positive sera from the residents of Uttar Pradesh.

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Gel Precipitation with ECHO 4 and Other Enteroviruses.* (30766)

ROBERT M. CONANT, ALMEN L. BARRON AND FELIX MILGROM

Department of Bacteriology and Immunology, School of Medicine, State University of New York at Buffalo, Buffalo

The method of double diffusion gel precipitation has been used in recent years for investigations on human enteroviruses(1,2,3). Studies on ECHO viruses have been reported by Middleton *et al*(4) and Balayan *et al*(5). In the present investigation, this procedure was used for the study of serologic variation occurring in ECHO virus type 4. Other ECHO serotypes as well as poliovirus and the Coxsackie virus were included for comparative purposes.

Materials and methods. Preparation for viral antigens. The following strains of viruses were used: ECHO 4—Pesascek strain (prototype), Shropshire strain, isolated in 1956 during an epidemic of aseptic meningitis in Buffalo, N. Y., and found by neutralization tests to be a serologic variant of ECHO 4(6); ECHO 14—Tow; Coxsackie B6—Schmitt; poliovirus II—MEF-1. Viruses were propagated on primary cultures of rhesus monkey kidney. The cultures were grown at 36°C in Blake bottles using a medium consisting of 0.5% lactalbumin hydrolysate in a base of Hanks' balanced salt solution (BSS) with 2% monkey serum. After 3 days of growth, the medium was exchanged with medium 199 plus 2% monkey serum.

Monolayers were usually complete after 6 days of incubation. At this time the cultures were inoculated with 1.0 ml of virus and the inoculum was allowed to adsorb for 90 minutes at 36°C. The fluid was then removed from each bottle and the monolayers were rinsed 3 times. The cultures were then maintained on medium 199 without serum. This procedure was employed because the viruses used for inoculation were grown in a medium containing bovine serum components. The presence of bovine serum antigens in viral preparations interfered with

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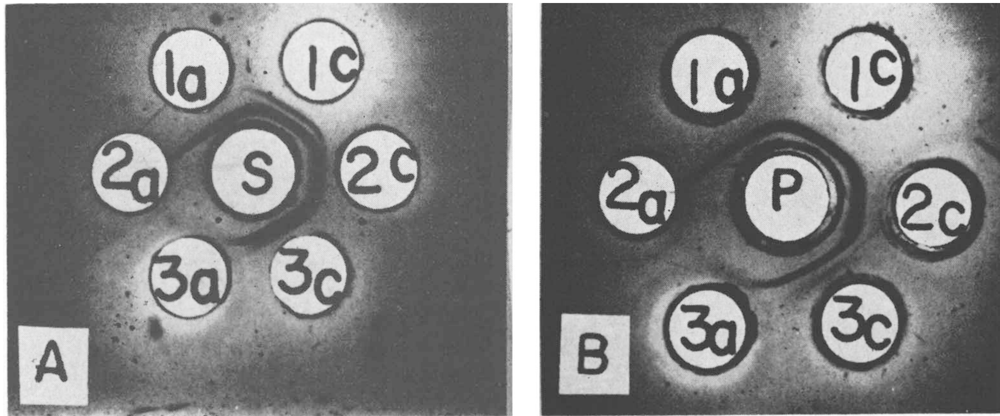

FIG. 1. Double diffusion gel precipitation. Central wells contain ECHO 4 antigens: in plate A, Shropshire strain, in plate B, prototype strain. Peripheral wells in both plates contain sera of patients (No. 1, 2 and 3) taken at acute (a) and convalescent (c) phase of illness.

evaluation of some experiments using animal immune sera since in many of these antisera, antibodies to bovine serum were present. In later experiments, special stock virus preparations were made consisting of virus grown in medium 199 alone. By using these preparations the rinsing procedure was avoided.

When viral cytopathic effect was maximal, the cultures were harvested by freezing and thawing 3 times; approximately 50 ml of material were collected from each bottle. Tissue debris was removed by centrifugation at 2000 rpm in a PR-2 International centrifuge and the supernatant fluid was recentrifuged at 30,000 rpm for 5 hours in a Spinco model L preparative ultracentrifuge using a #30 rotor. The pellet was resuspended in BSS to a volume corresponding to 1/200 of the original material. This procedure usually resulted in an increase of at least 2.0 logs of infectious virus as determined by TCD_{50} titrations. Uninoculated cell cultures were processed in a similar way for use as controls.

Antisera. Acute and convalescent phase sera were collected from individuals suffering from ECHO 4, ECHO 6 or ECHO 14 infections. The ECHO 4 sera were obtained during the epidemic in Buffalo mentioned above and additional sera were obtained from Dr. K. L. Herrmann, CDC, Atlanta, Georgia. Immune sera were produced in African green monkeys (*Cercopithecus aethiops*) and rabbits. For immunization viral antigen mixed with complete Freund adjuvant (Difco) was injected.

Double diffusion gel precipitation. The micro-method of gel precipitation was carried out on 1 $\times$ 3″ microscope slides using 0.8% Ionagar #2 (Consolidated Laboratories Inc.) in 0.15 M NaCl, as described by Grasset *et al* (7), and Middleton *et al*(4). Antigen wells were usually 5 mm in diameter. Serum wells were 4 mm in diameter for human sera and 3 mm in diameter for animal immune sera. The diffusion distance between antigen wells and serum wells was usually 2 to 3 mm. All antigens were used undiluted, as in most cases lines of reaction were not obtained at antigen dilutions greater than 1:2. After wells were filled with reagents, slides were placed in a humid chamber and incubated at 37°C. Photographs were taken usually after 15 to 20 hours of incubation.

Results. Antigen prepared from the Shropshire strain of ECHO 4 was reacted with acute and convalescent phase sera collected from 3 patients of the Buffalo epidemic (Fig. 1A). Two lines of reaction were observed which were designated A and B. The A line which was closer to the antigen well, and appeared usually within 4 hours of incubation, was formed with one acute phase serum and with all 3 convalescent phase sera. The B line which was closer to the serum well and appeared later than the A line, was formed with all 3 convalescent and no acute phase sera. When antigen prepared from strain Pesascek, the prototype strain of ECHO 4, was tested against the same sera, only one convalescent serum, refer 3c, produced a B

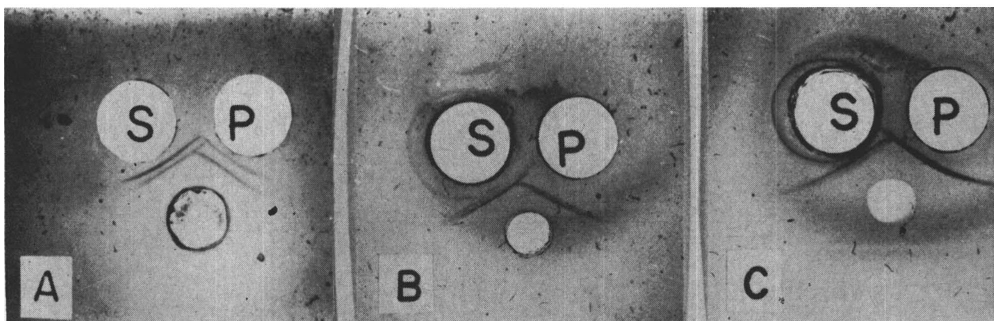

FIG. 2. Double diffusion gel precipitation. Upper wells in all plates contain ECHO 4 antigens: S, Shropshire strain; P, prototype strain. Lower wells contain anti ECHO 4 sera: plate A, convalescent serum of a patient from Buffalo epidemic; plate B, monkey anti-Shropshire serum; plate C, monkey anti-prototype serum.

line and this line was rather faint (Fig. 1B). The A lines, however, were very similar to those found with the Shropshire strain. All tests with control material prepared from uninoculated cultures were negative.

In total, Shropshire antigen was tested against 14 paired human sera from the Buffalo epidemic. The A line was formed by 10 acute phase sera, whereas B line was produced by only 2 acute phase sera. All 14 convalescent phase sera produced both A and B lines. When prototype antigen was tested against the same 14 paired sera, no difference was noted in the formation of A lines. On the other hand, B line was detected in only 8 convalescent phase sera. In addition, B lines formed with prototype antigen were fainter in most cases than those produced with Shropshire antigen.

Acute and convalescent phase sera of 10 other patients with ECHO 4 infections, obtained from Dr. Herrmann, were also examined. Here again, A lines were found in both acute and convalescent phase sera, whereas B lines were restricted to convalescent phase sera. However, with these sera the B line reactions were generally stronger with the prototype than with the Shropshire antigen.

A definite antigenic difference was observed between Shropshire and prototype strains when the two viruses were tested against one serum. Fig. 2A shows such a reaction with convalescent phase serum from a patient of the Buffalo epidemic. The A lines merged in a reaction of complete identity but B lines produced only a reaction of partial identity. The Shropshire line formed a spur extending over the prototype line, which indicated that this particular serum recognized more antigenic sites on the Shropshire preparation than on the prototype preparation. When human serum was replaced by monkey immune serum, only one line of reaction was noted, which apparently was analogous to the B line observed in reactions with human sera. Here again, reactions of partial identity were noted (Fig. 2B and 2C). Significantly, in the test with Shropshire antiserum the spur extended from the Shropshire line over the prototype line, and the reverse was the case with prototype antiserum. Similar results were obtained with rabbit immune sera.

The antigenic difference between Shropshire and prototype viruses could also be demonstrated in another way. Although human sera reacted only in low dilutions, some strong animal immune sera could be diluted as high as 1:1000. It was found that in titrating immune sera, the titers of the homologous reaction were always higher than those of the heterologous reaction.

To further ascertain the nature of the A and B lines, paired sera were examined which originated from three patients from whom ECHO 4, ECHO 6 or ECHO 14 virus had been isolated during a clinical infection. ECHO 4, ECHO 14, Coxsackie B6 and poliovirus II antigens were tested (Fig. 3). Shropshire antigen produced A lines with all sera except the acute phase serum of the patient suffering from ECHO 14 infection. The B line was found only in reaction with the convalescent serum from the patient with ECHO 4

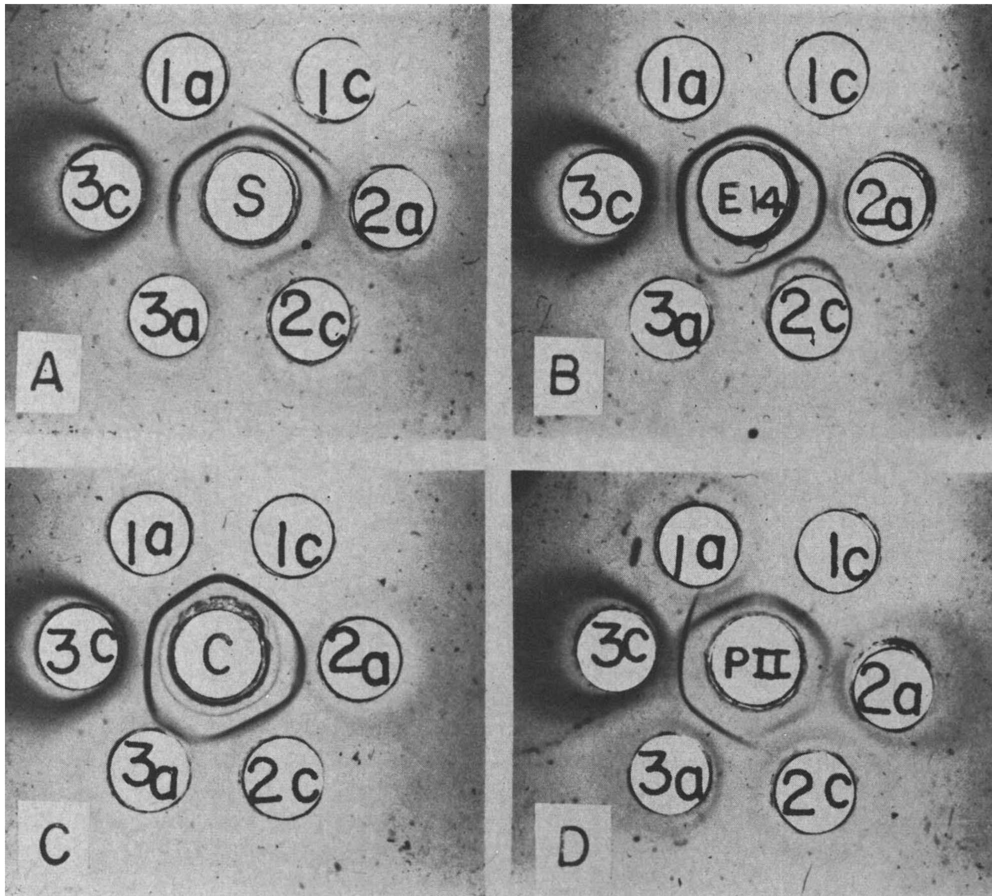

FIG. 3. Double diffusion gel precipitation. Central wells contain enterovirus antigens: plate A, ECHO 4—Shropshire; plate B, ECHO 14; plate C, Coxsackie B6; plate D, poliovirus II. Peripheral wells contain sera of 3 patients suffering from ECHO infections (No. 1, ECHO 4; No. 2, ECHO 6; No. 3, ECHO 14) taken at acute (a) and convalescent (c) phase of illness.

infection. Analogous results were obtained with ECHO 14 antigen. The B line appeared only with the convalescent phase serum of the patient with ECHO 14 infection. Neither ECHO 4 nor ECHO 14 antigens produced B lines with the sera from the patient with ECHO 6 infection. Although not shown in the Figure, ECHO 6 antigen yielded a B line with the convalescent phase serum of the ECHO 6 patient. Coxsackie B6 and poliovirus II failed to form B lines but they produced A lines similar to those formed by ECHO viruses. In addition, poliovirus formed a precipitation line with both serum samples of the patient with ECHO 14 infection. It would appear that this latter line was specifically related to poliovirus. This question will require further clarification.

Discussion. A serologic difference between prototype and Shropshire strains of ECHO 4 was demonstrated by double diffusion gel precipitation in several ways. The most significant result was the spur formation observed when the two viruses were diffused against the same serum. Of the two lines formed by human sera, the B line was interpreted as serotype specific. With serum from the Buffalo epidemic, the B line produced by Shropshire antigen extended over the B line formed by prototype antigen. Spur formation was also noted in studying animal immune sera to Shropshire and prototype strains. The arc produced by the homologous antigen always extended over the arc produced by the heterologous antigen. Other data which indicated a serologic difference

were obtained in examining sera from the Buffalo epidemic. While no difference was noted in the incidence of A lines, it was found that more convalescent sera formed B lines with Shropshire than with prototype antigen. This is consistent with the fact that the Shropshire variant predominated in the Buffalo epidemic. Finally, when animal immune sera were titrated by gel precipitation, the titer was always higher with the homologous antigen. The latter data were analogous to results which had been obtained previously by neutralization test(6).

The incidence of A and B lines in acute and convalescent phase sera would suggest that the A line was produced by antibody from a previous infection or antibody rapidly formed as a result of current infection. The B line would appear to result from current infection. It might be that in those rare instances in which a B line was obtained in "acute phase," sera were collected later in the course of infection and were not truly representative of acute phase of illness.

At present, data collected would suggest that the A line corresponds to a group antigen shared by human enteroviruses. Schmidt and Lennette(2) reported that human sera formed 2 lines with Coxsackie viruses. They considered one of the lines to correspond to a group antigen of Coxsackie virus. The relationship of this group antigen to poliovirus and ECHO viruses was not defined. It is not possible to relate the results of the present study to those reported by Middleton *et al*(4) and Balayan *et al*(5) since only a single line was observed in their studies on ECHO antigens.

As already mentioned some immune sera could be diluted as high as 1:1000 and they still gave a definite precipitation line. On the other hand, the concentration of the viral antigen was quite critical and more than 2-fold dilution resulted in complete disappearance of precipitation lines even in those instances in which undiluted preparations gave very strong precipitation. These results are quite different from those usually observed in double diffusion gel precipitation tests with protein or carbohydrate antigens where the concentration of serum but not of antigen is very critical. In this connection, one would be led to conclude that the viral antigens employed consisted of particulate rather than soluble material. Apparently the particles were small enough to diffuse in agar gel but large enough to give a visible line of reaction with a relatively small amount of antibody. If this is the case, the reactions observed in this study could be placed on the borderline between precipitation and agglutination.

Summary. Double diffusion gel precipitation was used to detect antibodies in sera of patients suffering from ECHO infections. Two lines of precipitation were observed. One of them was apparently group specific and represented an antigen shared by human enteroviruses. The other line was serotype specific. The pattern of reactions with patient sera as well as animal immune sera confirmed the previous observation on serologic difference between prototype and Shropshire strains of ECHO 4.

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