MICRO DIFFUSION PRECIPITIN TESTS FOR ENTEROVIRUSES AND INFLUENZA B VIRUS

GORDON K. MIDDLETON, JR., HENRY G. CRAMBLETT, HUGH L. MOFFET, JOYCE P. BLACK, AND HELEN SHULENBERGER

Virology Laboratory, Department of Pediatrics, Bowman Gray School of Medicine, Winston-Salem, North Carolina

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ABSTRACT

MIDDLETON, G. K., JR. (Bowman Gray School of Medicine, Winston-Salem, N.C.), H. G. CRAMBLETT, H. L. MOFFET, J. P. BLACK, AND H. SHULENBERGER. Micro diffusion precipitin tests for enteroviruses and influenza B virus. J. Bacteriol. 87:1171-1176, 1964.—A simple micro precipitin gel diffusion test has been adapted to the study of viral antigens. As far as is known from a review of recent literature, this is the first use of the ECHO viruses in precipitin tests and the first attempt to demonstrate by the gel diffusion technique precipitins in a patient’s serum after natural virus infection rather than artificial immunization. The principal value of this technique in virology is the rapid identification or qualitative analysis of viral antigen preparations by use of pooled or specific hyperimmune sera. Virus concentrations of $10^7$ TCD$_{10}$ per 0.1 ml are required for reliable results, but only 0.015 ml of serum is necessary for each test. Virus-serum precipitin reactions were type-specific except for reciprocal precipitation of ECHO 1 and ECHO 8 by their hyperimmune sera. No viral antigens have been found common to two or more virus types among those tested. Precipitins for viral antigens occur frequently in serum of patients after a viral infection and are readily detected by micro precipitin gel diffusion tests. However, this precipitin test remains at present a tool for virus and antigen identification and offers an approach for research appraisal of host response to infections.

Precipitin testing in gel medium has been shown to be a useful technique in the qualitative analysis of microbiological antigens (LeBouvier, Scherdt, and Schaffer, 1957; Hennisch, 1960; Crowle, 1961). Precipitin tests using viral antigens such as influenza (Jensen and Francis, 1953; Hennisch, 1960), vaccinia (Gispen, 1955), and polioviruses (LeBouvier, 1957; LeBouvier et al., 1957; Balaian, 1960) have already been described. The use of these techniques has been of limited value in virology, since a high concentration of antigen is required for visible reaction (Balaian, 1960). Furthermore, macro tests using petri dishes require several days of incubation for the development of visible precipitation (LeBouvier et al., 1957), offering no time advantage over neutralization tests for virus identification.

Micromodifications (Crowle, 1958; Grasset, Bonifas, and Pongratz, 1958; Mansi, 1958; Yakulis and Heller, 1959) of double-diffusion techniques have made precipitin tests practical for viral identification and demonstration of antibodies. The present study was undertaken to assess the value of a micro gel diffusion precipitin technique in the rapid identification of enteroviruses, to attempt to demonstrate antigenic relationships among various enteroviruses, and to study precipitins in sera of patients with enterovirus and influenza type B infections.

MATERIALS AND METHODS

Antigens. Viral antigens for precipitin tests were usually propagated in monolayer cultures of rhesus kidney cells grown in medium A (Melnick, 1955) and maintained in medium 199 (Morgan, Morton, and Parker, 1950) until the cytopathic effect was complete. The cell-virus suspensions were alternately frozen and thawed twice between -20°C and +20°C before harvesting.

The cell-virus culture suspension in four milk dilution bottles was centrifuged twice at 2,000 rev/min for 20 min at 4°C. Then, the supernatant suspension of virus (40 ml) was centrifuged in a Spinco model L centrifuge (×40 rotor) at 38,000 rev/min at 4°C for 2 hr. The sticky sediment was resuspended in 1 ml of Hank’s solution (Weller et al., 1952), producing an opalescent suspension adequate for approximately 20 double-diffusion tests.

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Concentrated antigens were prepared from prototype viruses including ECHO viruses 1 to 9, 19, and 20, and polioviruses 1, 2, and 3. Later, antigens from field strains of influenza B and Coxsackie B4 viruses were prepared. These antigens had a concentration of $10^8$ to $10^9$ TCID$_{50}$ per 0.1 ml and were used undiluted in identification tests and serum antibody titrations to insure reliability of negative results.

In some experiments, a continuous cell culture of human skin maintained in Eagle's (1955) basal medium with 5% horse serum was used for virus propagation to avoid calf and monkey kidney antigens and their possible resultant nonspecific precipitation. Control antigens were made using suspensions of tissue culture without added virus.

**Sera.** Human and animal sera were used in precipitin tests. Sera were collected from patients during the acute and convalescent phases of illness. Twofold dilutions to 1:16 of serum in saline from patients were used to semiquantitatively estimate precipitin titer.

Hyperimmune animal sera were used for identification of unknown viruses and as reference in tests for precipitins in human sera. Specific antisera against each virus were produced for routine use by repeated injection of prototype virus into rabbits. Limited amounts of reference antisera were supplied by the National Foundation. Monkey kidney cells grown in a 2% calf serum medium were used to immunize a rabbit against nonspecific antigens for use as a control in precipitin tests.

Precipitins for calf protein were present in all hyperimmune prototype antisera tested (Le-Bouvier, 1957). When agar contained a low concentration (3%) of calf serum, anticalf precipitins in the reference sera precipitated in a small ring around the serum well (Fig. 1 to 5) and were easily distinguished from test precipitants.

**Gel.** The gel diffusion medium was isotonic saline containing 0.8% Ig to Ag No. 2 (Oxoid), a concentration most satisfactory in minimizing both gel density and reaccumulation of water in the wells. The melted agar was filtered through paper. To each 100 ml of agar solution at 56°C, 3 ml of calf serum were added to remove calf precipitins from hyperimmune serum used in the tests.

Ordinary clean glass microscope slides (3 by 1 in.) were coated on one side with an agar film. When this had dried, 3 ml of the agar-calf serum mixture were applied by pipette. When cool, seven wells were cut with an agar gel cutter (Fig. 1 to 5). Virus antigen (0.04 ml) was placed in the 5-mm center well, and specimens of sera (0.015 ml) were added to the small 3-mm peripheral wells. A diffusion distance of 2 mm between serum and antigen wells afforded maximal sensitivity without loss of definition.

**Precipitin tests.** One or two patterns of seven wells were cut in the agar on each slide. To compare two antigen preparations for common components, two 5-mm wells were cut 2 mm apart for diffusion against their respective sera (Fig. 3).

The slides were incubated at 37°C for 48 hr in petri dishes containing a strip of moistened blotter paper, and were inspected at intervals with dark-field illumination. No staining was required for satisfactory photographic recording of precipitin reactions.

**Results**

**Sensitivity and stability.** Prototype ECHO 1 antigen ($10^7$ TCID$_{50}$ per 0.1 ml) was tested against its hyperimmune rabbit serum (neutralizing antibody titer, 1:960). Serial dilutions of each were made to determine sensitivity of the reaction. With the antigen undiluted, precipitins were barely detectable in a 1:64 dilution of serum. Conversely, no precipitation was visible when this serum was used undiluted against ECHO 1 antigen diluted to $10^4$ TCID$_{50}$ per 0.1 ml. Comparable precipitin titers were present in all the animal sera used.

In other tests, undiluted enterovirus antigen failed to show visible precipitation when pools of serum were used with an approximate activity of 20 neutralizing antibody units (Committee on the Enteroviruses, 1955) per 0.1 ml. Accordingly, hyperimmune sera were pooled so that final dilution of each serum was no greater than 1:4 (approximately 50 to 150 neutralizing antibody units per 0.1 ml). By using 0.015 ml of each pool in diffusion precipitin tests, positive identification was made of several unknown viruses prepared from a suspension of 10 ml of a culture of monkey kidney cells showing an advanced cytopathic effect (Fig. 1 and 2).

Precipitin tests with influenza B antigen and human reference serum also produced visible
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Fig. 1. Precipitation of ECHO virus type 27 in center well by an antiserum containing its respective homotypic reference antiserum. Note the monkey kidney antigen precipitated in a nonspecific ring around the center well. (A) ECHO virus type 27; (B) rabbit serum pool 1 containing antiserum against ECHO types 2, 5, 9, 12; (C) rabbit serum pool 2 containing antiserum against ECHO types 1, 3, 7, 13; (D) rabbit serum pool 3 containing antiserum against ECHO types 2, 6, 8, 9, 14; (E) rabbit serum pool 4 containing antiserum against ECHO types 15, 16, 17, 18, 19; (F) rabbit serum pool 5 containing antiserum against ECHO types 20, 21, 22, 23; (G) rabbit serum pool 6 containing antiserum against ECHO types 24, 25, 26, 27.

Precipitation. Repetition of these tests after storage of the antigen for 3 months at −20 C produced no visible precipitation. Titration of the antigen indicated an infectivity of only 10^4 TCID_{50} per 0.1 ml after storage. Precipitin activity of enterovirus antigens, however, could be maintained for 6 months or longer by storage at −20 C.

Specificity. Experiments were performed to determine specificity of the precipitin reaction of prototype viral antigens and hyperimmune rabbit antisera to specific enteroviruses. For each virus prototype, a specific reaction occurred with homotypic antisera. The precipitate became clearly visible after as little as 5 hr of incubation and was complete by 48 hr. The precipitate occurred in a single delicate arc, and in every case it was possible to determine virus type by such tests.

Fig. 2. Field strain of Coxsackie virus group B, type 4, grown in human skin cell culture in 8% horse serum precipitated by a serum pool containing homotypic antiserum. Note the absence of monkey kidney precipitate near the center well. (A) Coxsackie B4 virus antigen; (B) rabbit serum pool against viruses polio 1, 2, 3; (C) rabbit serum pool against viruses Coxsackie B1, 2, 3, 6; (D) rabbit serum pool against viruses Coxsackie B4, B5, A8; (E) rabbit serum pool against viruses ECHO 24, 25, 26, 27; (F) rabbit serum against calf serum antigen; (G) blank well (empty).

Fig. 3. Reciprocal cross-reaction between ECHO viruses types 1 and 8 and their respective antisera is demonstrated. ECHO 1 (ECHO virus type 1 antigen) and ECHO 8 (ECHO virus type 8 antigen) both propagated in cell cultures with medium containing horse serum. S 1: rabbit reference antiserum to ECHO virus 1 (not diluted). S 8: rabbit reference antiserum to ECHO virus 8 (not diluted).
No common or “group” antigens were found in precipitin tests among preparations of different enterovirus prototypes. A cross-reaction occurred only between ECHO types 1 and 8 (Fig. 3). The cross-reaction precipitation zones were slightly more diffuse and appeared later than the homotypic reaction. Furthermore, despite this cross-reaction, the zones between the two center wells were not continuous, showing that the antigens were not identical (Fig. 3).

Tests of sera from patients. Micro precipitin tests were performed with sera from several groups of patients. Sera from eight patients over 1 year of age were collected during the acute and convalescent phases of illness due to poliovirus type 1 infection. The pairs of sera were examined by gel diffusion tests with poliovirus type 1 antigen. A definite increase in precipitin antibody was demonstrated in the convalescent serum of all eight patients.

For similar tests, an antigen was prepared of a field strain of influenza type B virus. Acute and convalescent sera from nine patients, ages 5 to 16, known to have an influenza B infection, were studied for influenza antibodies. No precipitins were present in any acute serum, but precipitins against influenza B were demonstrated in eight of nine convalescent sera tested.

More extensive tests were conducted with prototype ECHO 19 antigen by use of pairs of sera from 20 patients from whom ECHO 19 virus was isolated. Saline dilutions of the sera were tested, and some produced visible precipitation in titers up to 1:16. Each of the six adults had precipitins in the acute serum and in the preacute serum. Six patients, all children, had no precipitins in either specimen of serum. Four patients older than 1 year showed a precipitin titer rise (Fig. 4 and 5). Precipitins were found in decreasing titer in six of eight infants less than 6 months old. In all six infants, precipitins were present in significant titer in either cord or acute serum. In the four cord sera studied, a significant precipitin titer was present equal to that in the corresponding maternal serum.

Eight infants under 6 months of age excreting other ECHO viruses were tested for precipitin response to the infection. The results corresponded to those in the ECHO 19 group. Four were found to have a falling precipitin level. Only two infants developed a rise in precipitins, and neither had precipitins in acute serum.

![Fig. 4. Precipitin reaction between ECHO virus type 19 and dilutions of acute serum from a patient up to 1:4.](image1)

![Fig. 5. Precipitin reaction between ECHO virus type 19 and dilutions of convalescent serum from a patient up to 1:8. In both figures, the calf serum precipitates in a broad ring about the top, reference serum well. ECHO 19: center well virus antigen. S 19: rabbit reference antisera un-diluted. A S: patient's acute serum undiluted. C S: patient's convalescent serum undiluted. S 1:2, etc.: dilution of the respective acute or convalescent serum.](image2)
DISCUSSION

The double-diffusion test represents only one of the many techniques of immunodiffusion, and micromodification has allowed practical application of its principles to virology (Mansi, 1958; Yakulis and Heller, 1959). The slide gel diffusion test is simple and sensitive and may be used in the clinical virology laboratory, especially for the purposes of rapid identification of viral isolates and qualitative antigenic analysis.

For reliable identification by this method, viral antigen must be concentrated to at least $10^7$ TCID$_{50}$ per 0.1 ml. This is not difficult to achieve by means of ultracentrifugation, and usually 10 ml of tissue-culture suspension yield sufficient concentrated viral antigen for five or six tests. For each test, only 0.015 ml of hyperimmune serum is required, and this may be diluted 1:4 by pooling with two or three other sera. When potent antisera and satisfactory antigen are used, precipitation zones are visible after overnight incubation and frequently as early as 5 hr, an important consideration when rapid viral identification is desired. It is to be stressed that an antiserum with relatively low antibody titer may not be satisfactory for precipitin testing.

It is not necessary to add a preservative to the gel with this technique, since precipitation is complete within 48 hr of incubation, thus allowing ample opportunity for staining or photography without gel deterioration. The sharpness of precipitin bands does not change appreciably with storage at 4 C as long as gel moisture is maintained.

During these studies, two kinds of nonspecific precipitates were encountered. First, almost all hyperimmune rabbit sera were found to contain precipitins for calf serum which had been adsorbed to or included in the immunizing viral antigen. These precipitins could be removed only by the addition of calf serum to the agar-diffusion medium. This caused their precipitation near the serum well and excluded them from consideration and possible confusion with test precipitates (Fig. 1 to 5).

A second nonspecific precipitate was that resulting from the use of monkey kidney cell cultures for viral propagation. Cellular antigens diffuse more slowly than viral ones and precipitate in a diffuse band near their origin. Elimination of these nonspecific precipitates was accomplished by propagation of viral antigen for precipitin tests in tissue culture not requiring either the monkey or calf products commonly used in preparation of viral antigens for immunizing rabbits (compare Fig. 1 and 2.)

Cross-precipitation reactions between prototype ECHO viruses were anticipated in view of a report of heterotypic neutralization by certain antisera prepared against prototype ECHO viruses (Committee on Enteroviruses, 1957). In a series of tests involving several different prototype viruses, only the reciprocal cross-precipitation of ECHO 1 and ECHO 8 (Fig. 3) with the respective hyperimmune sera occurred. In this test, the precipitation lines between antigen wells were not continuous, and in each case the heterotypic precipitation band was somewhat more diffuse and less well developed than the homotypic band when examined after 5 hr of incubation. It is pertinent that, since this work was completed, the Committee on Enteroviruses (1962) has classified ECHO viruses 1 and 8 together because of their close antigenic relationships. Therefore, despite this cross-reaction, no precipitating antigen common to two or more types was found. All other cases initially regarded as possible cross-reactions were subsequently found to be caused by precipitins against monkey kidney, since they did not occur when the precipitating viral antigens were prepared in a continuous cell culture of human skin, maintained in 5% horse serum (compare Fig. 1 and 2).

The possibility that demonstration of a precipitin response would enhance the etiological importance of a virus isolated during a clinical illness stimulated the application of precipitin tests to sera from human subjects. Precipitins had been described previously in the sera of patients after immunization against poliomyelitis (LeBouvier, 1957; Grasset et al., 1958). These findings, confirmed in our studies with the double-diffusion precipitin test, were extended to include sera from patients with acute infections with poliovirus type 1 and influenza type B. In these groups, all of whom were over 1 year of age, a precipitin rise occurred in every patient except one.

A precipitin response to ECHO 19 and other ECHO virus infections was not always present in tests on convalescent sera from patients. The occurrence in acute and convalescent sera from adult patients of specific precipitins for ECHO 19, which remain unchanged throughout the con-
valescent period, is an interesting but unexplained observation, suggesting possible previous experience with the virus. It would seem that the presence of precipitins in acute serum does not always denote immunity to reinfection. In the young infant, moreover, their presence, passively conferred, may interfere with precipitin production during infection by the same virus.

Acknowledgments

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Literature Cited


