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Attempts to Detect Homologous Autointerference In vivo with Influenza Virus and Vesicular Stomatitis Virus

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Von Magnus particles of influenza virus and defective interfering T particles of vesicular stomatitis virus were unable to provide significant protection of mice from disease or death when inoculated intranasally or intracerebrally along with moderate or high doses of homologous infectious challenge virus. However, yields of infectious virus from the affected organs were reduced as compared to controls inoculated with infectious virus alone. Serial intracerebral passage of vesicular stomatitis virus in mouse brain at high doses failed to produce T particles detectable by in vitro autointerference assays on BHK_{21} cells, whether or not T particles were introduced along with B virions at the first passage. When very low challenge doses of infectious B virions were inoculated intracerebrally along with high doses of homologous defective particles, there was significant prolongation of life, although most mice died eventually of slowly progressing disease. Also, the virus yields in the brains of these mice were significantly reduced, and virus was no longer detectable in the brains of "protected" mice surviving for 10 days or more. Our results suggest that although homologous autointerference does occur in vivo, it is a more complex phenomenon than in vitro cell culture experiments might indicate.

Homologous autointerference was first described by Von Magnus in 1951 (20, 21) in embryonated chicken eggs infected with high multiplicities of influenza virus in serial undiluted passage. Since then, numerous other viruses with ribonucleic acid (RNA) or deoxyribonucleic acid genomes have been shown to produce similar autointerfering virus-like particles upon serial, high multiplicity passage (1, 3, 4, 14, 16, 19). By far the best-understood system of homologous autointerference is that of vesicular stomatitis virus (VSV; reference 4), where the defective interfering T particles are truncated in length and are readily separable from infectious B virions by rate zonal centrifugation (6, 8, 10). T particles are truncated because they contain only a portion of the RNA genome of B virions, and they are homologous in nucleotide sequence to at least part of the B genome (17, 18). The mechanism by which defective particles interfere with virus production has not been elucidated in any system, but it appears that T particles of VSV interfere by competition at the level of RNA replication rather than at the level of transcription or translation (5, 9, 15, 17).

Even though defective interfering particles were first described by Von Magnus (20) in embryonated eggs, very little is known about homologous autointerference in vivo. Von Magnus, in his earliest studies of this phenomenon, reported that egg-grown, incomplete virus provided slight protection of mice against very small challenge doses of influenza virus when both were inoculated intranasally (20). There is no virus-host system in which autointerference has been thoroughly examined in vivo. Huang and Baltimore have reviewed evidence suggesting that homologous autointerfeernce occurs in living animals, and that it may play an important role in disease processes (7). There are several viral diseases of animals in which it appears very likely that homologous autointerference is strongly affecting virus replication in vivo. For example, lymphocytic choriomeningitis virus infection of mice and cells in culture shows multiplicity-dependent fluctuations in virus titer which are precisely what would be expected if defective interfering particles are controlling virus production (11). There is as yet no proof that defective interfering particles play a significant role in virus infection in vivo in any animal or human disease, although the work of Mims with Rift Valley fever virus provides very good evidence for such a role (12). The present

study is a preliminary attempt to observe homologous autointerference in mice by using two viruses which exhibit marked autointerference in vitro.

MATERIALS AND METHODS

Cells. The BHK_{21} line of baby hamster kidney cells was employed for all VSV production and plaque assay. The Maden-Darby canine kidney cell line was employed for influenza virus plaque assay. The cell culture medium was Eagle minimal essential medium containing 5% calf serum.

Viruses. The mouse neurotropic Wilson-Smith strain of influenza A_0 was employed for all influenza virus work. It was grown by dilute passage in 10-day embryonated chicken eggs. Homologous autointerfering particles (Von Magnus particles) of influenza virus were prepared as second serial undiluted passage of the virus on Maden-Darby canine kidney cells. The second passage contained 1% of the infectivity but all of the hemagglutinating titer of the first passage. This pool of Von Magnus particles was used throughout this study. It produced over 99.5% interference with infectious virus when frozen samples were thawed once before use.

The Indiana serotype of VSV B virions was cloned and passed at high dilution on BHK_{21} cells. One pool of B virions was employed throughout this study. It was free of detectable T particles. T particles were purified from the yield of the third undiluted passage (at a multiplicity of 1,000) of this virus in BHK_{21} cells. Only one pool of T particles was used throughout this study. Samples were stored at −60 C, and upon thawing these T particles interfered with an approximately equal number of B virions to suppress virus yield by 99.8% or more. T particles were purified by differential centrifugation, exclusion chromatography on 2% agarose beads, and banding on a 5 to 30% sucrose gradient, as described elsewhere (14, 15).

Mice. The mice employed were outbred young adult white Swiss mice of both sexes. Intranasal inoculation of approximately 0.03 ml of virus-containing fluid was carried out under light ether anaesthesia, as was intracerebral inoculation of 0.02 ml of virus suspension. Virus content of lungs or brains of dead mice was determined by plaque assay after the tissues were homogenized in a Potter tissue grinder in 5 ml of Eagle medium.

RESULTS

Intranasal prophylaxis with Von Magnus particles. We carried out several experiments attempting to determine the protective potential of Von Magnus particles inoculated intranasally along with infectious influenza virus. The results of a typical experiment are shown in Table 1. Eighteen mice were employed. Nine control mice were inoculated intranasally with 2×10^4

TABLE 1. *Intranasal infection of mice with influenza virus alone or in the presence of Von Magnus particles*

Inoculum	Mouse no.	Day of death	HA titer[a] yield per lung	PFU yield per lung	Lung pathology[b]
Influenza virus only[c]	1	3	160	10^8	4+
	2	3	160	4×10^7	4+
	3	3	320	2×10^8	4+
	4	4[d]	80	2×10^7	4+
	5	4	160	2×10^7	4+
	6	4	160	2×10^7	4+
	7	4	80	6×10^7	4+
	8	4	160	2×10^7	4+
	9	4[d]	160	3×10^7	4+
Influenza virus plus Von Magnus virus[c]	10	3	160	6×10^6	4+
	11	3	160	2×10^6	4+
	12	4	320	4×10^6	4+
	13	4	80	9×10^5	4+
	14	4	80	10^6	4+
	15	4	160	4×10^6	4+
	16	5[d]	80	4×10^5	4+
	17	5	320	10^7	4+
	18	5[d]	80	8×10^5	4+

[a] HA titer is hemagglutination titer of lung homogenate on human group O red blood cells.
[b] 4+ equals nearly total lung consolidation.
[c] Approximately 2×10^4 PFU were inoculated into each mouse. In mice 10 to 18, the same amount of virus was inoculated along with approximately 1×10^8 to 2×10^8 Von Magnus particles.
[d] Indicates mice killed when moribund. All others died of virus infection.

plaque-forming units (PFU) of neurotropic Wilson-Smith strain influenza virus contained in 0.02 ml. All mice died on days 3 or 4 postinfection or were killed when moribund. Their lungs showed extensive consolidation with great inflammation, edema, and hemorrhage. Virus titers in the total homogenized lung tissue ranged between 10^7 to 10^8 PFU.

Nine test mice were inoculated with 2×10^4 PFU of virus mixed with concentrated Von Magnus particles (more than 10^3 particles) in 0.02 ml. As Table 1 shows, most of these mice died on day 3 or 4, and three died or were moribund on day 5. Thus, there was no significant protection, although the defective interfering particles may have prolonged the time until death by a day or two for several of the mice. The lungs of "protected" mice exhibited consolidation which was equally as severe as that seen in control mice. Virus yields from lungs of treated mice were generally much lower than in control mice, suggesting that the Von Magnus particles did interfere with virus growth even though they did not prevent disease and death. The hemagglutinin titers of control and treated mice were approximately equal even where virus PFU was reduced, suggesting that defective interfering particles were produced in the lungs of treated mice.

Similar results (not shown here) were obtained when mice were inoculated intracerebrally with this neurotropic Wilson-Smith strain of influenza virus in the presence or absence of Von Magnus particles. All mice died within a week, although the presence of defective interfering particles apparently prolonged the lives of some mice by 1 or 2 days.

Intranasal inoculation of mice with VSV in the presence and absence of T particles. We next carried out a study of in vivo interference by using VSV B and T particles, because these T particles can be purified and concentrated. Intranasally administered VSV B virions killed mice within 3 to 7 days. Mice were inoculated by intranasal installation of virions contained in 0.03 ml of isotonic saline. The seven mice inoculated with 2×10^7 PFU of B virions only died on days 3, 4, 4, 5, 5, 6, and 7 postinoculation, respectively. The seven mice inoculated with 2×10^7 PFU of B virions mixed with approximately 5×10^{10} purified T particles died on days 3, 3, 4, 5, 5, 6, and 7 postinoculation, respectively. The mice died of a generalized infection, with obvious involvement of the central nervous system. The lungs of dead mice appear normal, with no signs of consolidation as with influenza virus. The presence in the inoculum of concentrated, purified T particles did not protect the mice from death and did not even significantly prolong survival time. However, since this route of infection with VSV causes disseminated infection rather than localized respiratory tract disease, it might be expected that locally administered T particles would be ineffective in preventing disease and death. Therefore, we did detailed studies of T-particle protection of mice inoculated intracerebrally.

Intracerebral inoculation of mice with VSV in the presence and absence of T particles. Table 2 shows a typical experiment in which mice were inoculated intracerebrally with dilute B virions only (5×10^4 PFU inoculated), with concentrated B virions only (10^8 PFU inoculated), and with a mixture of dilute B virions (5×10^4 PFU inoculated) and concentrated T particles (approximately 10^{10} particles inoculated). All mice died before 2 days (and all died within a 10-h period). Again, as was observed with influenza virus, the presence of T particles in the inoculum greatly reduced the final yield of virus from the brain, even though it did not confer significant protection against disease. Presumably this reduction of yield was due to homologous autointerference, yet none of the mouse brain homogenates contained sufficient T particles to cause detectable interference with B virion growth in 10^6 BHK_{21} cells.

Attempts to induce homologous autointerference in vivo by serial undiluted intracerebral passage of B virions alone or B virions mixed with T particles. It is known that host cell type can greatly influence the interfering ability of defective interfering viruses (2, 7, 14), so we tried to generate T particles in vivo or to select T particles which replicate preferentially in vivo by carrying out serial undiluted passage of VSV B virions (and of B virions mixed with T particles). The results are shown in Table 3. The first two "high multiplicity" passages of VSV B virions in mouse brain gave maximal virus yields in brain tissue (over 10^9 PFU per brain), and the yield was reduced after the third undiluted passage. However, none of the brain homogenates contained T particles capable of depressing B virion yield from 10^6 BHK_{21} cells, and mice died within 2 days at each passage level.

Serial undiluted mouse brain passage of VSV B virions mixed with T particles before the first passage failed to confer any better protection of mice (Table 3). Again the mice died 2 days after inoculation at each passage level, although yields were reduced by about 1 log at each passage. But again the brain homogenates contained no interfering T particles able to depress virus yields from 10^6 BHK_{21} cells. The large

TABLE 2. *Intracerebral inoculation into mice of dilute VSV B virions alone, concentrated B virions alone, or dilute B virions plus concentrated T particles*

Inoculum	Mouse no.	Day of death	PFU yield per brain	Interfering ability[a]
Ca. 5×10^4 PFU B virions	1	2	1.4×10^9	—
	2	2	1.8×10^9	—
	3	2	1.4×10^9	—
	4	2	1.4×10^9	—
	5	2	1.8×10^9	—
Ca. 10^8 PFU B virions	6	2	1.2×10^9	—
	7	2	4.0×10^8	—
	8	2	1.6×10^9	—
	9	2	1.4×10^9	—
	10	2	1.4×10^9	—
5×10^4 PFU B and ca. 5×10^{10} T virions	11	2	4.0×10^7	—
	12	2	6.0×10^7	—
	13	2	4.0×10^7	—
	14	2	10^8	—
	15	2	8.0×10^7	—

[a] Homologous antointerfering ability of virus in brain homogenates was tested by adsorbing 2 ml of the undiluted brain homogenate to monolayers of 10^6 BHK_{21} cells for 1 h, followed by challenge with 2×10^8 B virions. The — sign indicates that the B virion yield from challenged cells was not reduced, as compared to controls without brain virus (over 10^4 PFU per cell yield in all cases).

TABLE 3. *Serial undiluted intracerebral passage of VSV in mice starting with B virions alone or B and T virions in the first inoculum*

First passage inoculum	Day of death	PFU yield per brain	Interfering ability[a]
2×10^8 B virions			
1st passage	2	1.4×10^9	—
2nd passage	2	10^9	—
3rd passage	2	1.2×10^8	—
2×10^8 B virions plus 5×10^{10} purified T virions			
1st passage	2	6×10^7	—
2nd passage	2	8×10^7	—
3rd passage	2	2×10^8	—

[a] Interfering ability of brain homogenates was tested on BHK_{21} cells, as for Table 2.

quantity of membranous debris in brain homogenates made purification and physical detection of T particles very difficult, so we were unable to rule out the presence of in vivo grown T particles which might be unable to interfere in BHK_{21} cells.

Intracerebral inoculation of mice with infected cells replicating B virions only, or B virions and T particles. It was felt that the poor protective capacity of T particles in vivo might be due to failure to localize input B virions and T particles in the same cells during inoculation. It is well known that a portion of intracerebrally inoculated materials quickly appears in other parts of the body because of damage to the blood-brain barrier, so some of the input B particles may have infected cells which did not also become infected by T particles. Therefore, we devised an inoculation method which might maximize the opportunity for T particles to superinfect those brain cells infected by B particles. Instead of inoculating free B and T particles, we inoculated 10^4 BHK_{21} cells infected 4 h previously with B virions and T particles at multiplicities such that each cell yielded an average of about 10 to 20 T particles for each B virion produced (14). After inoculation of these living, virus-producing cells, it would be expected that the initial virus infection would be better confined to one area of the cerebral hemisphere because the inoculated cells would diffuse less than viruses, and that most brain cells infected by B virions would also be infected by T particles. The results are shown in Table 4. Once again all mice died within 2 days, and virus yields from mice inoculated with doubly infected cells were irregularly reduced as compared to mice inoculated with cells that had been infected with B virions only.

Intracerebral inoculation of mice with large numbers of T particles and very small challenge doses of B virions. In his earliest

TABLE 4. *Intracerebral inoculation into mice of living cells infected with VSV B virions only or of cells infected with B and T virions*[a]

Inoculum	Mouse no.	Day of death	PFU yield per brain
Cells infected with B virions only	1	2	2×10^9
	2	2	2×10^9
	3	2	2×10^9
	4	2	7×10^8
Doubly infected cells (B and T virions)	5	2	2×10^8
	6	2	10^9
	7	2	10^9
	8	2	2×10^7

[a] Mice were not inoculated with free virus but with infected cells budding virions. BHK_{21} cells were infected with B virions only at a multiplicity of 20 or with B virions at a multiplicity of 20 plus T particles at a multiplicity of approximately 100. The cells were incubated in cell culture for 4 h at 37 C and then washed thoroughly to remove free virus. Approximately 2×10^4 washed infected cells were inoculated intracerebrally into each mouse.

TABLE 5. *Effect of T particles in mice inoculated intracerebrally with very small doses of infectious B virions of VSV*

Inoculum	Mouse no.	No. of B virions inoculated	Day of death	PFU yield per brain
4 or 40 PFU B virions alone	1	4	2–3	1.1×10^8
	2	4	2–3	3.6×10^8
	3	4	2–3	3.2×10^8
	4	40	2–3	2×10^8
	5	40	2–3	2.4×10^8
	6	40	3	4×10^8
4 or 40 PFU B virions plus ca. 5×10^{10} T particles	7	4	6	1.4×10^7[a]
	8	4	6	2×10^6[a]
	9	4	14(s)[b]	<10
	10	40	7	10^7
	11	40	7	1.4×10^7
	12	40	10(s)	<10

[a] Brain yields from animals 7 and 8 lacked significant interfering ability in vitro when tested as in Table 2.

[b] s, Sacrificed. Mouse 9 was sacrificed 14 days after infection, after recovering from severe illness. Mouse 12 was sacrificed 10 days after infection at which time total hind limb paralysis was evident.

studies, Von Magnus (20) demonstrated that defective particles of influenza virus could protect mice inoculated intranasally only when the challenge dose of infectious virus was very low. Therefore, we examined the protective effect of purified T particles introduced intracerebrally along with very low doses of B virions. Table 5 shows that B virion doses of 4 or 40 PFU killed all the mice in less than 3 days when inoculated intracerebrally in the absence of T particles. When the same doses of B virions were inoculated along with large numbers of T particles, survival times were greatly extended and virus yields were significantly reduced. Most of the animals died in 7 days, but one mouse was sacrificed when moribund and paralyzed at 10 days and another after apparent recovery at 2 weeks. Both of these two mice had cleared infectious virus from their brains after exhibiting massive symptoms of central nervous system infection between 6 and 10 days after infection. Whereas mice inoculated with B virions alone died quickly within 1 day of the onset of symptoms, mice "protected" by T particles exhibited slowly progressive disease and died many days after the development of hind limb paralysis, semicomatose behavior, or other severe symptoms.

DISCUSSION

These are very preliminary results, and speculation at this stage is unwarranted. It does appear that prophylactic or therapeutic applications of defective interfering particles as antiviral agents may not be as straightforward as had been expected. It has been well established that defective interfering particles can behave quite differently in different cell types (2, 7, 14), and certain cell types in vivo may well be refractory to interference by certain defective interfering particles. To protect animals against significant doses of virus, interfering particles must be capable of greatly retarding cell to cell spread of the virus until an immune response develops. It is obvious that the mere reduction of virus yield does not necessarily protect against disease. The nearly total lung consolidation observed in lungs producing reduced influenza virus yields (Table 1) indicates that inflammatory responses, cytopathology, and other pathological changes may be maximal even where infectious virus production is reduced. A cell which replicates mainly defective particles may still die and release large amounts of viral antigens and inflammatory products.

The results of experiments with very low doses of B virions (Table 5) indicate that at least some protection can be afforded by defective interfering particles under the low input conditions of infection which usually prevail in nature. The results of Von Magnus (20) with very low doses

of influenza virus administered intranasally to mice are in agreement with this.

Obviously, much more work is needed to assess the in vivo potential of defective interfering particles, their capacity for in vivo prophylaxis, and their possible role in chronic viral disease. More extensive experiments than those reported here or in the previous literature appear to be required for adequate exploration of homologous autointerference in vivo. We are presently attempting to detect the presence of T particles in mouse brain by physical purification after serial undiluted passages in vivo.

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ADDENDUM IN PROOF

The purified T particle preparation employed in this study was very slightly contaminated (less than 0.01%) with infectious B virions, but this is able to alter the results with low challenge doses. We recently achieved a hundred billionfold purification of T particles, so that concentrated T particle preparations totally free of infectious virus can be employed in mouse protection tests. These do provide strong protection of mice against low challenge doses of infectious virus (Doyle and Holland, manuscript in preparation).

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