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# In Vivo Interference in Vesicular Stomatitis Virus Infection

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Inactivated defective interfering and complete particles of vesicular stomatitis virus given intracerebrally to adult mice protect them against challenge with homologous virus whether this is given at the same time or several days later. Two separate protective processes appear to be involved. The first, which comes into operation immediately after inoculation, is also effective against heterologous strains of vesicular stomatitis virus, rabies (another rhabdovirus), and a neurotropic strain of foot-and-mouth disease virus. The second, later effect, which is strain specific, appears to be correlated with the appearance of circulating neutralizing antibody. Our results suggest that the protective effect that Holland and his colleagues described using defective interfering particles of vesicular stomatitis virus may also be accounted for by an immunological mechanism rather than one involving interference.

Many animal viruses produce defective interfering (DI) particles, which interfere with the replication of the virus from which they are derived (16, 17). In 1970, Huang and Baltimore suggested that DI particles may be important in determining the outcome of infection in the intact animal (17), but until recently there were only the experiments of von Magnus with influenza virus (30) and those of Mims with Rift Valley Fever virus (24) to support this idea. However, in the last few years Holland and his colleagues have made use of the ease with which the DI particles of vesicular stomatitis virus (VSV) can be separated from the intact virus particles to study the effect of these particles on the course of infection in tissue culture and in mice (8, 11-14).

This group of workers has recently provided evidence that DI particles of VSV are necessary for the establishment and maintenance of persistent, noncytocidal infections of BHK-21 cells (12). They have also examined the effect of DI particles on the outcome of an otherwise fatal challenge to mice with various doses of homologous virus and achieved a significant prolongation of life, and in some cases complete protection, when DI particles were administered together with virus (8, 11, 14). Varying degrees of protection were obtained when large amounts of highly purified DI particles ($5 \times 10^{10}$/mouse) were inoculated with either small (25 to 200 plaque-forming units [PFU]) or large ($10^4$ to $10^6$ PFU) doses of homologous virus. In experiments in which small challenge doses were given, the majority of the mice survived, the remainder dying 8 to 11 days after inoculation (controls died in about 2 days). Even after large doses of challenge virus, 16% of the mice survived and the remainder died 9 to 14 days after infection (14). Virus replication in the brains of the treated mice was depressed (8, 11, 14). Furthermore, the effect of the DI particles was specific; particles from the Indiana strain of VSV did not provide protection against the New Jersey serotype or influenza virus, nor was interferon production involved (8). Holland and his colleagues (8, 11, 14) described in detail the altered pattern of disease in the treated animals; instead of dying rapidly after the first signs of encephalitis, they exhibited a gradual increase of paralysis, starting in the hind limbs, and wasted deterioration, with arched back and ruffled fur. It was concluded that DI particles had slowed down the viral infection and altered the nature of the disease, as Huang and Baltimore (17) had suggested might happen.

The purified DI particles alone had no obvious ill effects on mice when inoculated intracerebrally. As anticipated, the particles, which contain the same proteins as the infective virus, stimulated strong immunity against massive doses ($10^6$ PFU/mouse) of homologous challenge virus given intracerebrally 10 days later. Doyle and Holland (8) regarded this effect as completely different from the protection conferred when the particles and virions were inoculated together, an effect that they regarded as true homologous interference. Holland and Doyle found that, in vitro, approximately equal numbers of DI particles and virions were required to suppress virus yield by 99.8% or more (11). However, with our VSV preparations, 100 to 1,000 times more DI particles than virus

particles were required to ensure a similar effect (7). For this reason we have found it difficult to understand how in vivo interference can operate in an experimental system where it is difficult to locate input virus and DI particles in the same cells during inoculation (11). We have sought, therefore, to find an alternative explanation for Holland and Doyle's results which is based on an immunological response of the host and not on interference.

## MATERIALS AND METHODS

**Mice.** All animals used in these experiments were P-strain (PFD) mice. For interference experiments, 6- to 8-week-old male mice were used. They were inoculated intracerebrally or intraperitoneally without anesthetic.

**Virus growth and titration.** The Indiana, Brazil, and New Jersey strains of VSV and the Flury LEP strain of rabies virus were grown in BHK-21 cells. A single pool of each virus, passaged at low multiplicity (0.05) to ensure a high yield of infective virus and stored in 50% glycerol at −20°C, was used for all the challenge tests. The Indiana strain of VSV was also passaged at higher multiplicity (1.0) for the production of DI particles. Encephalomyocarditis virus was grown in L cells and also stored in 50% glycerol at −20°C.

A neurotropic strain of foot-and-mouth disease virus (FMDV) (serotype A, originally received from C. Levaditi, Paris, in 1951) was passaged intracerebrally in 6- to 8-week-old mice. The brains were removed when the animals were paralyzed 2 to 3 days after infection and stored in buffered glycerol (pH 7.6) at −20°C. Suspensions were made in 0.04 M $PO_4$, pH 7.6, as required.

Infectivity determinations of the Indiana serotype of VSV were made by intracerebral inoculation of 5- to 7-day-old mice or 6- to 8-week-old mice. The titer was essentially the same in each group of mice. All end points were calculated by the method of Reed and Muench (26), and the titers were expressed as log 50% infective doses per milliliter. Encephalomyocarditis virus was assayed by plaque titration in L-cell monolayers, and the titers were expressed as PFU per milliliter.

**Neutralization tests.** Serum samples were obtained by anesthetizing mice with ether and completely exsanguinating from the jugular veins. Serum was removed from the clot and stored at −20°C until required.

Serial dilutions of virus were mixed with equal volumes of 1/10 serum and inoculated intracerebrally into 5- to 7-day-old mice. The difference in titer between virus alone and virus mixed with antiserum provided a measure, the neutralization index, of virus-neutralizing activity in each serum.

**Interferon assays.** Sera were examined for the presence of interferon by a method similar to that described by Stebbing et al. (29). Dilutions were prepared in Eagle medium, and 0.2-ml amounts were added to monolayers of L cells grown in petri dishes. After overnight incubation at 37°C, a $10^{-6}$ dilution of stock encephalomyocarditis virus (containing about 30 PFU) was added to each plate and 30 min later overlayed with a mixture of Eagle medium and 5% fetal calf serum containing 1% agarose. The monolayers were stained with neutral red after a further 48 h of incubation, and the plaques were counted.

Two preparations of mouse interferon were included as internal controls in each assay. These were prepared by giving intraperitoneally 60 and 100 μg, respectively, of polyinosinic acid·polycytidylic acid (lot 410A, Merck, Sharp and Dohme Research Laboratories, West Point, Pa.) to groups of 6- to 8-week-old male mice, which were anesthetized and bled 3 and 4 to 4.5 h later. The pooled serum from each group was separated and stored at −20°C until required. A standard preparation of interferon, kindly provided by C. J. Bradish, Microbiological Research Establishment, Porton, U.K. (preparation no. 93/71), was also used in the tests. In each case the dilution of serum or standard interferon giving a 50% reduction in plaque number was measured.

**Preparation of virus and DI particle concentrates.** Virus and DI particle concentrates were prepared as described by Crick and Brown (6). Virus harvests were clarified by low-speed centrifugation and centrifuged for 1 h at 20,000 rpm in the SW25 rotor of a Spinco ultracentrifuge, and the resuspended pellet was centrifuged for 2 h at 20,000 rpm in a 15 to 45% linear gradient of sucrose in NTE buffer [0.13 M NaCl, 0.001 M sodium ethylenediamine-tetraacetate, and 0.05 M tris(hydroxymethyl)aminomethane, pH 7.8 (28). The virion or DI particle bands were withdrawn with a syringe after piercing the tube wall at the appropriate position. In some experiments the virion or DI particle preparation was purified further by a second cycle of pelleting and gradient centrifugation.

**Inactivation of virus.** Virus infectivity was destroyed by treatment of virion or DI particle suspensions with 0.05% acetyl ethyleneimine (AEI) for 7.5 h at 37°C. Residual AEI was inactivated by the addition of one-tenth volume of 20% sodium thiosulfate (3). The preparations were tested for noninfectivity by intracerebral inoculation into 5- to 7-day-old mice, which were then examined daily for 10 days. Before use in "interference" tests, the preparations were diluted in buffer so that one mouse dose (0.03 ml) contained approximately $10^{10}$ particles.

**Measurement of "protection" or "interference."** Adult mice were inoculated intracerebrally with AEI-inactivated DI particles or virions. The mice were then divided into groups of four or five animals for intracerebral challenge with 0.03 ml of serial dilutions of homologous or heterologous virus. The challenge viruses used and the time of challenge varied with the experiment. Control mice that had received no prior inoculation or had been given buffer only were challenged at the same time. Animals challenged with VSV or FMDV were examined daily for 14 days, and all those that died on or after day 2, or were paralyzed by day 14, were counted as reactors. Animals challenged with rabies virus were

kept for at least 21 days, and those that died or became paralyzed after day 6 were regarded as positive. The difference in virus titer between test and control groups was taken as the measure of protection or interference achieved.

In experiments in which there was to be no delay between giving the "protective" dose and the challenge virus, virus dilutions were made in the buffer containing the inactivated particles. Groups of mice were given either virus diluted in this way or buffer alone. Protective or "interfering" activity was determined by measuring the difference in titer between test and control groups.

## RESULTS

**Homologous interference in vivo by AEI-treated DI particles.** Huang and Wagner (18) reported that ultraviolet irradiation of VSV-defective particles destroyed their ability to interfere with the replication of homologous virus in vitro and suggested, therefore, that the ribonucleic acid must be functional for the interference to occur. Our own finding (Crick and Brown, unpublished data), that AEI also destroyed the interfering activity of VSV-defective particles, had led us to a similar conclusion. For example, a DI particle preparation that lowered the yield of VSV in BHK-21 cells by 4 logs had no effect after inactivation with AEI, as described in Materials and Methods. Our first experiments were designed to show whether AEI-treated, defective particles would affect the growth of homologous virus in the intact animal.

Groups of 6- to 8-week-old mice were given, by intracerebral inoculation, $10^{10}$ inactivated DI particles. At intervals, mice were challenged with dilutions of homologous virus, except in one group in which the virus dilutions were made directly in the DI particle suspension. At each time interval the challenge virus was also titrated in a control group of mice. The results in Table 1 indicate that the protective or interfering effect of the DI particles increases the later the challenge and, of more importance, that there is some protection from the DI particles even when the infective virus is given at the same time.

TABLE 1. *Effect of intracerebral inoculation of DI particles[a] of the Indiana serotype of VSV on the titer of homologous virus in adult mice*

| Time of challenge (h) | Virus titer ($\log_{10}$ $ID_{50}$/0.03 ml)[b] | |
|---|---|---|
| | Test | Control |
| 0 | 6.3 | 8.3 |
| 24 | 6.3 | 8.0 |
| 48 | 3.8 | 7.8 |
| 144 | 1.0 | 7.7 |

[a] The DI particles were treated with AEI before inoculation.

[b] $ID_{50}$, 50% infective dose.

TABLE 2. *Effect of intracerebral inoculation of DI particles of the Indiana strain of VSV on the titer of homologous and heterologous viruses in adult mice*

| Challenge virus[a] | Virus titer ($\log_{10}ID_{50}$/0.03 ml)[b] at: | | | |
|---|---|---|---|---|
| | 48 h | | 144 h | |
| | Test | Control | Test | Control |
| VSV | | | | |
| Indiana | 4.7 | 7.0 | 1.3 | 7.8 |
| Brazil | 6.3 | 7.3 | 4.8 | 7.0 |
| New Jersey | 5.7 | 7.7 | 4.8 | 7.5 |
| Rabies | 4.0 | 5.0 | 4.0 | 5.0 |
| FMDV, type A | 3.5 | 5.3 | 4.3 | 5.0 |

[a] Challenge was made intracerebrally 48 or 144 h after inoculation of DI particles inactivated with AEI.

[b] $ID_{50}$, 50% infective dose.

**Homologous interference in vivo by AEI-inactivated virions.** The experiments described in the previous paragraph were repeated using similar amounts (ca. $10^{10}$ particles/mouse) of AEI-inactivated virions instead of DI particles. Essentially the same degree of protection was obtained, showing that protection against infection may be achieved with virions as well as DI particles after inactivation of the nucleic acid with AEI.

**Heterologous and homologous interference in vivo with AEI-treated DI particles.** To determine whether or not the protection conferred by inactivated DI and complete virus particles was specific, mice were given AEI-treated DI particles intracerebrally, and 48 and 168 h later separate groups were challenged by the same route with serial dilutions of (i) homologous virus, (ii) the Brazil and (iii) New Jersey strains of VSV, (iv) rabies virus, and (v) a neurotropic strain of FMDV. At each time the viruses were titrated in control mice. Forty-eight hours after the inoculation of DI particles, the mice showed some resistance to heterologous as well as homologous virus (Table 2). By 144 h, however, there was a massive increase in the resistance of the mice to homologous virus, whereas resistance to the heterologous virus remained at the level observed at 48 h (Table 2). Essentially the same results were obtained when the mice were challenged at 24 h instead of 48 h, and 168 h instead of 144 h, after inoculation of the DI particles.

As part of the experiment summarized in Table 2, four mice were killed at 48 and 144 h after the administration of DI particles, and their sera were tested for neutralizing antibody against the homologous Indiana strain of VSV

and the heterologous New Jersey strain. The results in Table 3 show that the increase in specificity against challenge shown by the mice can be correlated with the presence of homologous neutralizing antibody in the serum.

**Role of neutralizing antibody in in vivo interference.** The next series of experiments was designed to determine whether neutralizing antibody was important in the protective effects we have described. Cyclophosphamide (6 mg/animal; Koch-Light Laboratories, Colnbrook, Bucks, U.K.) was used to suppress antibody production in the mice (5, 25) and was given intraperitoneally in phosphate-buffered saline 2 h before, 48 h after, or at both times relative to intracerebral inoculation of AEI-treated DI particles. Groups of mice (and controls) were challenged with serial dilutions of the homologous Indiana strain of VSV 48 h after injection of the DI particles. The results in Table 4 show that the drug had no effect on the protection against challenge conferred by DI particles at this time. On the other hand, when cyclophosphamide was given 48 h after the DI particles, but 120 h before challenge, the level of protection observed was reduced to that found at 24 to 48 h (Table 5). Examination of sera in control animals of each group at the time of challenge (i.e., 168 h after DI particle inoculation) showed that cyclophosphamide had suppressed the formation of neutralizing antibody. Therefore, these results support the view that neutralizing antibody is important in the late protection of mice by defective particles against challenge but plays little, if any, part in the immediate or early sparing effect we have observed.

**Has interferon a role in early protection by inactivated DI particles against virus challenge?** Because the early protective effect of inactivated DI particles against virus challenge is nonspecific and apparently is not mediated by neutralizing antibody, we examined the possibility that interferon was involved. Mice given either AEI-treated virions or DI particles intracerebrally were killed 48 h after inoculation, and the sera were examined for the presence of interferon. However, we were unable to detect its presence in any sera even at dilutions as low as 1/4, although the two control mouse interferon preparations had titers of >1/286 when tested against encephalomyocarditis virus in L cells and a standard preparation provided by C. J. Bradish had a titer of 1/128. It would appear, therefore, that circulating interferon is unlikely to be involved in the protective effect we have observed.

TABLE 3. *Homologous and heterologous serum neutralizing antibodies in mice 48 and 144 h after intracerebral inoculation of AEI-treated DI particles of the Indiana serotype of VSV*

| Time after inoculation (h) | Depression of virus titer ($log_{10}$ per 0.03 ml of 1/20 serum) | |
|---|---|---|
| | Indiana strain | New Jersey strain |
| 48 | 0.2 | −0.2 |
| | 0.8 | −0.2 |
| | 0.3 | 0.5 |
| | 1.1 | 0.2 |
| 144 | 2.7 | 0.1 |
| | 2.7 | 0.5 |
| | 1.7 | 0.1 |
| | 3.9 | −0.3 |

TABLE 4. *Effect of cyclophosphamide on the protection conferred in adult mice at 48 h postinoculation by DI particles of the Indiana serotype of VSV*

| Parameter | Virus titer ($log_{10}ID_{50}$/0.03 ml)[a] |
|---|---|
| Drug given at (h): | |
| −2 | 6.8 |
| +48 | 6.8 |
| −2 and +48 | 6.8 |
| No drug | 6.8 |
| Control titers | |
| No drug and no DI particles | 7.8 |
| Drug given at −2 and +48 h, no DI particles | 7.8 |

[a] Challenge was made intracerebrally 48 h after inoculation of DI particles that had been inactivated with AEI. $ID_{50}$, 50% infective dose.

TABLE 5. *Effect of cyclophosphamide on the protection conferred in adult mice at 168 h postinoculation by DI particles of the Indiana serotype of VSV*

| Conditions | Virus titer ($log_{10}$ $ID_{50}$/0.03 ml)[a] |
|---|---|
| DI particles, no drug | 2.3 |
| DI particles, drug given at 48 h | 6.5 |
| No DI particles, drug given at 48 h | 8.5 |
| No DI particles, no drug | 8.0 |

[a] Challenge was made intracerebrally 168 h after inoculation of DI particles that had been inactivated with AEI. $ID_{50}$, 50% infective dose.

## DISCUSSION

In recent years, evidence has been provided that suggests that many slow diseases of previously unexplained etiology may be caused by persistent viral infection (see 14, 16, 17). Some of the viruses implicated in these disease processes, for example, measles and lymphocytic choriomeningitis viruses, produce DI particles (10, 31, 32). It is also known that the DI parti-

cles of some viruses are apparently responsible for the production and maintenance of persistently infected cells in tissue culture (12, 19, 22, 27, 31; S. J. Martin, Abstr. 3rd Int. Cong. Virol., 1975, p. 180). Information of this kind, together with their own observations with VSV and polimyelitis virus (see 17), led Huang and Baltimore to suggest that DI particles may be important in determining the outcome of viral disease and, in certain cases, in establishing delayed or persistent infections (17).

Whereas it is true that the first observations of viral interference were made not in vitro but in vivo (15, 20, 21, 23), there is no direct evidence that DI particles are responsible for persistent infections in animals. The results of Holland and his co-workers, in which they were able to alter the course of VSV infection with massive doses of DI particles, appeared to give some indication that interference by these particles could occur in the intact animal (8, 11, 14). However, our experiments showing that a similar protective effect can be exerted by inactivated DI particles that no longer cause in vitro interference or by inactivated virions suggest that a different explanation is possible.

The large numbers of DI particles used in Holland's experiments presumably elicited a rapid host response similar to that we have observed. The reduced virus yields that these workers obtained from the brains of mice given both DI particles and infective virus could be as readily explained on an immunological basis as by interference. We have found that the intracerebral route of inoculation is exceptionally efficient for experimental immunization (Crick and Brown, unpublished data). Furthermore, Holland et al. have stated that the production of DI particles in adult mouse brains can be demonstrated only with difficulty (13, 14).

Doyle and Holland (8, 11) pointed out that by simultaneous inoculation of the purified DI particles of VSV and small amounts of virion, mice that did not survive indefinitely died from a slowly progressing infection different from the rapid paralysis and death usually observed with VSV infections. This phenomenon they also attributed to interference. However, we noticed a similar pathogenesis in some of the mice that had received inactivated DI particles followed by massive challenge with infective virus ($10^6$ to $10^8$ 50% infective doses). Less frequently, control mice that had been given only 10 to 100 50% infective doses of virus also died very late after infection, having become paralyzed after the normal time for reaction, which is 2 to 6 days in our animals. In several instances mice have survived illness and apparently recovered to normal health, apart from one or both hind legs remaining paralyzed. Several mice are still alive after 6 to 10 months in this condition. We have also observed similar effects in mice infected with the Flury LEP strain of rabies, Lagos bat virus (2), and Chandipura virus (1).

One difference between our results and those of Holland and his co-workers is our finding that the early protective effect of inactivated DI particles is exerted not only against the homologous Indiana strain of VSV but also extends to the heterologous strains of New Jersey and Brazil and to viruses such as rabies and FMDV. It is interesting that this early protective effect does not appear to be due to interferon production. In this respect, our results are in agreement with those of Doyle and Holland (8).

We were able to distinguish this early effect, in which the role of cell-mediated immunity and macrophages remains to be investigated, from the later, more specific effect coinciding with the release of neutralizing antibody into the circulation. At this stage, 6 to 7 days postinfection, the nonspecific effect could still be detected and remained at the same level as at 24 to 48 h. This was shown in the experiments in which the humoral antibody response was suppressed by cyclophosphamide and measured by the difference in titers at these times of rabies virus and FMDV in test and control mice. Slightly increased protection at 6 to 7 days against the heterologous strains of VSV probably can be attributed to interrelationship within the VSV subgroup of viruses (4, 9).

Whereas it would appear that in vivo interference mediated by DI particles has not yet been demonstrated unequivocally, it remains an attractive and interesting possibility. It is possible that interference can occur spontaneously in certain individuals, and in these, because of local DI particle production, persistent or delayed infections may develop. This would be a situation very different from that in which known amounts of DI particles are introduced artificially. Clearly, as Holland and Doyle have commented, homologous interference in vivo is a more complex phenomenon than in vitro tissue culture experiments might indicate (11).

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