shown to hibernate overwinter and to circulate virus in high titer and for long periods the following spring. Normal mosquitoes became infected by feeding on the emerged snakes and transmitted virus to chicks after an extrinsic incubation period of approximately 3 weeks. These data demonstrate a possible overwintering mechanism of WEE virus, but this virus has not, as yet, been isolated from garter snakes collected in the field.

*Summary.* Garter snakes were inoculated in September and November with Western equine encephalomyelitis (WEE) virus and were caused to hibernate under simulated natural conditions. They emerged during March, April, May and June. After varying periods when no virus was detectable in their blood, virus was detected in concentrations as high as $10^{6.3}>$ and for a period up to 70 days following emergence of snakes. Normal mosquitoes became infected by feeding on these snakes and after an extrinsic incubation period of approximately 3 weeks transmitted WEE virus to 1-day-old chicks. These data demonstrate that snakes may serve as a natural overwintering mechanism for WEE virus.

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## ECHO-4 Viruses: Improved Methods and Strain Selection for Identification and Serodiagnosis.*† (26007)

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The majority of the Enteroviruses propagated in cell culture are readily identified by standard roller tube neutralization technics (1,2). A notable exception is ECHO-4(2,3). Plaque-forming strains of ECHO-4 virus may be identified by the plaque reduction technic (2,3). The applicability of the complement fixation (CF) technic for identification of the first 13 ECHO viruses except ECHO-4 was first demonstrated by Archetti *et al.*(4) using virus antigens prepared in HeLa cells. Halonen *et al.*(5,6) later found that the fluorocarbon procedure of Gessler *et al.*(7) as applied by Manson *et al.*(8) to viral antigens grown in cell culture removed anticomplementary activity of rhesus monkey kidney cell culture (RhMKCC) fluids and greatly reduced reactions between cell culture antigens and antibodies thereto, factors otherwise posing problems in interpretation of tests used for viral identification. This report, although limited to studies on ECHO-4 virus, describes a further modification of the CF test which virtually eliminates all antibody reactions with cell-culture antigens.

Another problem in diagnostic work involving ECHO-4 infections is the general inability to demonstrate neutralizing antibodies in the convalescent serum of the patient. A strain of virus is described which has been found to be readily neutralizable in tube culture tests.

*Materials and methods. 1. Cell culture.* Trypsinized RhMKCC were grown in 0.5% lactalbumin hydrolysate in Hanks' (LAH) balanced salt solution (BSS) containing 2% calf serum and maintained in lactalbumin

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hydrolysate in Earle's BSS (LAE) containing 0.5% calf serum. Primary cell cultures of hamster, guinea pig and cat kidney were grown and maintained in similar media with only minor modifications of serum concentrations. Monolayer cell cultures of a human fibroblast-like continuous cell line, Detroit-504§ (Det.-504)(9), grown in Eagle's Basal Medium (EBM) supplemented with 20% pooled human serum. Primary cell cultures of human amnion and continuous cell cultures of bovine kidney,‖ HeLa (Gey),‖ H.Ep. #2¶ and cynomolgus monkey heart¶ were grown in EBM supplemented with 10% calf serum. *2. Viruses.* Prototype ECHO-4 virus, strain Pesascek, was obtained from Dr. J. L. Melnick of Houston, Texas. Five ECHO-4 strains (2219, 2212, 2235, 3112, 5705) were isolated in this laboratory from patients ill with paralytic disease or aseptic meningitis (10). Two ECHO-4 strains (Shropshire and Gregory) were isolated by Dr. David T. Karzon of Buffalo, N. Y. and supplied by him. Dr. Karzon also supplied DuToit strain isolated by Dr. Malherbe in South Africa. *3. Serology.* Serum neutralizing antibody (NA) titration methods have been described(10). Fifty per cent endpoints were calculated by the method of Reed and Muench(11). CF tests were performed in 13 x 100 mm tubes using 0.2 ml of each reagent. Antigen (virus) and antiserum plus 2 full units of guinea pig complement were incubated 1 hour at 37°C. Sensitized sheep red blood cells were then added and the test incubated another hour at 37°C. Endpoints represented the highest 2-fold dilution showing no more than 50% hemolysis as determined by visual comparison with hemolytic standards. Viral CF antigens were undiluted cell culture fluids clarified by centrifugation at 2,000 rpm for 10 minutes and extracted with fluorocarbon once according to the method of Manson *et al.*(8). *4. Antisera.* Monkey ECHO-4 antiserum prepared by Dr. Herbert A. Wenner(2) was supplied through the courtesy of The National Foundation. Additional ECHO-4 antisera were produced, as follows, in guinea pigs and monkeys in this laboratory. Guinea pigs received 6 intramuscular injections of 0.5 ml of fluorocarbon-treated virus concentrate emulsified in 0.5 ml of Arlacel-Bayol F adjuvant at approximately 10 day intervals. Monkeys were immunized by a similar regime which employed 2.0 ml of purified virus concentrate and 2.0 ml of adjuvant per injection. The procedure for concentration of fluorocarbon-treated enterovirus antigens for immunization of animals has been described(12). Monkey and guinea pig antisera used in CF tests were inactivated for 30 min at 60°C and 56°C respectively. Certain antisera were adsorbed by mixing equal volumes of undilute or 1:5 dilution of antisera and 10% suspension of trypsinized RhMK cells, incubating 1 hour at 37°C and then centrifuging at 2,000 rpm for 30 minutes at 4°C.

*Results. 1. Tube neutralization identification tests.* Four ECHO-4 antisera were tested against 9 strains of ECHO-4 virus. Results (Table 1) represent reciprocals of 50% serum dilution endpoints obtained at the time the final $TCID_{50}$ endpoint in the control virus titrations was first reached. Three strains, 3112, 5705 and DuToit, were neutralized effectively by all 4 antisera; 2 strains, Shropshire and Gregory, were not significantly neutralized by any antiserum. None of the 3 antisera produced with prototype strain Pesascek (columns 1, 3 and 4) significantly neutralized strains 2212, 2219 and 2235. A monkey antiserum to strain 2219 (column 2), a non-plaque forming virus, neutralized these 3 strains and strain Pesascek to a slight degree, dilutions 1:40 to 1:120. In all but 2 instances there was a further significant drop (>4-fold) in NA titer of all antisera after the $TCID_{50}$ endpoint was noted initially, *i.e.*, when no further cytopathogenic effect (CPE) occurred in the virus titration controls. The exceptions occurred with DuToit and 5705 strains. Strain DuToit was solidly neutralized with all 4 antisera; slight decreases (≤4-fold) occurred with strain 5705. *2. CF identification tests.* Two major problems which arose during early CF studies with ECHO viruses were reactions of antisera with

§ Cell line obtained from Dr. Cyril S. Stulberg, Detroit, Mich.

‖ Cell line obtained from Microbiological Assoc., Washington, D.C.

¶ *idem* from Dr. Jonas Salk, Pittsburgh, Pa.

TABLE I. Neutralizing Antibody Titers of 4 ECHO-4 Antisera against 9 Strains of ECHO-4.

| ECHO-4 virus | | ECHO-4 antisera: Strain; cell source; animal source | | | |
|---|---|---|---|---|---|
| Strain | $TCID_{50}$ | Pesascek* RhMKCC Monkey† | 2219 RhMKCC Monkey | Pesascek* RhMKCC G. pig | Pesascek* Det.-504 G. pig |
| Pesascek* | 100 | *80*‡ | 120 | *60* | *30* |
| 2219 | 100 | 5 | *60* | 10 | 10 |
| 2212 | 100 | 20 | 40 | 10 | 5 |
| 2235 | 32 | 20 | 80 | 10 | 10 |
| Shropshire | 100 | <5 | <5 | 5 | <5 |
| Gregory | 320 | 5 | 5 | 7.5 | 7.5 |
| 3112 | 200 | 240 | 240 | 160 | 80 |
| 5705 | 200 | 5120 | 5120 | 640 | 320 |
| DuToit | 200 | >640 | 640 | 320 | 320 |

* Prototype ECHO-4 virus. † Wenner antiserum. ‡ Reciprocal of 50% serum dilution endpoint.

RhMKCC = Rhesus monkey kidney cell culture; Det.-504 = Detroit-504 cell culture; G. pig = guinea pig.

RhMKCC antigens and anti-complementary activity of RhMKCC virus antigens. The latter problem was minimized when EBM without serum was employed as the medium for virus growth followed by a single fluorocarbon extraction of the virus cell culture fluids, as described by Halonen *et al.*(6). This treatment, however, does not remove all cell culture antigens. Thus, low dilutions of antisera produced with virus grown in RhMKCC will still react with virus grown in homologous cell culture. Adsorption of these antisera with trypsinized RhMKCC generally removes these non-viral reactions.

A more desirable means to eliminate cell culture antibody reactions would be to propagate virus for CF antigen in a cell culture heterologous to that used to grow virus for immunization, as had been done with certain ECHO viruses in HeLa cells by Archetti *et al.* (4). Unfortunately, ECHO-4 does not propagate in HeLa cells. Nonetheless, with this premise in mind, attempts were made to adapt Pesascek and 2219 strains to primary cell cultures of hamster, guinea pig and cat kidney, human amnion and to continuous cell lines of bovine kidney, HeLa (Gey), H.Ep. #2, and cynomologus monkey heart, all with no success. During these studies, Stulberg *et al.*(9) reported propagation of the first 16 ECHO viruses, including ECHO-4, in a human fibroblast-like cell line. A similar line, Det.-504 was obtained from Dr. Stulberg. Two ECHO-4 strains, Pesascek and 2219, were readily adapted to Det.-504 in this laboratory. After 6 passages in Det.-504, a titer of $10^{5.7}$/ml was obtained with the Pesascek strain. A pool of virus was prepared, extracted twice with fluorocarbon, concentrated 10-fold and used to immunize 8 guinea pigs and 1 monkey. Tube NA tests with the resultant guinea pig antisera are shown in Table I; similar low levels of NA were obtained with the monkey antiserum (not shown). These 2 antisera from Det.-504 cultures and 4 other ECHO-4 antisera produced with RhMKCC grown virus were titrated for CF antibodies against antigens of 9 strains of ECHO-4 virus. Results are shown in Table II. Titers of Wenner's monkey antiserum, strain Pesascek (column 1) ranged from 1:160 (strain DuToit and Shropshire) to 1:2048 (strain 2212) whereas a similarly prepared antiserum to strain 2219 (column 2) ranged from 1:512 to 1:2048. Titers of antisera produced in guinea pigs immunized with Pesascek (column 3) and 2219 (column 4) viruses grown in RhMKCC ranged from 1:120 to 1:2560 and from 1:80 to 1:2560 respectively. Guinea pig and monkey antisera produced with Pesascek virus grown in Det.-504 cells (columns 5 and 6 respectively) showed somewhat lower titer ranges of 1:24 to 1:1024 and 1:32 to 1:1024 respectively. Strain 2212 showed strongest reactions (1:1024 to 1:2560) with all antisera. In contrast, strains Shropshire and DuToit produced reactions ranging from 1:24 to 1:640 and

TABLE II. Complement-Fixing Titers of 6 ECHO-4 Antisera against 9 Strains of ECHO-4.

| ECHO virus* | | ECHO-4 antisera: Virus strain; cell source; animal source | | | | | |
|---|---|---|---|---|---|---|---|
| Type | Strain | Pesacek† RhMKCC‡ Monkey§ | 2219 RhMKCC‡ Monkey | Pesacek† RhMKCC‡ G. pig | 2219 RhMKCC‡ G. pig | Pesacek† Det.-504 G. pig | Pesacek† Det.-504 G. pig |
| 4 | Pesacek† | 1024 | 1024 | 1280 | 640 | 512 | 256 |
| 4 | 2219 | 1024 | 1024 | 640 | 640 | 128 | 512 |
| 4 | 2212 | 2048 | 2048 | 2560 | 2560 | 1024 | 1024 |
| 4 | 2235 | 512 | 512 | 640 | 1280 | 256 | 128 |
| 4 | Shropshire | 160 | 640 | 120 | 80 | 24 | 64 |
| 4 | Gregory | 640 | 1920 | 960 | 640 | 128 | 128 |
| 4 | 3112 | 1024 | 1024 | 320 | 640 | 128 | 128 |
| 4 | 5705 | 1024 | 2048 | 960 | 320 | 256 | 256 |
| 4 | DuToit | 160 | 1280 | 320 | 120 | 48 | 32 |
| 12 | Travis† | <10 | <10 | <10 | <10 | <4 | <4 |
| | RhMKCC¶ | <10 | <10 | <10 | <10 | <4 | <4 |
| | RhMKCC *vs* unadsorbed antiserum | 20 | 40 | 40 | 160 | | |

* Virus antigens grown in Eagle's Basal Medium, extracted once with fluorocarbon.
† Prototype virus strain.
‡ Antiserum adsorbed with trypsinized RhMKCC.
§ Wenner's ECHO-4 antiserum.
‖ Represents reciprocal of 50% serum dilution endpoint.
¶ Prepared from twice frozen and thawed RhMKCC, clarified by centrifugation only.

from 1:32 to 1:1280 respectively. No cross-reaction occurred with ECHO-12 strain Travis and with RhMKCC control fluids at 1:10 dilution of 4 antisera produced with virus grown in RhMKCC when adsorbed with RhMKCC. Prior to adsorption these 4 antisera showed cross reactions with RhMKCC which ranged from 1:20 to 1:160. In contrast, 2 antisera produced with virus grown in Det.-504, even though not adsorbed, did not cross react with RhMKCC at 1:4 dilution.

*Discussion.* It has been our experience that the tube neutralization technic is an unreliable method for serologic identification of ECHO-4 viruses. Apparently other investigators have encountered similar difficulties (2,3). The lack of a readily neutralizable strain of ECHO-4 has presented serious problems in titration of serum NA to ECHO-4 viruses. In this laboratory it had been found that NA in patients' sera could be detected best with the patients' homologous virus(10). Recently a strain (DuToit) was reported available by Barron and Karzon(3) which was readily neutralized in tube tests. These investigators have employed the DuToit strain to titer NA in sera of persons infected with other ECHO-4 strains. As shown herein, the DuToit and 5705 strains are nearly equally neutralizable in tube tests. It would appear that both strains may be used to titrate NA.

To our knowledge, no ECHO-4 strain has been isolated which produces in immunized laboratory animals an antiserum that effectively neutralizes in the tube test the majority of strains isolated. Thus, the search is still on for a strain of ECHO-4 which will stimulate production of NA effective against all strains of ECHO-4 in the tube neutralization test.

Lacking a simple tube neutralization test, the most reliable method for laboratory identification of ECHO-4 viruses has been the CF test(2,4,5,6). Although in the studies reported here 2 strains, Shropshire and DuToit, did not react with the higher dilutions of all antisera, specific identification of these strains and the 7 other strains tested is assured by the lack of cross-reactions at even a 1:4 dilution with controls. Reactions between RhMKCC antigens and antibodies directed thereto, which were stimulated by immunization of animals with virus grown in RhMKCC, were obviated by fluorocarbon treatment of virus CF antigens and adsorption of the antisera with RhMKCC. This latter procedure was of absolute necessity with those antisera produced in guinea pigs

since CF titers of 1:40 to 1:160 to RhMKCC had been noted in these same antisera prior to adsorption. Habel *et al.*(13) have produced CF titers of 1:3840 to both HeLa and RhMKCC when rabbits were immunized with ECHO-4 grown in RhMKCC that had not been extracted with fluorocarbon. In the work reported here there can be no doubt concerning the specificity of the CF reactions obtained with the 2 antisera produced with Pesascek virus grown in Det.-504 cells since a 1:4 dilution of both antisera, even though not adsorbed with RhMKCC, did not react with either undiluted RhMKCC fluids clarified by centrifugation or with other ECHO viruses grown in RhMKCC and extracted once with fluorocarbon. This specificity may be due to lack of antigenic relationship between RhMKCC and Det.-504 or to removal of sufficient cell culture antigens by fluorocarbon treatment prior to animal immunization, and use of the guinea pig instead of the rabbit for source of antiserum.

Recently Sweet and Hilleman(14) reported that a very high percentage of RhMKCC contains high titers of a virus cytopathogenic for *Cercopithecus aethiops* (African green) MKCC. The presence of this agent in RhMKCC grown virus may account in part for cross-reactions obtained with CF virus grown in RhMKCC and antisera produced with similarly propagated heterologous virus. The lack of cross-reactions in studies reported here indicates that adsorption of antisera with RhMKCC apparently containing large amounts of this virus may remove much of the antibody for this agent.

The obvious advantage of employing ECHO-4 virus grown in Det.-504 for animal immunization and virus grown in RhMKCC for CF antigen is that adsorption of antisera is not necessary to assure reaction specificity even with virus antigens of low antigenicity. Another advantage of this system is the use of the guinea pig to replace the more expensive monkey as source of immune serum for the CF identification test. In addition, these studies have demonstrated that the ECHO-4 antibody produced in guinea pigs immunized with virus grown in Det.-504 is specific and does not react with the RhMKCC antigens remaining in virus-containing fluids after a single fluorocarbon treatment. Although no attempt has been made in this laboratory to extend this particular "heterologous CF" system to identification of other ECHO viruses, it seems reasonable to expect that extension is possible since Stulberg *et al.*(9) have demonstrated that 15 other ECHO virus types tested will grow in a similar human fibroblast-like cell line.

*Summary.* These studies have shown that the tube neutralization identification test for ECHO-4 viruses is unreliable and that the most reliable identification method at present is the CF test. It has been demonstrated that immunization of guinea pigs and monkeys with ECHO-4 virus grown in Det.-504 human fibroblast-like cells produces an antiserum free of antibody which will react with RhMKCC antigens, thereby eliminating cell culture cross-reactions and yielding a highly specific diagnostic reaction even with virus antigens of low antigenicity. The ability of ECHO-4 antibodies to readily neutralize DuToit strain as reported by Barron and Karzon(3) has been confirmed and our strain 5705 was found to have similar properties. As suggested by these investigators, DuToit-like strains should probably be employed to detect NA in human patients' sera.

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## Hematologic Response of Monkeys to X-irradiation.* (26008)

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Previous reports from this institution have described pathology(1) and antibody response(2) in monkeys following total body x-irradiation. As part of the over-all project, the effect of irradiation on the peripheral blood picture was also studied. Most investigations into the effect of total body x-irradiation on cellular elements of the blood have been conducted in subprimate species. In the relatively few studies(3-5) concerned with monkeys, varying dose levels and schedules have been employed. This report describes hematologic response of monkeys following a single total-body exposure to 450 r.

*Materials and methods.* A total of 37 young adult monkeys (1½-3 years old, *Macaca mulatta*) received total body irradiation with 450 r by a previously described method (1). Group II (18 monkeys) was irradiated 6 months after Group I (19 monkeys). Seven control monkeys were handled in an identical manner, but received no irradiation. Blood counts done from ear lobes were obtained 4-5 times over a 2-week base-line period, and then at 2, 4, 10 and 12 days after irradiation; subsequent counts were then made twice weekly for 2 months and weekly up to 6 months. Hemoglobin content was determined with a Leitz-Rouy Photrometer. Differential counts were made of 200 cells stained with Wright's stain.

*Results.* The effect of irradiation in the 37 monkeys was first reflected by the leukocytes, which were depressed significantly at 2 days, markedly at 4 days, and maximally between 10-17 days (Fig. 1). At the latter intervals, mean leukocyte count was only 5% of base-line level. Beginning recovery of leukocytes, noted at 19 days, was followed by a marked leukocytosis in 17 of 37 monkeys between 4 and 7 weeks post-irradiation. Recovery occurred at variable rates in the remaining animals, the low mean leukocyte count at 60 days reflecting the fact that half the animals had not attained base-line levels. By 3 months mean leukocyte count had returned to pre-irradiation level.

The effect of irradiation on erythrocytes (Fig. 1) was noted later and to a less marked degree than the effect on leukocytes. Erythrocytes were not significantly decreased at 2 and 4 days, and lowest levels were not reached until 17-19 days post-irradiation, at which time mean count was about half pre-irradiation value. Beginning recovery at about 4 weeks was followed by a gradual increase to base-line level at about 3½ months. In general, depression and recovery of hemoglobin paralleled that for erythrocytes. A gradual decline in hemoglobin concentration was noted in control, non-irradiated monkeys, but all other elements remained stable during the observation period.

The effect of irradiation on differential leukocyte count in 18 monkeys (Group II) is presented in Table I, with data on erythrocytes included for comparison purposes. Lymphocytes showed early reflection of irradiation with a rapid decline noted by the second day and a mean level of only 9% of base-line at 12 days. Gradual recovery began about the 20th day, with return to pre-

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