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Blocking of the Virus-Enhancing Activity of 5-Methyl-2-D-ribobenzimidazole (MRB)[1] (37811)

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5-Methyl-2-D-ribobenzimidazole (MRB) restores the ability of the chorioallantoic membrane from older chicken embryos to produce, *in vitro,* large amounts of influenza B (Lee) virus after a small inoculum (1, 2). To obtain a maximal effect, prolonged and continued treatment of the chorioallantoic membrane with MRB is necessary. MRB does not appear to act through the interferon mechanism, but rather by blocking another restrictive mechanism, which becomes operative during embryological development and does not require induction. As cyclic AMP has been shown to play a broad role in the regulation of cell metabolism, morphology, and growth (3), it was of great interest to determine its effect on influenza virus multiplication in control and MRB-treated tissues.

Materials and Methods. 5-Methyl-2-D-ribobenzimidazole (MRB), mol wt 252.3, was obtained through the courtesy of Merck, Sharp and Dohme Research Laboratories, Rahway, NJ. N^6,$O^{2'}$-Dibutyryl adenosine 3′:5′-cyclic monophosphoric acid, monosodium salt (db-cAMP), mol wt 491.3; adenosine 3′:5′-cyclic monophosphoric acid, crystalline (cAMP), mol wt 329.2; and adenosine 2′:3′-cyclic monophosphoric acid, sodium salt (2′:3′-cAMP), mol wt 351.4, were purchased from Sigma Chemical Co., St. Louis, Missouri. Theophylline, mol wt 180.2, was also obtained from Sigma. Influenza B virus, Lee strain, was prepared and stored as described previously (1). Fertilized chicken eggs were purchased from Shamrock Farms, North Brunswick, NJ.

Suspension cultures of chorioallantoic membrane from 13-day-old embryonated chicken eggs (1, 2) were prepared from groups of 6 eggs, randomly selected. From the chorioallantoic membrane of each egg eight 1.8 cm^2 portions were punched out and distributed among 4 cultures as follows: control; MRB (3.5 m*M*); db-cAMP (1 m*M*); and MRB (3.5 m*M*) + db-cAMP (1 m*M*). As set up, each culture contained 3.8 cm^2 of membrane in 2.0 ml of medium (1) and 6×10^4 EID_{50} of Lee virus as the inoculum. After incubation at 35° for 41 hr (1, 2), the medium from each culture was collected and virus yield was determined by hemagglutination titration using a modified fractional dilution procedure in which each sample was assayed in triplicate. The yields were expressed in hemagglutinating units per milliliter of medium. The index of inhibition of virus yield in a db-cAMP-treated culture was a decrease of at least 0.2 log unit compared to the yield in the corresponding untreated control culture. The index of enhancement of yield in an MRB + db-cAMP-treated culture was an increase of at least 0.2 log unit over the yield in the corresponding db-cAMP-treated culture. The experiment outlined above was carried out 4 times with chorioallantoic membranes from a total of 24 embryonated eggs. As the reproducibility of virus yields among portions of membrane from the same egg is high (1, 4), this experimental design permits reliable determination of the effects of MRB and db-cAMP in membranes from individual chicken embryos. The factor of

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host variation can be fully documented and taken into account. As the embryonated eggs came from a random breed flock of white leghorn hens, host heterogeneity is known to be marked (1, 4).

In some experiments, each culture contained twelve 1.8 cm^2 pieces of membrane from 12 different eggs, and 9 such equivalent cultures were set up in each experiment (2). In such experiments the results reflect

TABLE I. The Effects of Dibutyryl-cyclic AMP (db-cAMP) and 5-Methyl-2-D-ribobenzimidazole (MRB) on Influenza B (Lee) Virus Multiplication in Chorioallantoic Membranes from Individual Embryonated Chicken Eggs.

	Yield of virus in chorioallantoic membrane *in vitro* (HAU/ml)			
Egg no.	Control	MRB (3.5 m*M*)	db-cAMP (1 m*M*)	MRB (3.5 m*M*) + db-cAMP (1 m*M*)
A. db-cAMP inhibits, MRB enhances in presence of db-cAMP				
1	13	63	7.9	28
3	50	100	32	63
4	32	130	13	20
7	20	25	5.0	13
9	10	79	3.2	16
13	50	140	25	100
14	13	89	6.3	20
15	63	200	20	63
16	40	160	3.2	25
19	63	130	20	50
23	22	63	3.2	7.9
Mean[a]	28	93	8.9	28
% of control	100	330	32	100
B. db-cAMP inhibits, MRB does *not* enhance in presence of db-cAMP				
10	13	40	7.9	6.3
11	40	50	20	16
17	25	160	10	7.9
18	160	320	63	32
Mean[a]	36	100	18	13
% of control	100	280	50	36
C. db-cAMP does *not* inhibit, MRB enhances in presence of db-cAMP				
2	10	63	10	25
5	7.9	50	7.1	13
6	7.9	100	10	63
21	10	79	10	25
24	13	110	16	32
Mean[a]	9.6	78	10	28
% of control	100	810	100	290
D. db-cAMP does *not* inhibit, MRB does *not* enhance in presence of db-cAMP				
8	16	50	16	20
12	50	100	40	40
20	3.2	63	5.0	6.3
22	40	160	40	25
Mean[a]	18	85	19	19
% of control	100	470	110	110

[a] Geometric mean.

the mean response of a randomly selected group of embryos.

Results. Effects of MRB and db-cAMP. As expected, the chorioallantoic membranes from individual chicken embryos showed marked variation in their responses to the effects of MRB and db-cAMP on Lee virus multiplication. In Table I the results obtained are grouped into 4 groups according to whether db-cAMP inhibited (groups A and B) or did not inhibit (groups C and D) Lee virus yield, and whether MRB enhanced (groups A and C) or did not enhance (groups B and D) in the presence of db-cAMP. No systematic variation was found from experiment to experiment, and therefore, membranes from the 24 embryonated eggs were treated as one population. The dates of the experiments were as follows: eggs 1–6: 2/22/72; eggs 7–12, 2/29/72; eggs 13–18, 4/11/72; and eggs 19–24, 5/2/72.

As illustrated in Fig. 1, percentage increase in virus yield in MRB-treated cultures is inversely related to the yield in control cultures (1, 4). Comparison of the mean yields in control and in MRB-treated cultures in groups A, B, C, and D in Table I shows that MRB made possible the production of an additional mean amount of 65 HAU of virus.

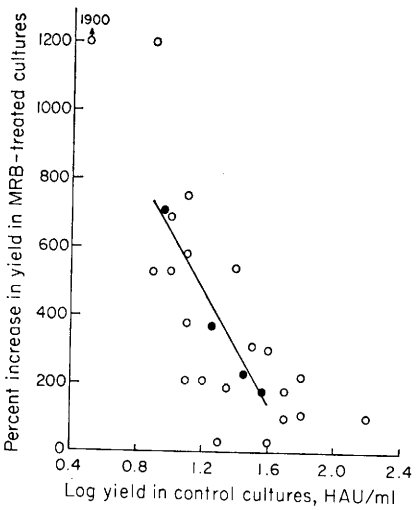

FIG. 1. Relationship between yield of Lee virus in control cultures and percentage increase in the presence of 3.5 m*M* MRB. (○) Results with chorioallantoic membranes from individual chicken embryos; (●) mean results (see Table I).

The mean inhibitory effect of db-cAMP on Lee virus yield is best illustrated by pooling the results in groups A and B, and in groups C and D. Table II shows that in membranes from the 15 eggs in which db-cAMP had an inhibitory effect, the mean yield in treated cultures was 14 HAU/ml, equivalent to 41% of that in controls from the same group of eggs. This reduced yield is closely similar to the mean yields in control or db-cAMP-treated cultures derived from the 9 eggs in whose chorioallantoic membranes db-cAMP did not inhibit Lee virus multiplication. These results suggest that db-cAMP inhibits the production of virus above a certain level, but has no effect on virus production below that level. The level to which db-cAMP restricts multiplication varies among chicken embryos.

db-cAMP markedly limits enhancement of Lee virus yield by MRB. As shown in Table I, regardless of whether the yield is reduced by db-cAMP alone, in its presence MRB shows only partial enhancing action (groups A and C) or no enhancing action at all (groups B and D).

Effects of cAMP and 2′:3′-cAMP. Table III shows that cAMP and 2′:3′-cAMP also inhibited influenza virus multiplication to a moderate extent. Thus the virus-inhibitory effects of db-cAMP cannot be considered as related to the known specific actions of cyclic AMP.

Effects of enriched media. Table IV shows that Eagle's spinner medium (5), while not itself affecting virus yield (6), markedly reduced the enhancing effect of 3.5 m*M* MRB. This effect was dependent on the total concentration of spinner medium ingredients, and could be reproduced with a solution of spinner salts and glucose alone, provided that $NaHCO_3$ was included. Eagle's MEM (1959) also did not itself affect virus yield, however, enhancement by MRB was somewhat reduced in MEM as well as in the MEM salts and glucose solution. Blocking of the enhancing effect of MRB was not related to pH, which was maintained between 7.1 and 7.3, or to the presence or absence of $CaCl_2$ per se. Rather, it appears that the

TABLE II. The Mean Effects of db-cAMP and MRB on Lee Virus Multiplication in the Chorioallantoic Membrane.

Mean yield[a]	Yield of virus (HAU/ml)			
	Control	MRB	db-cAMP	MRB +db-cAMP
Groups A and B	32	98	13	19
Groups C and D	13	81	14	23
All embryonated eggs	22	91	12	23
% of control	100	410	55	105

[a] See Table I for definition of groups. Geometric mean yields are given.

markedly reduced enhancing effect of MRB in spinner medium is referable to the presence of $NaHCO_3$ (2 g/liter) in the milieu of this medium, which includes a relatively high concentration of $NaH_2PO_4 \cdot H_2O$ (1.5 g/liter). No serum was used in any of the media.

Discussion. In membranes from 13-day-old embryonated eggs virus yield is limited to a varying extent by an endogenous restrictive mechanism, which MRB is capable of overcoming. Virus yield can be further reduced by treatment of cells with double-stranded RNA, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), or cycloheximide, or by dextrose deprivation (2). MRB is capable of increasing virus yield even under these exogenously imposed restrictive conditions, but the yield does not reach as high levels under such conditions as it does when MRB alone is used to treat the chorioallantoic membrane. This would be expected if MRB does not directly reverse the effects of interferon inducers, inhibitors of RNA or protein synthesis or dextrose deprivation, but acts on an endogenous restrictive mechanism (2). Examination of the effects of MRB and db-cAMP in chorioallantoic membranes from individual embryonated eggs has shown that db-cAMP blocks partially or completely the enhancement of virus yield by MRB. By itself, 1 m*M* db-cAMP reduces virus yield in membranes from many, but not all chick embryos. The moderate virus-inhibitory activity of db-cAMP may not be related to any specific effects of cAMP as 2′:3′-cAMP shows similar activity. There is no basis for suggesting that db-cAMP limits and MRB increases virus yield through the same mechanism.

The effect of spinner medium is noteworthy in that virus yield in spinner medium or in spinner salts and glucose is closely similar to that in the simple control medium (1), yet MRB shows little enhancing activity in spinner medium. There are several possibilities involving the transport and activity of MRB, the state of the target for MRB, and the tissue capacity for production of additional quantities of virus in spinner me-

TABLE III. Effects of db-cAMP, cAMP, and 2′:3′-cAMP on Lee Virus Multiplication in the Chorioallantoic Membrane.

Egg no.	Yield of virus (HAU/ml)			
	Control	db-cAMP[a]	cAMP[a]	2′:3′-cAMP[a]
1	40	40	18	22
2	25	14	16	18
3	56	40	25	63
4	3.2	2.5	2	2
5	126	79	32	40
6	16	10	5	8
Geometric mean	25	18	11	16
% of control	100	72	44	64

[a] 1 m*M*.

TABLE IV. Effect of Spinner Medium on Enhancement of Lee Virus Multiplication by MRB.

Medium	Yield of virus (HAU/ml)[a]		
	No MRB	MRB (3.5 m*M*)	% Increase with MRB
Control	16	89	456
Spinner[b]	18	32	78

[a] Geometric mean yields from five experiments. In each of these experiments cultures contained portions of membrane from 12 eggs.

[b] Spinner medium components were present at a concentration equivalent to 90% of the concentration in spinner medium (5).

dium. Further experiments are needed to explain the present findings.

Summary. Dibutyryl-cyclic AMP (db-cAMP, 1 m*M*) limits the yield of influenza B (Lee) virus in the chorioallantoic membrane from 13-day-old embryonated chicken eggs. In membranes treated with 5-methyl-2-D-ribobenzimidazole (MRB, 3.5 m*M*), db-cAMP partially or completely blocks enhancement of virus yield by MRB. Eagle's spinner medium does not by itself affect virus yield compared to the yield in a simpler control medium, but it reduces the enhancing effect of MRB by more than 75%.

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