaque Formation

Mycoplasm reproduction	Length of treatment (in days)	Mycoplasm reproduction	Cytopathic effect of mycoplasms	Percentage suppression of virus titer		Percentage suppression of plaque-forming particles	
				VEE	EEE	VEE	NDV
M. laidlawii	1	+	+	50	48-82	42-60	32
	2	+	+++	99,9	78-95	NP	NP
	3	+	-	NP	NP	NP	NP
M. agalactiae	1	+	-	52-97	20	59	21
	2	+	±	84	98	80-86	42
	3	+	+	NP	99,9	90-98	73
M. hominis 1	1	+	-	NP	0	0	21-38
	2	+	-	NP	0	72-88	51
	3	+	±	NP	1	96	80-84
M. salivarium	1	+	-	0	0	NP	NP
	2	+	-	0	0	0	0
	3	+	±	62	48	28-63	54
M. from SOTs	1	+	-	0	0	NP	NP
	2	+	-	0	0	0	0
	3	±	+	25-37	10-42	0	20
M. from NEr-2	2	+	-	0	0	0	0
	3	+	-	20-32	28	0-18	0
M. from HeLa	1	+	-	0	0	0	0
	2	+	-	0	0	0	0
	3	+	-	0	0	0	0
M. from A-1	2	+	-	0	0	0	0
	3	+	-	0	0	0	0

Legend: ±, +, +++ = various degrees of cytopathic effect; - = absence of cytopathic effect; NP = experiment not performed.

Percentage suppression of plaque formation and virus reproduction is computed in each experiment by comparison with the control (cells not infected by mycoplasms).

Data regarding the effect of mycoplasms on interferon production in mixed mycoplasm-virus infection are given in table 2.

Table 2

Stimulation of Interferon Production by Cells Previously Infected by Various Species of Mycoplasms[1]

Manner of treatment	Length of contact[2] (in days)	Interferon titers[3] 1:2	1:5	1:10	1:30	1:40	1:80	1:160	1:320
EEE Virus	1	+++	+++	+++	++	-	-	-	-
	2	+++	+++	+++	+	-	-	-	-
	3	+++	+++	++	-	-	-	-	-
M. laidlawii	1-3	-	-	-	-	-	-	-	-
M. laidlawii + EEE	1	+++	+++	+++	+++	++	+	-	-
	2	+++	++	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
M. agalactiae	1-3	+	-	-	-	-	-	-	-
M. agalactiae + EEE	1	+++	+++	+++	+++	+++	+++	-	-
	2	+++	+++	+++	+++	+++	-	-	-
	3	+++	+++	+	+	-	-	-	-
M. from KV	1-3	+	-	-	-	-	-	-	-
M. from KV + EEE	1	+++	+++	+++	+++	+++	+	-	-
	2	+++	+++	+++	+++	+	-	-	-
	3	+++	++	++	-	-	-	-	-
M. from HeLa	1-3	-	-	-	-	-	-	-	-
M. from HeLa + EEE	1	+++	+++	+++	+++	+++	++	+	-
	2	+++	+++	+++	+++	+++	+	-	-

[1] Mean data are given from 3 repeated experiments in each medium.

[2] After contact between chick embryo fibroblasts and mycoplasms, the cells were infected with EEE virus.

[3] Interferon titers were determined as percentage suppression of plaque-formation in comparison with the control: below 25% -; 25-50% +; 51-75% ++; 76-100% +++.

As table 2 shows, the mycoplasm strains investigated did not in practice induce interferon production. At the same time, interferon formation was not noted when cells were infected with EEE virus. As the culture aged, a certain reduction was observed in the interferon-forming activity of EEE virus.

Prior treatment of cells with mycoplasms stimulated interferon production by a factor of approximately 4. This stimulating effect was particularly manifest when mycoplasms were introduced 24 hours prior to infection of the cells. Increasing the period of contact between cells and mycoplasms regularly led to a gradual decrease in interferon titers. This was especially marked with respect to M. laidlawii (first group), which causes alsmost complete destruction of the cell culture 72 hours after it is introduced.

The phenomenon we observed is similar to the method described by Mandy and Ho [10] of double contact of cells with the whole inactivated virus. The existence of synergism in the action of mycoplasms and viruses may be postulated; however, the nature of this phenomenon has not yet been explained.

Comparison of the data presented in tables 1 and 2 permits us to conclude that an inverse relationship exists between the degree of interferon production and the intensity of cytopathic effect of the mycoplasms.

Discussion

Study of the behaviour of mycoplasms in tissue culture permitted us to divide them into two groups. The first group includes mycoplasms which have a cytopathic effect, causing cell destruction similar in some features to the cytopathic effect of viruses. Representatives of this group are Nelson-type catarrhal mycoplasm, Shepard-type M. hominis, M. Gallisepticum and, as we have shown, M. laidlawii and M. agalactiae. Along with the cytopathic effect, intensive reproduction of these mycoplasms and marked disruption of cell metabolism are noted. The second group of conditionally pathogenic mycoplasms includes mycoplasms which exhibit no cytopathic effect but actively reproduce in tissue. The absence of manifestations of infection enables us to view this as a group of latent mycoplasm-infections. Representatives of this group, according to our data, are M. hominis 1, M. salivarium and mycoplasm contaminants isolated from HeLa, NEr-2, SOTs, KV and other tissue cultures.

In analyzing the associative interrelationships between mycoplasms and viruses, two completely different phenomena are noted: an increase in the pathogenic effect of mycoplasms and the suppression of virus reproduction by mycoplasms.

Conclusions

Our investigations demonstrated the presence of a selective inhibitory effect of mycoplasms on the reproduction of viruses, depending on the species to which the former and latter belonged.

The inhibitory effect of mycoplasms may be caused by: 1) the cytopathic effect of mycoplasms (first group); 2) acidification of the medium, leading to rapid inactivation of the viruses; 3) competition for various components in the medium (amino acids, nucleotides, growth factors) essential both for development of mycoplasms and for fully active reproduction of viruses; 4) changes in cell metabolism; 5) production of macromolecular substances such as Kraemer's exotoxin; 6) stimulation of the production of interferon, which in its turn suppresses virus reproduction.

It is interesting to note that interferon production is increased by cells infected with group A arboviruses after previous treatment with first- and second-group mycoplasms. It is important to emphasize that mycoplasms by themselves did not induce interferon synthesis. This phenomenon may apparently lie at the basis of latent viral infections in tissues chronically infected with conditionally pathogenic species of mycoplasms, which, as we have shown, promote the activation of interferon formation.

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