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Brief Communication

Localization of Mammary Tumor Virus inside Red Blood Cells of Mice[1]

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Summary

Mammary tumor virus activity in the red blood cells of mammary tumor virus-infected BALB/cfC3H mice was found to be localized intracellularly rather than to be adsorbed on the surfaces of red blood cells.

Introduction

In adult nontumor-bearing mice infected with MTV,[2] blood-borne viral activity has been shown to be associated primarily with cellular fractions (3, 7). However, in tumorous females, Bittner (3) observed similar MTV activity in both plasma and cellular fractions of blood. A few years ago, on the basis of our studies (13) of blood drawn from the tail veins of MTV-infected BALB/cfC3H (C^+) mice, we suggested that blood-borne MTV activity is primarily in the red blood cell fraction and only occasionally in plasma. Subsequent studies (10, 14, 15) prompted us to suggest that RBC's carry MTV activity and that this activity is not due to adsorption of virus onto the surfaces of RBC's. Differences observed in the biological behavior of R-MTV and M-MTV led us to suggest that R-MTV activity may not be due to B particles (10, 11).

In a recent report, Moore *et al.* (9) have observed that B particles can adsorb to RBC surfaces when milk from infected mice is centrifuged together with RBC's; 5 to 20% of RBC's showed B particles on their surfaces in electron micrographs. On the basis of these and other studies, the authors suggested that B particles may be the only infectious form of MTV, occurring in both blood and mammary tissues.

The present investigation was undertaken to determine whether MTV activity is inside or on the surface of the red blood cells and to test whether adsorption of B particles to the RBC surface could account for R-MTV activity of RBC's.

Experiments, Results, and Discussion

The source of M-MTV was MTV-infected lactating BALB/cfC3H (C^+) females; that of R-MTV was 6- to 8-week-old male or virgin female C^+ mice. High-speed pellets of MTV from lactating mammary tissues were prepared according to the method described previously (14). As controls, pellets of lactating mammary gland extracts were prepared in the same way from MTV-free BALB/c (C^-) mice. B particles used for the immunization of adult rabbits were prepared by isopycnic and rate-zonal sedimentation in sucrose gradients (8). Immunization methods for rabbits have been described previously (4) and antibodies against B particles were found to cause precipitation of intact B particles in immunodiffusion (4). R-MTV-carrying RBC's from C^+ mice and control RBC's from C^- mice were processed by methods described previously (11). Hemolysis of RBC's was always done with 10 mM $MgCl_2$ in distilled water, pH 7.4. The enzymes used were RDE (Microbiological Associates, Albany, Calif.), phospholipase C (Worthington Biochemical Corporation, Freehold, N. J.), and trypsin (Worthington). RDE was diluted with 0.85% NaCl solution; phospholipase and trypsin were diluted in $MgCl_2$ (5 mM). All enzymes were preincubated in a 37° water bath for 1 to 2 hr before use.

MTV activity of M-MTV and R-MTV preparations was detected by modified nodule assay (14) following inoculation of 0.05 to 0.1 ml equivalent of packed RBC's or 10^{-3} to 10^{-5} g equivalent of mammary tissue into 3-week-old C^- test mice.

In the 1st experiments, attempts were made to determine whether MTV activity could be eluted from R-MTV-carrying RBC's by means of RDE treatment. Intact C^+ RBC's in 0.85% NaCl solution were incubated with 1/6 volume of RDE (titer, 1:800) at 37° for 1 hr; control RBC's were incubated similarly in 0.85% NaCl solution. The mixture was centrifuged in a Sorvall refrigerated centrifuge at 150 × *g* for 10 min. The supernatant from the 1st washing and the RBC's that were washed 4 more times were injected separately into test mice. The results summarized in Table 1 show that all MTV activity was associated with the RBC fraction. Thus, unlike influenza virus particles that can be eluted from the RBC surface by RDE treatment (1), MTV activity cannot be eluted from R-MTV-carrying RBC's with the same enzyme.

In the 2nd experiment, attempts were made to adsorb M-MTV onto RBC's from MTV-free C^- mice. The incubation mixture consisted of 0.1 ml of 10^{-3} dilution of M-MTV, 1 ml of RBC's and 1.9 ml of 0.85% NaCl solution. The incubation was carried out at 37° for 1 hr or at 4° for 18 hr. After incubation, the RBC mixture was washed 6 times with large volumes of 0.85% NaCl solution. Test mice were given injections of washed RBC's and supernatants after the 1st and

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[2] The abbreviations used are: MTV, mammary tumor virus; R-MTV, red blood cell-borne MTV; M-MTV, mammary tissue-borne MTV; RDE, receptor-destroying enzyme.

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Table 1
Nodule incidence in C⁻ mice inoculated with RDE-treated RBC's from C⁺ mice

Control RBC's (1 hr at 37°)	RDE-treated RBC's	Supernatant from RDE-treated RBC's
80 (10)–28[a]	93 (14)–29	0 (19)–0

[a] Percentage of mice with nodules (no. of mice used) – average no. of nodules per infected mouse.

Table 2
Nodule incidence: attempts to adsorb M-MTV onto RBC's of MTV-free C⁻ mice

Inoculum	Nodule incidence[a]
M-MTV extract control	100 (8)–16
RBC's incubated with M-MTV (37°, 1 hr)	0 (8)–0
RBC's incubated with M-MTV (4°, 18 hr)	0 (4)–0
Supernatant, 1st wash[b]	75 (12)–18
Supernatant, 6th wash[b]	0 (14)–0

[a] See Table 1, footnote *a*.
[b] Data pooled from 2 incubation groups.

6th washing. The results, summarized in Table 2, show that quantities of MTV detectable by our bioassay did not become adsorbed to RBC's.

In the 3rd set of experiments, attempts were made to use sequential treatment with enzymes to determine whether R-MTV is inside or on the surfaces of RBC's. Sequential treatment at 37° with a low concentration of phospholipase C (75 to 100 μg/ml) for 1 hr followed by trypsin (1 mg/ml) inactivates R-MTV in hemolyzed RBC's but not M-MTV (12); such enzymatic treatment does not cause hemolysis of RBC's. If these enzyme combinations were to inactivate MTV from both hemolyzed and intact RBC's, the R-MTV activity would appear to be on the RBC surface. In this experiment, M-MTV extracts and hemolyzed or intact RBC's from C^+ mice were incubated with phospholipase C followed by trypsin. The incubation mixtures were injected into C^- test mice. The results summarized in Table 3 show that the enzymatic treatment can inactivate viral activity only in hemolyzed RBC's. Viral activity in M-MTV extracts and in intact RBC's was not affected by this enzymatic treatment. The results also indicate that M-MTV and R-MTV activity is not due to identical particles, since only R-MTV in hemolyzed RBC's was affected by the enzymes. One previous study (5) has, however, shown that high concentrations of phospholipase C treatment followed by trypsin incubation cause disruption of B-particle nucleoids; no infectivity test was carried out in this experiment.

In the last series of experiments, attempts were made to determine whether adsorption of B particles onto the RBC surface could account for the R-MTV activity in RBC's of C^+ mice. Intact RBC's of C^+ mice, incubated for 1 hr at 37° with heat-inactivated (56°, 30 min) and adsorbed rabbit anti-B particle antiserum, were inoculated into C^- mice. If B particles were absorbed onto RBC's as has been suggested previously (9), the incubation with antiserum should neutralize the R-MTV activity of intact RBC's. The results summarized in

Table 3
Nodule incidence in C⁻ mice given injections of M-MTV and R-MTV from C⁺ mice; mixtures incubated in phospholipase C followed by trypsin

Inoculum	Nodule incidence[a]	
	Control	Enzyme treated
M-MTV in $MgCl_2$	80 (10)–32	50 (10)–21
R-MTV; hemolyzed RBC's in $MgCl_2$	82 (17)–35	0 (28)–0
M-MTV diluted with C^- hemolyzed RBC's in $MgCl_2$	100 (9)–26	90 (10)–27
R-MTV; hemolyzed RBC's diluted with C^- lactating extract	80 (10)–24	0 (10)–0
R-MTV; hemolyzed RBC's in $MgCl_2$[b]	75 (20)–26	0 (18)–0
R-MTV; intact RBC in 0.85% NaCl solution[b]	100 (18)–30	89 (18)–36

[a] See Table 1, footnote *a*.
[b] Same RBC preparation used in these 2 experiments.

Table 4
Nodule incidence: effect of rabbit anti-B particle antiserum on intact RBC's of C⁺ mice

Inoculum	Nodule incidence[a]
Control RBC's in 0.85% NaCl solution	67 (9)–9
RBC's in normal rabbit serum	60 (10)–8
RBC's in anti-B serum	80 (10)–11
Control M-MTV in 0.85% NaCl solution	89 (9)–13
M-MTV in normal rabbit serum	86 (7)–17
M-MTV in anti-B serum	0 (10)–0

[a] See Table 1, footnote *a*.

Table 4 show that, although the antiserum neutralized M-MTV (assumed to be B particles), neither this serum nor normal rabbit serum had any effect on R-MTV. The results demonstrate that R-MTV activity of RBC's from C^+ mice could not be due to B particles adsorbed onto their surfaces.

Results from the 4 sets of experiments taken together suggest that: (*a*) MTV activity cannot be eluted from infected RBC's by means of RDE; (*b*) detectable quantities of M-MTV cannot be adsorbed by means of incubation alone onto RBC's; (*c*) R-MTV is inside the RBC's and not on their surfaces; and (*d*) R-MTV activity cannot be due to adsorption of B particles onto the surfaces of RBC's. Thus, R-MTV appears to be like Colorado tick fever virus (6) and various lower vertebrate viruses (2) which have been found inside RBC's. Precise intracellular localization of R-MTV inside RBC's will necessitate purification of this form of MTV and its identification by immunological and/or biochemical procedures. Such studies are now in progress.

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