Heat-Labile Factor Necessary for Hemagglutination-Inhibition Testing of Horse Sera

JOSEPH L. DeMEIO AND ARMAND N. DeSANCTIS

Department of Research, The National Drug Company, Division of Richardson-Merrell Inc., Swiftwater, Pennsylvania 18370

Received for publication 29 October 1970

Normal and immune sera were obtained from horses immunized with either aqueous, alum, or adjuvant bivalent vaccines containing Milford equine 2 virus. Upon heating at 56°C for 30 min, a factor, required for hemagglutination-inhibition but not complement fixation or neutralization testing, was destroyed. This factor which is present in normal sera does not appear to be complement.

During the evaluation of an experimental bivalent equine influenza virus vaccine, it was noted that heated sera from immunized horses lost the ability to inhibit Milford equine 2 virus from agglutinating chicken red cells (3). This report describes the presence of a heat-labile factor found in horse serum which is essential for the detection of antibody to the Milford equine 2 virus in the performance of hemagglutination-inhibition (HI) tests but not complement fixation (CF) or in ovo neutralization tests.

MATERIALS AND METHODS

Virus. The Milford strain of equine influenza virus was used. It was cultivated in the allantoic sac of chicken embryos by standard methods previously described (3).

Sera. Horse sera were obtained as previously noted (3).

Serological techniques. Hemagglutination and HI tests were performed as outlined by the Committee on Standard Serological Procedures in Influenza Studies (2). Four hemagglutinating units of virus were used in HI tests, and titers are expressed as reciprocal of initial serum dilution. Complement fixation tests were performed by using allantoic fluid antigen and two full units of complement. Neutralization tests in ovo employed approximately 200 egg-infectivity doses of virus and serial dilutions of serum previously inactivated at 56°C for 30 min. Virus-serum mixtures were incubated for 1 hr at 4°C and inoculated intra-allantoically. Inoculations were made into 11-day-old embryos, which were incubated an additional 2 days before allantoic fluids were tested for presence of hemagglutinins. The 50% infectivity end points were calculated by the method of Reed and Muench (7).

RESULTS AND DISCUSSION

Postimmunization sera from six horses given various equine influenza virus vaccines lost their ability to react in the HI test when heated at 56°C for 30 min as shown in Table 1. This capacity was not lost if sera were unheated, heated at 37°C for 30 min, or heated at 56°C for 30 min followed by the addition of preimmunization serum from the same animal.

However, immune sera which had been heated at 56°C for 30 min yielded significant serological antibody rises when tested by in ovo neutralization and CF techniques (Table 2). In this study, for comparative purposes, the HI test was performed simultaneously by using unheated sera.

Investigators working with herpes simplex (4), Rous sarcoma (6), and Western equine encephalitis (8) viruses noted a drop in neutralization titers when a heat-labile factor was destroyed.

More recently, while studying parainfluenza 2 infections in infants, Chanock (1) noted the possible existence of a "heat-labile accessory factor." This factor was required for maximum neutralization but not HI activity.

Since evaluation of the vaccine in our study involved testing all sera at 1:8 or greater, it is not known how far below 1:8 the reactivity may have decreased with heating.

The heat-labile accessory factor described in this paper differs from those previously reported in that it appears to be essential for the HI test but not the neutralization test system. The factor does not seem to be whole complement. When sera from immunized horses were treated with ammonia to inactivate C'4, they were still able to react with the Milford virus in the HI test provided they had not been heat-treated. The possibility remains, however, that a heat-labile fraction of complement may be essential.

McQueen (5) was able to detect HI antibody
Table 1. Hemagglutination-inhibition (HI) titers in postimmunization sera treated in four different ways and tested against Milford equine virus

| Horse no. | Vaccine | HI titer in Sera heated 56 C, 30 min | Unheated sera | Sera heated 37 C, 30 min | Sera heated 56 C, 30 min + normal sera<sup>a</sup> |
|-----------|---------|-----------------------------------|---------------|------------------------|-----------------------------------------------|
| 1         | Aqueous | <8                  | 32            | 32                     | 32                                            |
| 2         | Aqueous | <8                  | 64            | 32                     | 64                                            |
| 3         | Alum    | <8                  | 32            | 16                     | 16                                            |
| 4         | Alum    | <8                  | 32            | 64                     | 32                                            |
| 5         | Adjuvant| <8                  | 16            | 16                     | 16                                            |
| 6         | Adjuvant| <8                  | 16            | 16                     | 16                                            |
| 7         | Placebo | <8                  | <8            | <8                     | <8                                            |
| 8         | Placebo | <8                  | <8            | <8                     | <8                                            |

<sup>a</sup> An equal amount of preimmunization unheated serum was added to heated postimmunization serum from the same horse. All preimmunization sera were <8.

Table 2. Demonstration of antibody responses by three different serological techniques in horses immunized with Milford equine influenza virus vaccine

| Horse no. | Vaccine | Neutralization in ovo<sup>a</sup> | Hemagglutination-inhibition<sup>b</sup> | Complement fixation<sup>c</sup> |
|-----------|---------|-----------------------------------|----------------------------------------|-------------------------------|
|           |         | Pre-immunization | Post-immunization | Pre-immunization | Post-immunization | Pre-immunization | Post-immunization |
| 1         | Aqueous | <8<sup>e</sup>   | 92               | <8              | 32              | <2              | 16              |
| 2         | Aqueous | <8<sup>e</sup>   | >128             | <8              | 64              | <2              | >32             |
| 3         | Alum    | <8<sup>e</sup>   | >128             | <8              | 32              | <2              | 16              |
| 4         | Alum    | <8<sup>e</sup>   | >128             | <8              | 32              | <2              | 16              |
| 5         | Adjuvant| <8              | 53               | <8              | 16              | <2              | 8               |
| 6         | Adjuvant| <8              | 25               | <8              | 16              | <2              | 8               |
| 7         | Placebo | <8              | <8               | <8              | <8              | <2              | <2              |
| 8         | Placebo | <8              | <8               | <8              | <8              | <2              | <2              |

<sup>a</sup> Sera were heated.
<sup>b</sup> Sera were unheated.
<sup>c</sup> Dilution of sera.

in heated horse sera tested against Milford virus. These results would seem to be at variance with those obtained by us. The experimental techniques, however, differed in two important respects. The sera used by McQueen were obtained from a pool of naturally infected horses, and the antibody titers were of greater magnitude than those reported here.

It would seem beyond question that the HI technique is measuring Milford equine virus antibody. This was revealed by demonstrating activity in the heated sera by the CF and neutralization tests and by restoration of HI antibody activity after addition of negative preimmune horse serum to the inactivated preparation. In future vaccine evaluation studies, where no significant HI antibody rises can be elicited with heated sera, it might be well to test unheated paired sera against the homologous virus. In addition CF or neutralization testing should be employed.

LITERATURE CITED

1. Chanock, R. M. 1956. Association of a new type of cytopathogenic myxovirus with infantile group. J. Exp. Med. 104:555–576.
2. Committee on Standard Serological Procedures in Influenza Studies. 1950. An agglutination-inhibition test proposed as a standard of reference in influenza diagnostic studies. J. Immunol. 55:347–353.
3. DeMeio, J. L., D. E. Gutekunst, J. M. Beller, I. M. Paton, and A. N. DeSanctis. 1969. The evaluation of an experimental bivalent equine influenza virus vaccine. J. Amer. Vet. Med. Ass. 155:278–281.
4. Geller, P., V. R. Coleman, and E. Jawetz. 1953. Studies on herpes simplex virus. V. The fate of viable herpes simplex virus administered intravenously to man. J. Immunol. 71:410–418.
5. McQueen, J. L., F. M. Davenport, and E. Minuse. 1966. Studies of equine influenza in Michigan, 1963. I. Etiology. Amer. J. Epidemiol. 83:271–279.
6. Mueller, J. H. 1931. The effect of alexin in virus-antivirus mixtures. J. Immunol. 20:17–23.
7. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 27:493–497.
8. Whitman, L. 1947. The neutralization of Western equine encephalitis virus by human convalescent serum. The influence of heat labile substances in serum on the neutralization index. J. Immunol. 56:97–108.