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Prophylaxis and Immunization in Mice by Use of Virus-Free Defective T Particles to Protect Against Intracerebral Infection by Vesicular Stomatitis Virus

(plaque-forming assay/influenza virus)

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ABSTRACT **Defective interfering T particles of vesicular stomatitis virus provide remarkable protection against viral disease and death when introduced intracerebrally in large numbers along with an otherwise rapidly fatal low dose of standard infectious virus. This profound prophylactic effect of defective T particles is due to homologous autointerference since it is serotype-specific and interferon is not induced. This protective effect can be demonstrated only with preparations of T particles that have been purified completely free of infectious virions. When pure T particles are injected intracerebrally along with large doses of infectious virus, they convert an otherwise rapidly fatal disease process to a slowly progressing virus infection that generally terminates in death after many days of wasting disease and paralysis. Intracerebral injection of virus-free T particles alone is apparently innocuous to mice and stimulates immunity to massive doses of homologous infectious virus.**

***In vitro*, virus-free T particles at extremely high multiplicities depress cellular RNA and protein synthesis and kill BHK21 cells in culture, but do not exhibit such effects at moderately high multiplicities.**

Defective, homologous, autointerfering viral particles containing subgenomic RNA or DNA fragments instead of an entire viral genome have been described for nearly all classes of animal virus (1–7) since they were first observed by von Magnus (8) with influenza virus. In all cases, these defective interfering particles arise after repeated passages of virus at high multiplicity of infection, and the maintenance of these defective viruses requires coinfection of cells with standard infectious virus to support their replication.

It was recognized several decades ago (9) that interfering viral particles may have potential for prophylaxis against virus disease. von Magnus in 1951 (10) and Bernkopf in 1950 (11) showed that defective influenza virus particles provided slight protection of mice against very low doses of infectious influenza virus, but these results were complicated by the fact that purified defective virus preparations were not available. Definitive studies of the prophylactic potential of T particles have not been performed with any virus.

Huang and Baltimore suggested recently (12) that defective interfering virus particles may play a significant role in viral disease *in vivo*. Although there are several animal virus infections in which defective virus particles seem to be important [e.g., lymphocytic choriomeningitis virus (13) and arbovirus infections (14)], *in vivo* effects of defective interfering particles remain obscure. Vesicular stomatitis virus (VSV) offers several advantages for the study of *in vivo* effects of defective viruses. Its regular, bullet shape allows sucrose velocity sedimentation to be used for separation of the shorter, defective T particles from standard length, infectious virions (15). Furthermore, the physical and biological characteristics of the defective T particles of VSV have been studied extensively *in vitro*. T particles of VSV contain virion proteins identical to those of infectious virions (16); they lack the transcriptase activity of infectious virus and seem not to express their genetic information; they apparently interfere with infectious virus by competition for replication of RNA rather than transcription or translation (17–21).

We have reported (22) that T particles reduced yields of standard VSV in the brains of mice, but conferred only marginal protection against death from even low doses of infectious virus. In the present report, we show that T particles that have been rigorously purified to completely free them of infectious virus provide strong protection of mice against challenge with low doses of virus. Furthermore, they profoundly alter the disease process resulting from challenge with higher doses of virus and stimulate effective immunity when injected alone.

MATERIALS AND METHODS

Cells and Virus. BHK21 hamster kidney cells were used for all virus growth and assay. Cells were grown in Eagle's minimal essential medium (MEM) plus 7% calf serum. Vesicular stomatitis virus was the Indiana serotype and was kindly provided by Dr. John Mudd (23). Infectious B virus pools were prepared by passage of diluted virus (diluted 1/10,000), and defective interfering T particles were prepared as the yield from the third serial undiluted passage of infectious virus on BHK21 cells. Only one size class of T particle was produced. This is the "standard" T particle containing an RNA genome fragment about $^1/_3$ the size of the virus genome (15).

Mice. All animals used in this study were Swiss Webster outbred mice 1–4 weeks after weaning. They were inoculated intracerebrally (see footnotes of Table 1) with a 25-gauge needle while under ether anesthesia. These very young mice are susceptible to smaller doses of VSV than are older mice.

Abbreviations: VSV, vesicular stomatitis virus; PFU, plaque-forming units.

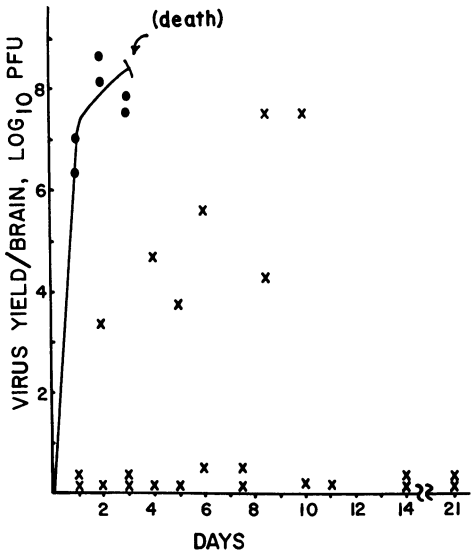

FIG. 1. Yields of infectious VSV from brains of mice inoculated intracerebrally with virus alone or with virus plus T particles. ●——●, six mice were each inoculated intracerebrally with 250 PFU of infectious virus alone. Two mice were killed on day 1, two mice died on day 2, and two mice died on day 3. The brain of each mouse was homogenized in MEM and plaque-assayed on BHK21 cell monolayers. ×——×, 23 mice were each inoculated intracerebrally with a mixture of 250 PFU of infectious virus plus 5×10^{10} T particles. Individual mice were killed on the indicated days for plaque assay of infectious virus in the brain, and each × indicates the virus titer in each mouse brain. *Points just above the abscissa* denote mice with no detectable virus (less than 10 PFU per brain). B and T particles were Indiana serotype.

TABLE 1. *T-particle protection of mice inoculated intracerebrally with VSV*

Number of mice	Virus inoculum*	Days after inoculation at which individual mice died†
6	25 PFU virus alone	2,3,4,5; 2 mice survived
14	25 PFU virus plus T particles	All 14 mice survived
7	127 PFU virus alone	2,2,2,2,2,3,5
12	127 PFU virus plus T particles	1 Died on day 12; 11 mice survived
4	10^3 PFU virus alone	2,2,2,3
5	10^3 PFU virus plus T particles	1 Died on day 8; 4 mice survived
5	10^4 PFU virus plus T particles	6,8,8; 2 mice survived
5	10^5 PFU virus plus T particles	6,6,6,7,9
5	10^6 PFU virus plus T particles	5,8,9; 1 mouse survived

The T-particle pool used here contained no infectious virus.

* Virus was inoculated intracerebrally (0.02 ml) after appropriate dilution into medium free of T particles or containing about 5×10^{10} T particles per 0.02 ml. B and T particles were Indiana serotype.

†All mice described as surviving lived for more than 30 days and were apparently healthy when killed.

Purification of T Particle to Remove All Infectious Virus. In most experiments, the virus yield from 60 to 80 32-oz bottles was pooled and clarified at 9000 × *g* in a Sorvall GSA rotor for 5 min. The supernatant was sedimented for 3 hr in a Beckman no. 19 rotor. The pellet was suspended in 0.05 M Tris·HCl (pH 7.4)–0.1 M NaCl–0.5 mM EDTA (TEN buffer), sonicated gently at 0° for 30 sec (Branson model LS75 W140D at a setting of 2), layered onto 5–40% sucrose gradients containing TEN buffer, and centrifuged for 40 min at 37,000 rpm in a Spinco SW41 rotor. The top two-thirds of the well-separated T-particle band was carefully aspirated with a pasteur pipette from all tubes and pooled (15–30 mg average total T-particle yield). T particles were diluted in MEM, concentrated by sedimentation onto 0.2-ml glycerol cushions in an SW41 rotor, resuspended in TEN buffer–1%

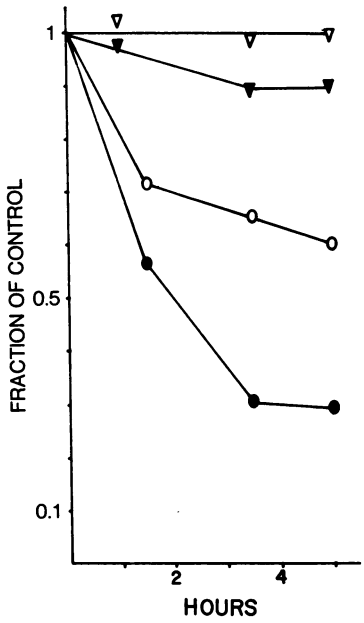

FIG. 2. Inhibition of rates of cellular RNA and protein synthesis in BHK21 cells at various times after exposure to high multiplicities of virus-free T particles. ●——●, incorporation of [^{3}H]-aminoacid into cell protein during a 30-min pulse label at indicated times after a 30-min exposure to 10^6 T particles per cell; ○——○, [^{3}H]aminoacid incorporation after exposure to 10^5 T particles per cell for 30 min; ▼——▼, incorporation of [^{3}H]-uridine into RNA during a 30-min pulse label at indicated intervals after a 30-min exposure of cell monolayers to 10^6 T particles per cell; ▽——▽, incorporation of [^{3}H]uridine after exposure to 10^5 T particles per cell.

Matched cell monolayers containing about 10^6 cells were allowed to adsorb T particles for 30 min, then rinsed to remove unattached particles. Cells were pulse-labeled for 30 min with either MEM containing 100 μCi of [^{3}H]uridine or MEM containing 100 μCi of [^{3}H]valine in place of unlabeled valine ([H^3]uridine, specific activity 28 Ci/mmol, [^{3}H]valine 17 Ci/mmol; Schwarz). All incorporation is expressed as fraction incorporated into 10% Cl_3CCOOH-insoluble material; uninfected control cell values were taken as 1.0. Uridine incorporation into the Cl_3CCOOH-soluble pool was equal in control and treated cells.

Monolayers exposed to 10^6 T particles per cell showed extensive cytopathic effects at 24 hr, but the small fraction of cells that survived gave rise to normal-appearing cell cultures that were fully susceptible to homologous virus after 8 days. Monolayers exposed to 10^5 T particles showed only transient pathology and most cells survived.

calf serum, sonicated, and sedimentated as before on a single 5–40% sucrose gradient. The top two-thirds of the T-particle zone was carefully recovered with a pasteur pipette and immediately layered on top of a 20–40% sucrose gradient in TEN buffer and sedimented for 3 hr at 37,000 rpm in an SW41 rotor. The T-particle zone was recovered and again concentrated as above by dilution in MEM and sedimentation onto a glycerol cushion, resuspended in TEN buffer plus 1% calf serum, sonicated as above, layered onto a 5–40% sucrose gradient with TEN buffer, and centrifuged 40 min at 37,000 rpm. The T-particle band was carefully recovered. Maximum final yields were 1–2 mg of purified T particles containing no infectious virus detectable by serial daily passage on BHK21 [addition of 5 plaque-forming units (PFU) of infectious virus to 0.2 mg of these pure T particles followed by serial daily passage led to rapid cell destruction and virus production on the second and subsequent passages, indicating that the presence of minimal amounts of virus can be detected by this method]. Since this represents greater than 10^9-fold purification of T particles, great care must be exercised in the final stages to avoid virus contamination (the swinging bucket rotor and its cap must be thoroughly cleaned after each use, sterile forceps must be used to remove the tubes from the rotor, etc.). T particle loss by aggregation at each step is unavoidable, but the use of MEM and 1% serum at the stages indicated above minimizes aggregation. Contaminating virus was always found after two sucrose-gradient steps, it was usually present after three steps, and was not found after the four gradient steps outlined above.

RESULTS

Preparation of T particles completely free of infectious virus

In previous studies (22) we used T particles purified essentially as described by Huang *et al.* (15), using one or two successive sedimentations in sucrose gradients to remove infectious virus. When assayed by plaque tests, such preparations exhibited no ability to form virus plaques. This does not mean that virus is absent, since any virus present is suppressed on first passage by the much greater amounts of T particles. We could show that significant amounts of virus are in fact present in all of these preparations by serial passage on BHK21 monolayers as described in *Methods*.

TABLE 2. *Specificity of T-particle protection*

Number of mice	Virus inoculum*	Days after inoculation at which individual mice died
3	10^3 PFU NWS influenza virus alone	7,7,8
3	10^3 PFU NWS influenza virus plus VSV (Indiana serotype) T particles	7,7,8
3	10^3 PFU VSV (New Jersey serotype) virus alone	2,3,4
3	10^3 PFU VSV (New Jersey serotype) virus plus VSV (Indiana serotype) T particles	3,4;1 mouse survived

* T-particle preparation and the method of intracerebral inoculation was identical to that used for Table 1.

TABLE 3. *Induction of antiviral immunity by intracerebral inoculation of T particles*

Number of mice	Immunizing antigen*	Challenge virus*	Days after virus challenge at which mice died
5	None	10^6 PFU VSV†	2,2,2,2,2
6	5×10^{10} T particles†	10^6 PFU VSV†	5 mice survived; 1 died at 4 days
	5×10^{10} T particles	5×10^3 PFU NWS strain influenza virus	6,7,7,8‡

* T particles were injected intracerebrally, and 10 days later mice were challenged with virus into the same cerebral hemisphere.

† T particles and challenge VSV were Indiana serotype.

‡ Normal time of death for NWS influenza virus is about 7 days (Table 2).

We finally used a technique by which concentrated T-particle suspensions could consistently be prepared completely free of infectious virus. This method, described above, requires four consecutive rate zonal centrifugation steps in sucrose gradients and great care to avoid virus contamination, since more than a 10^9-fold purification of T particles from infectious virus is required. Preliminary *in vitro* biological characterization of these pure T-particle preparations is reported in the last section of this report.

Prophylactic effects of purified T particles

Table 1 shows that highly purified T-particle preparations provided excellent protection of mice when injected intracerebrally along with low but lethal doses of infectious virus, and that they significantly prolonged survival times even with very large doses of homologous virus. Note that with the lowest dose of virus alone, nearly all control mice died within several days and nearly all mice receiving the same amounts of virus along with T particles survived indefinitely. When these survivors were challenged intracerebrally several weeks later with large doses of infectious virus alone they were found to be solidly immune (data not shown).

Note also in Table 1 that purified T particles showed decreasing protective capacity as the dose of virus was increased from 100 PFU to 10^6 PFU. At the higher doses nearly all mice eventually died, but the presence of T particles converted an otherwise rapidly fatal disease into a slowly progressing disease in which the animals showed progressive paralysis and generalized deterioration for many days before death.

Fig. 1 compares the growth of virus in the brains of control mice given 250 PFU of infectious virus alone and of mice given the same dose of virus along with T particles. It can be seen that there was rapid growth of virus in the brains of control mice until their death at 2 or 3 days, but in the mice treated with T particles there was strong, early suppression of virus production. The brains of treated mice contained either no detectable virus or very little virus during the first days after infection. Over the next week, about half the mice showed increasing virus titers and half showed little or no virus. After 2 weeks no virus was detectable in any mice. It appears that T-particle interference suppresses virus production very strongly in most mice, and that it delays the produc-

tion of significant amounts of virus in the brains of other mice until immunologic reactivity develops.

Specificity of T-particle interference in mice

In contrast to the data of Table 1 showing homologous protection, the data in Table 2 show that purified T particles from the Indiana strain of VSV did not provide heterologous protection against challenge with New Jersey serotype VSV nor against challenge with influenza virus. This result indicates that the *in vivo* protection is true homologous autointerference and is not due to interferon, VSV-receptor saturation, or other nonspecific effects. Interferon assays on the brains of mice injected with T particles alone or with T particles plus infectious virus showed the presence of little or no interferon.

Immunization with highly purified T particles

Table 3 shows that virus-free T particles caused no apparent ill effects and no deaths when injected intracerebrally. Since T particles contain the same virion proteins as infectious virus (16), they might be expected to immunize at least as well as inactivated virions. Table 3 shows that in fact they do stimulate strong immunity. Within 10 days after intracerebral injection of virus-free T particle of Indiana serotype, mice developed immunity to challenge by large doses of infectious homologous virus, but they developed no immunity against the unrelated influenza virus.

Cytotoxicity and biochemical effects of virus-free T particles on BHK21 hamster cells in culture

Huang *et al.* (15), using partially purified T particles contaminated with low multiplicities of infectious virus, observed that T particles shut off host-cell RNA synthesis and caused cytopathic effects. Fig. 2 confirms that extremely high multiplicities (10^6/cell) of virus-free T particles depress BHK21 cell protein and RNA synthesis, and they kill the majority of cells within 24 hr. No infectious virus could be recovered from the dying cells, as detected by plaque assay or by further passage of virus dilutions in BHK21 cells. After the few surviving cells were washed and refed, they multiplied to give virus-free but fully virus-susceptible cultures within 8 days.

Lower, but still very high, multiplicities of purified T particles (10^5 T particles per cell) did not significantly depress cellular synthesis or damage most cells. Nevertheless, these 10-fold lower multiplicities of T particle caused over 99% interference with virus production. Since T particles do not appear to express the genetic information of their genome fragments (17–21), the cytotoxicity caused by massive multiplicities of T particles may be a result of membrane alterations after attachment and fusion (24) of many T particles, and probably does not require or reflect expression of a gene function.

DISCUSSION

Although these studies were confined to intracerebral inoculation rather than natural routes of infection, they do suggest that virus-free, defective interfering particles could offer considerable prophylactic and immunizing potential against animal and human viral disease wherever they can be purified free of all infectious virus. At present, only other rhabdoviruses such as rabies virus appear to offer practical possibilities for total removal of infectious virus from large quantities of defective virus. If methods could be devised for large-scale preparation of virus-free defective interfering particles from viral isolates during influenza virus pandemics or Newcastle disease epidemics in fowl, they might offer a definite prophylactic–immunizing potential not available in conventional vaccines. The poor protective effects of defective VSV particles observed in our previous study (22), in Mims' work with arbovirus (14) and in the earliest studies with influenza virus (10, 11) were undoubtedly due partly to the presence of harmful titers of infectious virus contaminating the defective particle preparations.

Our observation of slowly progressing virus disease in mice treated with T particles given higher doses of infectious virus reinforces the possibility raised by Huang and Baltimore (12) that defective interfering particles may play a role in the pathogenesis of some slow virus diseases. It also demonstrates the necessity for caution and for careful testing over wide dosages of virus before use of defective particles for prophylaxis and immunization of domestic animals and humans.

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1. Cooper, P. & Bellett, A. (1959) *J. Gen. Microbiol.* **21,** 485–497.
2. Atchison, R., Casto, B. & Hammon, W. (1965) *Science* **149,** 754–755.
3. Kingsbury, D. & Portner, A. (1970) *Virology* **42,** 872–879.
4. Nonoyama, M., Watanabe, Y. & Graham, A. (1970) *J. Virol.* **6,** 226–236.
5. Uchida, S., Watanabe, S. & Kato, M. (1966) *Virology* **27,** 135–141.
6. Cole, C., Smoler, D., Wimmer, E. & Baltimore, D. (1971) *J. Virol.* **7,** 478–485.
7. Mims, C. (1956) *J. Pathol. Bacteriol.* **91,** 395–412.
8. von Magnus, P. (1954) *Advan. Virus Res.* **2,** 59–79.
9. Henle, W. (1950) *J. Immunol.* **64,** 203–239.
10. von Magnus, P. (1951) *Acta Pathol. Microbiol. Scand.* **29,** 157–180.
11. Bernkopff, H. (1950) *J. Immunol.* **65,** 571–583.
12. Huang, A. & Baltimore, D. (1970) *Nature* **226,** 325–327.
13. Lehmann-Grubbe, F. (1971) *Virol. Monogr.* **10,** 1–173.
14. Mims, C. (1956) *Brit. J. Exp. Pathol.* **37,** 129–142.
15. Huang, A., Greenawalt & Wagner, R. (1966) *Virology* **30,** 161–172.
16. Wagner, R., Schnaitman, T., Snyder, R. & Schnaitman, C. (1969) *J. Virol.* **3,** 611–618.
17. Huang, A. & Manders, E. (1972) *J. Virol.* **9,** 909–916.
18. Perrault, J. & Holland, J. (1972) *Virology* **50,** 159–170.
19. Emerson, S. & Wagner, R. (1972) *J. Virol.* **10,** 297–309.
20. Roy, R. & Bishop, D. (1972) *J. Virol.* **9,** 946–955.
21. Reichmann, R., Pringle, C. & Follett, E. (1971) *J. Virol.* **8,** 154–160.
22. Holland, J. & Doyle, M. (1973) *Infection and Immunity* **7,** 526–531.
23. Mudd, J. & Summers, D. (1970) *Virology* **42,** 958–968.
24. Heine, H. & Schnaitman, C. (1971) *J. Virol.* **8,** 786–795.