Microbiology

THE POSSIBLE ROLE OF ANIMAL VIRUSES IN THE PATHOGENESIS OF MYCOPLASMA

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The stable and irreversible complexes formed *in vitro* between the cells of mycoplasma (DP and F_5) and Ranikhet disease virus (RDV) were found to be actively multiplying. The genome of the cell associated virus particles seemed to initiate sequence of events resulting either in the synthesis of mature viral progeny or appearance of other biological changes. As only one infective unit (ID_{50}) of the virus could be recovered from 4.5×10^4 to 9×10^5 Colony Forming Units (CFU) of such complexes it seems that the majority of cells become transfected. The progeny of RDV transfected mycoplasma cells could unlike the original mycoplasma cultures, induce specific pathogenic lesions in chick embryos, and chicken. The plants of sugarcane, maize, paddy and sorghum grown from the embryos (seedlings) infected with RDV translected mycoplasma DP showed more pronounced disease syndromes of mycoplasma-like infections than those infected by the original DP strain. The cells of the monolayers of chick embryo fibroblasts (CEF) infected by mycoplasma strains showed marked decrease in the rate of cell division and the pattern of growth was disorganised. This may explain the association of stunted growths or the appearance of various deformities both in animals and plants infected by these organisms.

INTRODUCTION

It has been suggested (Nelson 1950; Lench and Bulture 1966: Klienberger-Noble 1967; Nakamura and Hiroaki 1969; Ranck *et al.* 1970) that animal viruses may have some synergestic effect for the activation of virulence of apparently avirulent or latents infections of mycoplasma particularly of murine, human, and animal origin. In chronic respiratory disease (CRD) of poultry, mycoplasma appears to be the main causative agent, but the etiological implication of some unidentified viruses (possibly of myxovirus group) has not been ruled out (Lench and Bulture 1966; Fahey and Crawley 1954; Fahey 1956). The possible role of mycoplasma as the carrier of IB and CELO viruses in some outbreaks of respiratory diseases of poultry or the association of some viruses in mycoplasmal arthritis of chicken has also been suggested (Cummings 1969; Kerr *et al.* 1967). In cell cultures the presence of some strains of mycoplasma may activate the growth of some viruses or inhibit the replication of others (Gafford *et al.* 1969; Kagar *et al.* 1969). These observations clearly suggest the possibility of virus-mycoplasma interaction *in vivo*, but the nature of the reaction and its effect, if any, on the biological properties of the mycoplasma have not been studied. It has also been shown (Babbar 1968) that bacterial (*E. coli*) spheroplasts could be transfected by the genome (DNA/RNA) of at least

two animal viruses. Further progeny of the protista cells transfected by the genome of Ranikhet disease virus possessed new biological properties, including activation of virulence (Babbar *et al.* 1972). Therefore the mycoplasma cells, which resemble the spheroplasts of protista cells but otherwise are more closer to animal cells, may be more susceptible to transfection by animal viruses. This may occur *in vivo* in nature because different types of animal viruses have also been observed (Murphy *et al.* 1967) in the animal tissues from where these organisms are usually isolated. There are recent reports (Maramorosch *et al.* 1968; Hirumi and Maramorosch 1969; Sinha and Paliwal 1970) that a number of plant diseases, attributed to mycoplasma-like organisms, could only be transmitted after their passage through arthropod vectors, which are known to transmit or act as the carriers of plant or animal viruses. This suggests the possibility of virus-mycoplasma interaction in arthropods to activate their virulence. Therefore transfection of mycoplasma by an animal virus may induce a varying degree of malignancy in them. This phenomenon has not been investigated. This possibility was studied by allowing the two well characterized avian mycoplasma DP and F_5 (Babbar and Deo 1974), to interact *in vitro* with Ranikhet disease virus (RDV), a well-known avian pathogen, and the progeny of the transfected mycoplasma cells was tested for their ability to induce disease syndromes in different susceptible hosts (Babbar *et al.* 1973).

MATERIALS AND METHODS

The biological and the serological characters of the pure cultures of two avian strains of mycoplasma (DP and F5) and the procedure for preparing various media including PPLO nutrient broth (NP broth) and to obtain the concentrated suspensions and the lysates of these mycoplasma cells have been described in detail previously (Babbar and Verma 1974). The procedures to assay the virulence of the mycoplasma cultures in 9 days old chick embryos and in 3 to 4 days old plant embryos (seedlings of maize, sugarcane, paddy and sorghum) have also been described (Babbar and Varma 1974; Babbar *et al.* 1972 and 1973). To judge the cytopathic activity of mycoplasma suspensions in cell culture, 18 hr-old chick embryo fibroblast (CEF) monolayers were prepared by the method of Babbar *et al.* (1970), washed and then incubated at 37°C after adding maintenance medium (Babbar *et al.* 1970), to give titers of about 3 to 4×10^7 CFU/ml of these organisms. The monolayers were examined for cytopathic or any other change either in the morphology or in the rate and pattern of the growth of the cells.

To test the ability of a mycoplasma culture to induce some type of respiratory disease syndrome in birds, 0.2 ml of mycoplasma suspension, having 3 to 4×10^8 CFU/ml, was given intranasally to each of one day old chicks (white leghorn) obtained from mycoplasma free stocks.

The strain of Ranikhet disease virus used (RDV, a virus of myxovirus group) and methods of its purification, and calculation of its infective (ID_{50}/ml) and neutralization titres have been detailed earlier (Babbar *et al.* 1970).

For the transfection of the mycoplasma strains DP and F_5 by the viral genome, mycoplasma suspensions (in early exponential growth phase, 18 hr growth cultures) having 0.3 to 1.5×10^8CFU/ml and purified RDV (100 to 400 ID_{50}/ml the final

concentrations) were allowed to react at 37°C for 3 hr as described earlier (Babbar *et al.* 1972).

RESULTS

The suspensions of the stable and irreversible virus-mycoplasma complexes, formed *in vitro*, were thoroughly washed, re-suspended in NC broth and incubated at 37°C. Aliquots were drawn after different periods of incubation and tested for the concentrations of the viable mycoplasma cells as CFU/ml and of the infective virus particles as ID_{50}/ml. Each experiment was repeated 4 times. It was found that the mycoplasma cells of the complexes were actively multiplying up to 36 hr (Fig. 1) while the titre of infective units recovered from these complexes, fell sharply and either disappeared completely or reached the lowest level within 12 hr (Fig. 2). The RNase sensitive infective units, however, reappeared in the cell lysates made after 18 hr of incubation. On further incubation these units became RNase resistant and their titre increased gradually and reached the maximum level after 36 hr. The infective units then recovered, could be neutralized by anti-RDV sera, thus suggesting the formation of mature viral particles (Fig. 2). There was again a fall in the yield of infective units when these complexes were incubated beyond 36 hr. This was possibly due to the fall in the viability of the mycoplasma cells (Fig. 1). The sequence of the events described above, appeared to suggest that the genome of cell associated RDV was able to react with the metabolism of the mycoplasma cells so as to initiate the synthesis of viral components. This could only happen if the viral genome was closely associated with the cytoplasm of the mycoplasma cells. On calculation it was found that one infective unit (ID_{50}) could be recovered from 4.5×10^4 to 9.0×10^5 CFU of these complexes (Table I). This suggested that the

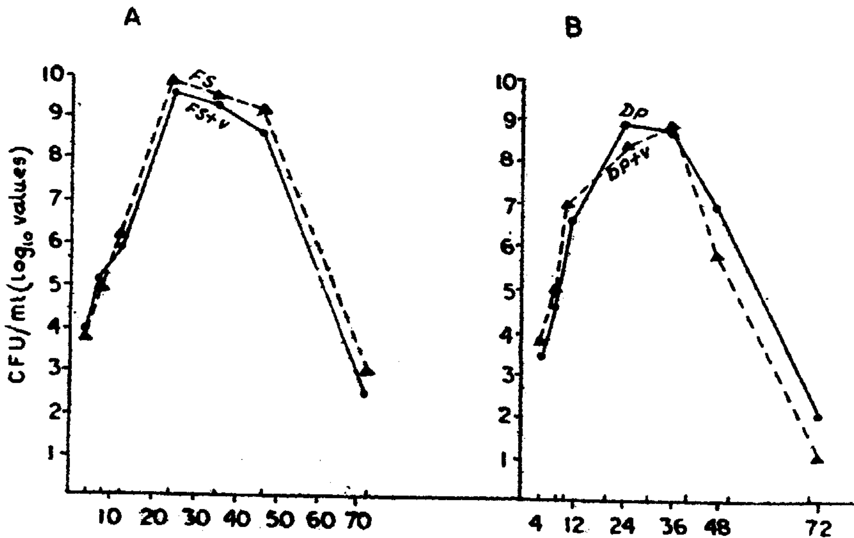

FIG. 1. Comparison of the growth curves of normal and virus transfected mycoplasma strains DP and F_5.

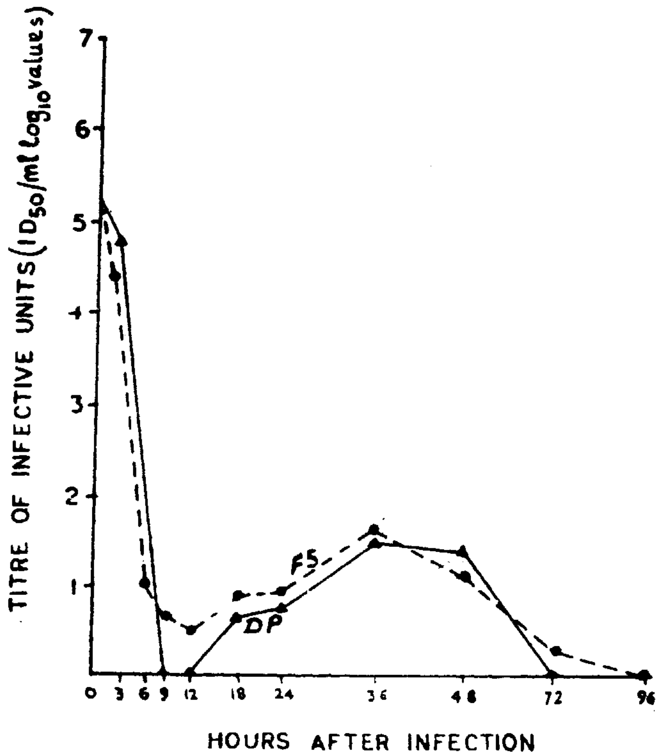

FIG. 2. The titres of the infective units (ID_{50}/ml) recovered from the suspensions of virus mycoplasma complexes incubated at 37°C for different periods. The infective units recovered between 18 to 24 hr of incubation could be destroyed by RNase (5 μg/ml for 20 min at 26°C). The infective units recovered after 36 hr. were resistant to this treatment but could be neutralized when treated *in vitro* for 3 hr at 37°C and 18 hr at 4°C with antisera for RDV.

TABLE I

Recovery of one infective unit from the RDV-mycoplasma complexes when incubated for different periods*

Mycoplasma isolates	Number of CFU of RDV-mycoplasma complexes releasing one infective unit (ID_{50}) of the virus after	
	0 hr	36 hr
DP	2 to 3 × 10^2	4.5 to 6.5 × 10^4
	3 to 4 × 10^2	5.5 to 8.5 × 10^5
F_5	1 to 4 × 10^2	7.0 to 9.0 × 10^5
	6 to 7 × 10^2	4.6 to 9.0 × 10^5

CFU = colony forming units
*Complexes formed after 3 hr at 37°C

genome of RDV could not initiate synthesis of viral components in most of the mycoplasma cells.

The virus-mycoplasma complexes were subcultured serially at 36 hr intervals. The resultant cell populations or their lysates were tested for their ability to induce lesions in chick embryos, (18 embryos/sample/experiment), as such or after their neutralization with anti-RDV (Babbar *et al.* 1970) anti-DP or anti-F_5 sera (Babbar and Varma 1974). The results of 4 such experiments (Table II) clearly showed that both cell suspensions or their lysates could produce mortality, haemorrhagic lesions, besides various types of deformities in chick embryos. No such property could be detected in controls. It was also found (Table III) that the lesions inducing properties of the suspensions or lysates of these RDV transfected mycoplasma cells could not be neutralized by anti-RDV or anti-mycoplasma (DP and F_5) sera and that such lesions occurred in normal chick embryos with frequencies varying from 0.006 to 0.12% (Table II). These observations seem to suggest that the various types of lesions described above were induced by

TABLE II

Appearance of lesions in chick embryos infected with IInd generation progeny of the mycoplasma cells transfected with RDV

Mycoplasma strain			% of embryos showing pathological lesions				
			M	UD	Hb/B	Ag	Ab
DP	CS	M + V	*58.33/84	25.5	**12.5(+4)	0	0
		M	0	0	0	0	0
	CL	M + V	51/72	16.6	51.0(+)	0	0
		M	0	0	0	0	0
F_5	CS	M + V	66.6/84	36.4	50(+2)	11.6	0
		M	0	0	0	0	0
	CL	M + V	66	22.2	20(+4)	0	0
		M	0	0	0	0	0

Not : 1. 1600 normal embryos were elected at random and examined for presence of lesions, showed UD=0.006% and Ab=0.012%.

2. Ranikhet disease virus, brought about 100% mortality of the infected embryos and the average death period was 42 hr.

M = % mortality; Hb/B haemorrhagic lesions and body and yolk sac ; *Ag* = aedematous outgrowth; *UD* = under developed or deformed embryos; *Ab* = abdomen open ; CS = The washed cell suspensions, having 5 to 6 × 10^8 CFU/ml. *CL* = The lysates of 2×9 10^9 cells in 2 ml of distilled water; M+V = Virus transfected mycoplasma DP or F_5, *M* = Uninfected mycoplasma DP or F_5

* = % mortality/mean death period (hr)

** = % of embryos showing lesions and average lesion scores within bracket.

TABLE III

Inability of the specific anti-RDV and anti-mycoplasma sera to neutralize lesions inducing property of the cells of the subcultures of RDV complexes with mycoplasma cultures DP and F_5

The cells of 2nd sub-culture of complexes of RDV with strain of mycoplasma	*In vitro*		% of embryos showing pathogenic lesions		
			M	UD	Hb/B
DP	Controls		63.2	2.2	*66/+4
	Neutralized with antiserum of	RDV	66.6	33.4	66/+4
		DP	66.6	33.4	66/+4
F_5	Controls		66.6	33.4	50(+2)
	Neutralized with antiserum of	RDV	66.6	33.4	66.6(+2)
		F_5	66.6	33.4	66.6(+2)

M = % mortality of chick embryos.
UD = under developed embryos.
Hb/B = Haemorrhagic lesions on the body and yolk sac of embryos

$\frac{\text{\% of embryos showing lesions}}{\text{* \% lesions scores}}$

RDV transfected mycoplasma cultures. The results of the infection of various plant embryos with normal or RDV transected mycoplasma cultures are given in Table V. It was found that both normal as well as RDV transfected mycoplasma cultures of DP induced various disease syndromes attributed to the infection by mycoplasma-like organisms (Sinha and Paliwal 1970) but RDV transfected mycoplasma culture caused either higher mortality as in sugarcane embryos or induced more acute disease as in maize, paddy and sorghum.

The results of 4 experiments of CEF monolayers infected with the suspensions of normal or RDV transfected mycoplasma cells, using 6 monolayers/suspension showed (Table IV) that the extent of the cytopathic changes measured as % cytopathic score (% CPS) (Babbar *et al.* 1970) was very low. The cells of the remaining portion of the monolayers were apparently of the normal appearance for 7 days when they became rounded and peeled off. The monolayers infected with RDV had the % CPS of 50 to 70 within next 24 hr. The monolayers infected with 1st and the 2nd sub-cultures of the RDV transfected mycoplasma had the maximum % CPS of 25 to 30 within 24 to 36 hr. This was followed by the period of 21 days when the cells remained normal and viable but there was a marked decrease in the rate and pattern of cellular division which also became disorganised. It was, however, not possible to have the secondary cultures of these cells.

The normal or RDV transfected mycoplasma cultures (DP) (maintained serially in chick embryos up to 30 generations) were tested for their ability to induce respiratory disease syndromes in one day old chick, using 50 chicks/experiment. It was found that 6 % of the infected chicks showed stunted growth while 15 % of them developed syndrome resembling chronic respiratory disease

TABLE IV

Effects of the subculture of the complexes of RDV with mycoplasma cultures on the monolayers of embryo fibroblasts (CEF)

Mycoplasma isolates	Effect on CEF monolayers	
	Cytopathetic changes (CPS)	Effect on the morphology and growth of cells in growth medium at 37°C
DP (Normal)	10–15 (24 to 36 hr)	Cells remained normal up to 7 days at 37°C and became rounded and peeled off.
DP RDV IG complexes IIG	25–30 (24 to 36 hr)	Cells remained normal up to 21 days and areas of random growth appeared on 6th day and increased up to 21 days.
RDV	50 to 75 (24 to 36 hr)	Cells degenerated within 72 hr and peeled off.
Normal CEF	monolayers	Cells grew rapidly and peeled off within 96 hr.

CPS = % of cytopathetic score (Babbar *et al.* 1970).

TABLE V

*Virulence of the normal and RDV transfected mycoplasma for different hosts***

Host	Mycoplasma	% mortality of the host	% showing different diseased symptoms			
			CRD-like	Stunted growth	Deformities†	Other changes‡
Day old chicks	M	0	0	0	0	0
	M + V	5	15	6		
4–5 days old plant embryos(seedlings) of sugarcane	M	45		60	3	30
	M + V	80		50	12.5	35
Maize	M	10.5		34.5	0	76
	M + V	12.5		23.6*	0	65*
Paddy	M	45		25	0	25
	M + V	50		33*	0	33
Sorghum	M	25		42	0	0
	M + V	33		25*	0	12*

CRD-like = Symptom identical to CRD
M = mycoplasma strain DP
M+V = mycoplasma strain DP transfected with RDV
*The stunted growth, deformities and other changes were more acute or pronounced
**Average of six experiments, using 50 embryos of each type/experiment
†Tumour formations or distorted growths
‡ Changes in the colour of foliage or abortive cab formaton

(CRD) (Table V) of fowls. The normal mycoplasma strains DP or RDV or alone failed to induce these types of syndromes.

Discussion

The results suggest that the mycoplasma cells transfected with genome of RDV may have been transformed into virulent cells, which could induce some typical disease syndromes attributed to these organisms in chick embryos and chicks (Babbar and Varma 1972, 1974; Fahey 1956; Fahey and Crawkey 1954). In plants induction of syndromes usually described to be specific to the infections of mycoplasma-like organisms were more acute when infected by RDV transfected DP strain of mycoplasma.

Interaction of mycoplasma with some of the animal viruses may be possible *in vivo*, in the tissues or the organs where both of the microbes have been shown to be present simultaneously (Murphy *et al.* 1967). Such interaction may result in the activation of the virulence in otherwise known avirulent mycoplasma or those causing latent infection only. Such activation may be of temporary or abortive nature, as the transfecting genome (Spizizen *et al.* 1966) would not be able to integrate into its genetic system and may, therefore, disappear when sub-cultured in artificial media. Therefore, the direct or indirect association of the mycoplasma with a number of diseases of unknown etiology may be explained on the possible activation of their pathogenic potentials by some animal virus which happens to transfect them *in vivo*. This may also help to understand the pathogenesis and epidemiology of the various diseases attributed to mycoplasma or mycoplasma-like organisms. The ability of mycoplasma cultures DP and F_5 to bring about a marked disorganisation of the pattern of growth of the chick embryo fibroblast cells in monolayers suggest that the factor(s) associated with virulence act at embroynic stage resulting in the appearance of various types of deformities

It has been recently reported that some animal viruses may transfect other protista and induce new biological properties in them (Babbar *et al.* 1972; Babbar and Varma 1972). This suggest the new role of animal viruses in determining the biological behaviour of the microbial population of a host and the part they may play in initiating the various types of disease syndromes of unknown etiology. This aspect of the pathogenesis of animal viruses is new and unexplored and mycoplasma, which lack cell wall and intracytoplasmic membranes may be highly susceptible to transfection by various viruses.

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