Anti-Virus Antibody Studies Following Induced Infection of Man with West Nile, Ilhéus, and Other Viruses

Chester M. Southam and Alice E. Moore

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This paper presents a study of antibody formation in man following intentional inoculation of live viruses. Ilih"us virus and two isolates of West Nile virus were extensively studied, and more limited studies were done with Bunyamwera, Br I, R, and Newcastle disease viruses.

This study is a by-product of an experimental clinical evaluation of the ability of these viruses to localize in and cause destruction of neoplastic tissue in patients with advanced inoperable cancer. The clinical course, virology, and anti-neoplastic effects of these virus infections in man have been reported elsewhere (1-3), and the pathologic lesions induced have been reported by Newman and Southam (4).

The objectives of this study were: (1) To determine the characteristics of the antibody production curve, including the time of first appearance of detectable antibodies, the time and degree of maximum response, and the persistence of serum antibodies. (2) To compare antibody titers as determined by complement-fixation technique and neutralization technique. A few studies with hemagglutination technique are also included. (3) To study relationships between the clinical severity of the virus infection and the rapidity and degree of antibody formation, and between amount of virus propagation and antibody production. (4) To observe the effects of various types of neoplastic diseases upon the antibody-forming capacity. (5) To investigate the possible existence of common antigenic factors among these viruses. (6) To determine whether residents of the United States had antibodies against these viruses prior to the experimental infections.

**MATERIALS AND METHODS**

**Patients.** All patients were volunteers who had advanced neoplastic disease of an extent, type, and stage which precluded the possibility of therapeutic benefit from surgery, x-ray, or anti-neoplastic chemotherapeutic agents. The general physical condition of these patients varied extremely, from apparently well to terminal. There was a wide range of diagnostic types, including epidermoid carcinomas, adenocarcinomas, lymphomas and leukemias, and other sarcomas. Blood for antibody studies was obtained immediately prior to each virus inoculation and usually at weekly intervals thereafter during the period of hospitalization, and as frequently as was practicable after patients had been discharged to their homes. Serum was separated and stored at 4°C until use. Sera stored in this way for over a year showed no change in antibody titers.

**Viruses.** Four isolates of West Nile virus were used in this study. The original isolate, which will be referred to in text merely as “West Nile”, was isolated by Smithburn and coworkers (5) from a native of Uganda whose only evidence of illness was slight fever. This isolate had been through at least 140 serial mouse brain passages prior to use in the present study. Viremia rarely occurred and there was no clinical reaction other than a slight fever in a few patients (1). The other 3 isolates of West Nile virus used in this study were isolated from sera of presumably well persons in Egypt (6) and will be designated as Egypt 101, Egypt 21, and Egypt 19. (These same viruses are referred to in some reports as the Egypt strains of West Nile virus. We have used the term *isolate* because it carries no implication as to identicalness of, or closeness of relationship between, these viruses isolated from 4 different
persons.) The 3 Egypt isolates were available as fourth-passage preparations in mouse brain or chick embryo. Egypt 101 is highly infective for man under the conditions of these studies, as indicated by a persistence of viremia for 3 days or more in 87% of the patients. It caused no clinical reaction, or no reaction other than fever, in 91% of patients, but suggestive or definite signs of transient encephalitis in 9% (3). Egypt 21 has similar clinical potentialities, as judged from trials in only 5 patients. Egypt 19 did not produce viremia or illness in the 3 patients studied. These 4 isolates of West Nile virus are antigenically identical or nearly so according to Melnick and coworkers (6). West Nile virus has been transmitted by mosquitoes (7-9) under laboratory conditions, and viruses which appear to be identical with West Nile have recently been isolated from Culicine mosquitoes in Egypt (9). Recently, West Nile virus has been isolated from sera of several other patients in Egypt (10) and Israel (11, 12), and antibodies against this virus have been detected in sera from natives of India (13).

Ilhéus virus was isolated from mosquitoes in Brazil by mouse brain passage (14) and was available to us as twenty-third passage brain suspension. This virus produced viremia of a few days' duration in approximately half of the patients studied. Most patients showed no clinical reaction, but transient clinical signs of encephalitis developed in 3 (1). Natural infection of man by Ilhéus virus has not been reported, but natives from the region where it was isolated have been shown to have anti-Ilhéus serum antibodies (14). Ilhéus virus can be transmitted by mosquitoes to mice under laboratory conditions (14).

Bunyanwera virus caused severe encephalitis in 1 of the 3 patients inoculated in these studies but produced neither viremia nor clinical evidences of infection in the other 2 (1). This virus was isolated from mosquitoes in Uganda (15). Natural infection of man has never been recognized but antibodies for this virus have been demonstrated in African natives (15).

Newcastle disease virus causes an enzootic and epizootic disease of fowl and has occasionally been observed to infect man (16, 17). Moolton and Clark have suggested that Newcastle disease virus may be a cause of serious illness in man (18). Intravenous inoculation of the Massachusetts strain of Newcastle disease virus (as infected chick embryo allantoic fluid) into 13 patients caused no clinical illness other than an immediate and transient fever (19), and no histopathologic changes (4).

Br I and R viruses were isolated from the brains of sick or paralysed mice during the course of intracerebral tumor passage studies by Dr. H. Koprowski. They were used in patients previously treated with other viruses because they are antigenically different from West Nile and Ilhéus viruses and because they inhibit growth of certain transplantable tumors in mice. R virus propagated in but caused no apparent illness and no histopathologic findings in the 1 cancer patient inoculated (4). Br I virus was not shown to propagate in the 6 patients inoculated (20).

Virus inocula. Newcastle disease virus inoculum was infected chick allantoic fluid dialyzed against distilled water to remove excretory solutes, and was administered intravenously in a single dose of 20 to 120 ml in 5% glucose. Some of the Egypt 101 inocula were crude chick embryo suspensions. All other inocula were mouse brain suspensions. Each patient received a single inoculation of 2 to 10 ml of 10% suspension. They were usually administered intramuscularly, but a few were given intravenously in 500 ml of 5% glucose containing 50 mg of heparin. Most tissue suspensions were crude, some were centrifuged to remove macroscopic particles, and a few of the Ilhéus inocula were "purified" by the protamine technique (21).

Virus propagation. Since the virus antigen in these studies was an infective agent, it is obvious that the total dose of antigen was not the amount of virus inoculated, but rather the total virus propagated. The total dose of antigen can therefore not be determined. However, the duration of viremia was a rough indication of the amount of virus propagation since it has been shown (1, 3) that these viruses (with the exception of Newcastle disease virus) are not excreted, and that the duration of viremia usually coincides with the persistence of virus in various body tissues. The neurotropic viruses were detected by the occurrence in mice (Swiss, Banks), after intracerebral inoculation with heparinized whole blood or 10% tissue suspensions, of paralysis and death at a time characteristic of the virus being studied. Newcastle disease virus was detected by inoculation of chick embryos and confirmed by hemagglutination of chicken red blood cells by the infected chick allantoic fluid.
Complement-fixation techniques. Lipid-free antigens were prepared from adult mouse brain by benzene extraction (22) and were used as a 1:40 or 1:80 suspension. The antigen used for the study of each patient's sera was prepared from the same virus isolate used for inoculation of that patient, unless stated otherwise specifically. The lowest dilution of serum tested was 1:8 (initial dilution). Appropriately diluted inactivated test serum, antigen, and complement (three 50 per cent-hemolysis units) (23) were added in that order in 0.2 ml volumes to Wasserman tubes and stored overnight at 4°C. Sensitive erythrocytes (0.4 ml of a 2% suspension) were then added and tubes were incubated for 20 minutes in a 37°C water bath. Reactions were graded visually as 0 (complete hemolysis) to +++++ (no hemolysis). Titters are recorded as the greatest serum dilution giving a reaction of +++. The +++ reaction showed definite hemolysis but a substantial residue of unlysed erythrocytes. All sera from each individual patient were tested simultaneously. Normal mouse brain (or chick allantoic fluid) antigens, serum controls, antigen controls, complement controls, and a known positive serum were included in each study.

In order to permit graphic presentation of all data and calculation of mean titers, serum antibody titers are presented in some of the figures in terms of the number of "tubes" (number of 2-fold dilutions) of specific antiviral antibody. If a serum showed no anti-complementary or anti-normal mouse brain activity a 1:8 titer is considered a "1-tube", 1:16 as "2-tube", etc. For sera showing non-specific reactions at low dilutions the anti-virus antibody titer is recorded as the number of tubes showing a reaction with virus antigen minus the number of tubes showing anti-normal brain or anti-complementary activity. With sera which showed such non-specific reactions, a difference of 2 ++ marks in the degree of complement-fixation with virus antigen as compared with normal mouse brain antigen or serum controls at a given serum dilution was considered as a significant difference; thus, a serum which with normal brain antigen showed a + reaction but with virus antigen showed a +++ reaction was accepted as showing a specific anti-virus reaction at that dilution. Anti-normal brain antibodies usually developed to titers of 1:8 or 1:16 in patients who were inoculated with more than 1 virus, and occasionally occurred following a single virus inoculation. The validity of these conventions, for comparison of sera tested simultaneously, was demonstrated by the fact that serial serum specimens graded by this method showed excellent agreement in antibody titers in spite of the erratic occurrence of anti-complementary activity, or the development of anti-normal brain antibodies following inoculation of a second or third virus. Furthermore, the antibody curves in patients whose sera showed non-specific reactions at a given serum dilution agreed both in general pattern and in degree of the maximum response with data from patients who did not show these reactions.

Neutralization technique. All neutralization tests presented in the figures and table were performed by mixing equal volumes of inactivated undiluted serum and serial 10-fold dilutions of virus suspensions, incubating for 2 hours in a 37°C water bath, and testing for persistence of virus by inoculation of a susceptible host. For Newcastle disease virus the test animal was the 10-day chick embryo inoculated intra-allantoically. For some studies with West Nile virus 10-day chick embryos were inoculated into the yolk sac. For all other neutralization studies mice were inoculated intracerebrally. Neutralizing antibody titers were expressed as the difference between the logarithms of the LD50 dilutions in pre-virus and post-virus sera of each patient. A control incubation and titration in 10% normal horse serum or with known negative undiluted human serum was included in each study to detect possible antibodies in pre-inoculation sera.

Anti-hemagglutination technique. The few anti-hemagglutination antibody titrations reported in this paper were performed by Dr. Robert Channock, using essentially the same technique described for St. Louis encephalitis virus (24).

Explanation of figures. In antibody curves of individual patients all except the latest negative reaction (less than 1:8 or less than 1.0 logs) are omitted, and patients who showed no antibody rise are omitted entirely. In Figures 5 and 9 patients whose sera showed anti-complementary or anti-normal mouse brain activity are omitted.

The authors are greatly indebted to Dr. Frank Maltaner and his associates for preparing and supplying all of the sheep erythrocytes and hemolysin used throughout these studies.
TABLE I

Development of serum antibody titers suggestive of or diagnostic of virus infection, following experimental inoculation of viruses in cancer patients. Summary of data.

<table>
<thead>
<tr>
<th>VIRUS INOCULATED AND ANTIBODY DETECTION TECHNIQUES</th>
<th>West Nile</th>
<th>Egypt 101</th>
<th>Egypt 19</th>
<th>Egypt 21</th>
<th>Ilhéus</th>
<th>N.D.V.</th>
<th>Bunyamwera</th>
<th>Br I</th>
<th>R virus</th>
<th>Semliki</th>
<th>Rabies</th>
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<tr>
<td>Total patients studied ..................</td>
<td>20</td>
<td>11</td>
<td>50</td>
<td>17</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>20</td>
<td>6</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Pts. with adequate series of sera ..........</td>
<td>17</td>
<td>11</td>
<td>30</td>
<td>17</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>18</td>
<td>6</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Pts. with diagnostic titers ................</td>
<td>12</td>
<td>8</td>
<td>15</td>
<td>13</td>
<td>2</td>
<td>—</td>
<td>0</td>
<td>8</td>
<td>5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Pts. with suggestive or diagnostic titers .</td>
<td>12</td>
<td>11</td>
<td>27</td>
<td>15</td>
<td>2</td>
<td>—</td>
<td>1</td>
<td>16</td>
<td>11</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Pts. with no antibody response .............</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>2</td>
<td>0</td>
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* Five additional patients were studied who received cortisone treatment concurrently with Ilhéus virus. See text and Fig. 17.

RESULTS

Serum was obtained from each patient prior to experimental inoculation of any virus. The total number of patients tested by each technique for antibodies against each virus is recorded in the top line of Table I. No antibodies were detected which indicate prior exposure to West Nile-like viruses in 70 patients studied. Neither were antibodies detected, in the few patients studied, against Bunyamwera, Br I, R, Semliki, or Rabies viruses. A 1:8 titer of anti-Ilhéus complement-fixing antibodies was demonstrated in the pre-inoculation sera of 2 patients out of 20. These 2 patients were not studied by the neutralization technique. Low titers (1:8 and 1:16) of complement-fixing antibodies against Newcastle disease virus were detected in 2 of 10 patients.

Table I summarizes the number of patients whose sera were studied after inoculation of each virus, the number of adequate series of sera, and the frequency of suggestive or diagnostic antibody rises. The term "adequate series" is defined as a series of serum specimens encompassing either (1) a period of 4 weeks or longer after inoculation of the virus under consideration, or (2) a shorter period if antibody response appeared in the specimens which were available. This definition presupposes that if antibody production is to become detectable at all it will be
Figure 1. Anti-West Nile antibody curves for individual patients following inoculation of West Nile virus. Complement-fixation technique.

Figure 2. Anti-West Nile antibody response following inoculation of West Nile virus. Averaged data from all patients. Complement-fixation technique. Lower curve shows antibody titers. Upper curves indicate percentage of patients having diagnostic and suggestive antibody rises.

detectable within 4 weeks of virus inoculation. The data to be presented indicate that this supposition is true for both complement fixing and neutralizing antibodies in the great majority of patients. A diagnostic antibody rise is considered to be 2 or more two-fold serial dilutions (2 tubes) by the complement-fixation technique, or 1.7 or more logs by the neutralization technique (neutralizing index of 50 or greater). Suggestive antibody rises are considered to be at least 1 tube and 1.0 log respectively.

West Nile Virus

From 20 patients inoculated with West Nile virus (original isolate of Smithburn et al.) 17 adequate series of sera were available. Five of these (29%) showed no complement-fixing antibody rise. The other 12 are individually presented in Figure 1. Significant antibody rises against West Nile virus often occurred within 2 weeks after inoculation, maximum titers of 1:16 to 1:64 appeared between 2 and 8 weeks, and most titers fell off slightly shortly after the maxima were reached. The over-all pattern of complement-fixing antibody formation is pictured in Figure 2 which includes all tested sera from all patients. Ignoring minor fluctuations, these data indicate that by 4 weeks after inoculation the mean antibody titer has reached its maximum of 13 tubes, and the percentage of patients showing antibody rises is at or near maximum. After 8 weeks a drop in mean antibody titer is noted.

Figures 3 and 4 show comparable data for neutralizing antibodies in a group of 11 patients. Two points of difference from the complement-
WEST NILE ANTIBODIES
NEUTRALIZATION TECH.
II PATIENTS

ANTIBODIES NO. LOGS. NEUTRALIZED

DAYS AFTER VIRUS INOCULATION

Figure 3. Anti-West Nile antibody curves for individual patients following inoculation of West Nile virus. Neutralization technique.

fixing data are noteworthy: (1) two patients who had not produced detectable neutralizing antibodies by 4 weeks did so by 6 weeks; (2) there is no tendency for neutralizing antibodies to fall from their maxima during the duration of this study.

In addition to the above neutralization studies which were done in mice in the routine manner with undiluted serum and serial dilutions of virus, an additional study with sera from 17 patients was carried out using the technique of serial dilutions of serum with a single dose of virus (50 to 100 LD50 doses per mouse). There was good agreement between these two neutralization techniques with respect to presence or absence of antibodies and the time of first appearance of antibodies.

Egypt 101 Virus

From 50 patients inoculated with the Egypt 101 isolate of West Nile virus 30 adequate series of sera were available. Of these, 3 patients (10%) developed no detectable antibodies. Figure 5 illustrates antibody curves in terms of serum-dilution titers for a selected group of 14 patients whose sera had no anti-complementary or anti-normal mouse brain antibodies at 1:8 dilution. Maximum titers ranged from 1:16 to 1:256. Figure 6 presents the over-all pattern of complement-fixing antibody formation in averaged data from the entire group of 50 patients. A diagnostic titer was demonstrated in only 2 patients at 3 weeks, but by 4 weeks 60% of patients had diagnostic titers and 80% had at least suggestive titers. The tendency for complement-fixing antibodies to fall after 6 to 8 weeks is again seen. In fact 2 patients had no detected serum antibodies 2 to 3 months after virus inoculation although diagnostic levels of antibody were present in the sera drawn at 3 to 5 weeks after inoculation.

Thirteen of the 17 patients studied produced diagnostic levels of neutralizing antibodies. Data are presented in Figures 7 and 8. Of the 4 patients who failed to show diagnostic levels of neutralizing antibody, one had a diagnostic titer of complement-fixing antibodies, but the other three patients had none. In one patient a significant drop in neutralizing antibodies occurred but the titer was still above the diagnostic level in serum collected 8 months after inoculation.

Of the 13 patients included in Figure 7, 4 were studied using Egypt 101 virus as antigen and mice as the test animal. These four are repre-
Figure 5. Anti-Egypt 101 antibody curves for individual patients following infection by Egypt 101 virus. Complement-fixation technique. No patient with anti-complementary or anti-normal mouse brain activity is included in this figure.

Figure 6. Anti-Egypt 101 antibody responses following infection by Egypt 101 virus. Averaged data from all patients. Complement-fixation technique. Lower curve shows mean serum antibody titers. Upper curves indicate percentage of patients having diagnostic and suggestive antibody rises.
Figure 7. Antibody curves for individual patients following infection by Egypt 101 virus. Neutralization technique. For patients indicated by circular symbols the antigen was Egypt 101 virus, titered in mice. For other patients the antigen was West Nile virus (original isolate) titered in chick embryos.

Figure 8. Neutralizing antibody responses following infection by Egypt 101 virus. Averaged data from all patients studied by serial virus dilution method in mice or chick embryos.

From 20 patients studied for complement-fixing antibodies after inoculation of Ilhéus virus, 18 had adequate series of sera. Of these, 2 developed no detectable antibodies against Ilhéus virus and 8 showed only suggestive (1 tube) rises. Antibody curves of 9 patients whose sera showed no anti-complementary or anti-normal mouse brain activity are presented in Figure 9 in terms of serum dilutions. Maximum titers ranged from 1:8 to 1:128. Two patients (not included in Figure 9) had a 1:8 titer of antibodies against Ilhéus virus prior to inoculation of this virus. Neither of these 2 had received experimental inoculations of other viruses prior to the date of these sera, nor was there any

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ILHEUS ANTIBODY PRODUCTION
C.F. TECHNIQUE 9 PATIENTS

Figure 9. Anti-Ilhéus antibody curves for individual patients inoculated with Ilhéus virus. Complement-fixation technique. No patient with anti-complementary or anti-normal mouse brain activity is included in this figure.

Figure 10. Anti-Ilhéus antibody responses following inoculation of Ilhéus virus. Averaged data from all patients. Complement-fixation technique. Lower curve shows mean serum antibody titers. Upper curves indicate percentage of patients having diagnostic and suggestive antibody rises.

known previous infection or immunization with viruses of this type. Averaged data for all 20 patients are presented in Figure 10.

Only 6 patients were studied for Ilhéus neutralizing antibodies by the routine virus-dilution technique. All showed an antibody rise. Data are presented in Figure 11. An additional group of sera from 11 patients was studied for neutralizing antibodies using undiluted serum and a single virus dose of approximately 100 LD_{50}. Each of these patients developed sufficient antibodies to be detected by this method.

Newcastle Disease Virus

Production of antibodies following inoculation of Newcastle disease virus was studied by the complement-fixation technique in sera from 10 patients, and by neutralization technique in sera from 5 patients. Both techniques showed diagnostic antibody rises in every patient studied. Data are presented in Figures 12, 13, and 14. All patients represented in Figure 14 are designated by the same symbols in Figure 12. Antibody titers are much greater than were seen with the viruses previously discussed. Maximum titers for complement-fixing antibodies ranged from 1:64 to 1:1024, and for neutralizing antibodies 2.4 to 6.5 logs. The difference in behavior of complement-fixing and neutralizing antibodies is
Figure 11. Anti-Ilhéus antibody responses following inoculation of Ilhéus virus. Neutralization technique.

Figure 12. Anti-Newcastle antibody curves for individual patients following inoculation of Newcastle disease virus. Complement-fixation technique. All patients studied are included in this figure.
striking. Complement-fixing antibodies rose very abruptly so that all patients had at least suggestive titers by 1 week after inoculation and the maximum mean titer was reached at 3 weeks, but they also fell rapidly in 3 of the 4 patients from whom sera adequate for such study were available. Neutralizing antibodies were not detected at 1 week after inoculation, did not reach a peak until 5 weeks or longer after inoculation, and no drop in titer was observed. Hemagglutination inhibition studies were not done.

Other Viruses

Adequate sera were available from three patients following inoculation of Bunyamwera virus. Complement-fixation could not be demonstrated in any of these using two antigen preparations made by the benzene extraction technique. However, by using crude mouse brain antigens, a rise of 1 and 2 tubes was demonstrated in the 6 and 9 week specimens respectively in one patient, but earlier sera were negative. Neutralization of 1.6, 2.1, 2.2, and 2.6 logs of virus was demonstrated in sera collected from this same patient at 4, 5, 6, and 9 weeks after inoculation. This patient was the only patient in whom this virus propagated and caused clinical illness (for case report see reference 1).

Adequate sera were available from one of two patients studied after inoculation of Br I virus. Only complement-fixation studies were attempted. Antibody titers were negative, 1:8, and 1:32 on days 21, 28, and 49 respectively. Virus propagation was not demonstrated in this patient.

R virus was inoculated into only one patient and sera spanned a period of 3 weeks only. No complement-fixing antibodies were demonstrable. A virus, assumed to be R virus, was recovered from the blood of this patient as late as day 10.

Sera were available from 1 patient for 31 days after inoculation of Semliki Forest virus. No complement-fixing antibodies were found. Virus propagation was not demonstrated.

Neutralizing antibodies sufficient to protect mice against at least 41.7 and 47.2 LD₅₀ doses of a street strain of rabies virus were produced by two patients following inoculation with the Flury strain (40th-50th egg passage) of rabies virus. No clinical indication of rabies virus infection occurred in these patients. These anti-
body studies were performed by Dr. Hilary Koprowski.

Comparison of Antibody Detection Techniques

The above data indicate that neutralization and complement-fixation techniques were equally satisfactory for demonstration of serum antibodies. The hemagglutination inhibition technique, judging by very limited studies, also seems satisfactory. The similarity of antibody curves as determined by four different techniques is illustrated in Figure 15. In this figure the ordinate scales have been so chosen that the antibody curves roughly coincide. These curves, and the data presented in previous figures, suggest that in the first few weeks following Egypt virus infection a neutralizing capacity of 1 log is roughly equivalent to a complement-fixing titer of 1:8 and a hemagglutination-inhibition titer of 1:20. Similarly a 3 log neutralizing titer is roughly equivalent to a 1:32 complement-fixing titer, and to a 1:320 hemagglutination-inhibition titer. Obviously considerable variation from these suggested equivalents would be expected, and has been observed in these sera.

With few exceptions, detectable titers of complement-fixing antibodies appeared slightly earlier than neutralizing antibodies with all viruses. Complement-fixing antibodies tended to fall 2-3 months after virus inoculation, but there was no decrease in neutralizing titers within the time period (8 months maximum) covered by this study.

Cross Reactions Between Viruses

The search for common antigenic components among viruses was approached in three ways: (1) sera shown to have antibodies against the virus with which the patient had been inoculated, were tested with other virus antigens to see if the antibodies would cross-react; (2) following inoculation of a patient with a second virus, sera were studied for changes in titer of antibodies against the first virus, coincident with the appearance of antibodies against the second virus; (3) after a patient had developed antibodies against two or more inoculated viruses, a repeat injection of one of the viruses was given and antibodies against both viruses were studied in order to see if there was a booster effect upon the antibodies against a virus which was not re-injected as well as upon antibodies against the re-injected virus.

The antigenic similarity of Egypt 101 and West Nile viruses was confirmed by repeated demonstrations of equal titers of complement-fixing and/or neutralizing antibodies against both of these viruses following infection with Egypt 101 virus (Fig. 15). The antigenic similarity of Egypt 101 and Egypt 21 viruses was indicated by the development of complement-fixing antibodies against Egypt 101 virus in serum of a patient who was infected with Egypt 21 virus. It was also suggested by the failure to induce infection by inoculation of Egypt 101 virus into a patient who had previously been infected with Egypt 21 virus. It was also suggested by the failure to induce infection by inoculation of Egypt 101 virus into a patient who had previously been infected with Egypt 21 virus (whereas inoculation of Egypt 101 virus into 85 other patients failed only 4 times to induce infection).

Sera from 5 patients who were inoculated with
Egypt 101 virus only, and who had developed neutralizing antibodies against this virus, failed to show any neutralizing antibodies against Ilhéus virus. Similarly, among ten patients who had been inoculated with Egypt 101 virus only, and whose sera showed complement-fixing antibodies against this virus, nine showed no complement-fixing anti-Ilhéus antibodies. The other patient however showed a convincing and progressively increasing complement-fixing antibody titer against Ilhéus virus following the inoculation of Egypt 101 virus (patient I. B. 49F in Figure 16). It is of interest that this same patient showed the promptest and highest antibody rise against Egypt virus that was seen among all of the patients studied (Figure 5, patient designated by open squares connected by solid lines). The possibility of a laboratory error in test antigen is excluded by occurrence of the expected antibody reactions in known Ilhéus positive, Ilhéus negative, and Egypt positive sera tested simultaneously with the sera of this patient. The only observed instance of a change in anti-West Nile (or Egypt) titer concurrent with development of anti-Ilhéus antibodies was the transient appearance of a 1:8 anti-West Nile titer in patient E. S. 58F (Figure 16) 2 weeks after Ilhéus inoculation. Furthermore, prior infection with Egypt 101 or West Nile virus did not prevent subsequent infection (indicated by viremia for 2 to 7 days) by Ilhéus virus in 5 patients. Two patients who were initially inoculated with West Nile virus and then with Ilhéus virus, were, after a period of several months, re-inoculated with the Egypt 101 strain of West Nile virus. In both patients there was a prompt production of antibodies against both the Egypt 101 and the original strain of West Nile virus, and in addition both patients developed a suggestive increase in antibodies against Ilhéus virus (Figure 16, patients E. S. 58F and S. C. 49M). These results indicate that there is no major degree of cross-antigenicity between Ilhéus virus and West Nile (and Egypt) virus, but a very minor antigenic component in common seems probable.

Sera from two patients who had been inoculated with Egypt 101 virus and later (one and 4 months later respectively) with Ilhéus virus, were studied for antibodies against Japanese encephalitis virus. One of these patients developed complement-fixing antibodies and both developed neutralizing antibodies against Japanese encephalitis virus. Significant titers (1:96 complement-fixing titer and between 2 and 3 logs neutralizing titer) were reached only after the Ilhéus inoculation, suggesting that both the Egypt virus and the Ilhéus virus have some antigenic component in common with Japanese encephalitis virus. Evaluation of the validity and significance of this observation must await further studies. These same sera showed no complement-fixing antibodies against western equine encephalitis virus.

Cross-reactions with antisera against other single viruses could not be studied in the material available because all patients from whom serial serum specimens were available had received Egypt or West Nile virus in addition to other viruses which may also have been inoculated.

4 Complement fixation studies against Western equine and Japanese viruses were performed by Dr. Joseph Melnick of Yale University. Neutralization studies against Japanese encephalitis virus were performed by Dr. William L. Pond of the Army Medical Service Graduate School.
However, indirect and fragmentary data are available which suggest a lack of antigenic relationship between several of the viruses studied. In no patient did the development of anti-Newcastle disease virus antibodies cause any significant change in the titer of previously formed West Nile or Ilhéus antibodies (Figure 16). Neither did inoculation of Bunyamwera virus affect West Nile or Ilhéus antibody titers. Vaccinia virus inoculation (one patient) did not affect anti-Egypt or anti-Ilhéus antibodies. In one patient who had a 1:8 titer of antibodies against Egypt 101 virus subsequent inoculations of Semliki Forest virus, rabies virus, and dengue virus, caused no change in the anti-Egypt antibody titers.

**Correlation of Serologic, Virologic, and Clinical Observations**

Scatter diagrams were prepared for comparison of maximum complement-fixing antibody titer with persistence of viremia, and with severity of clinical illness due to virus infection. The severity of clinical illness was graded on a scale of 0 to ++ + +, according to the definitions used in a previous report (3). West Nile, Egypt 101, and Ilhéus viruses were studied separately by this method, using all adequate series of sera. The resulting scatter diagrams showed random distribution of points, with no suggestion of correlation between these factors. During the course of our clinical studies we were struck by the fact that 4 of our patients in whom occurred definite or suggestive evidences of encephalitis produced no detectable serum antibodies. (Sera were available for only 3 weeks from 2 of these patients, but were available for at least 4 weeks from the other 2.) This suggested that patients with poor antibody producing ability might be less well able to fight off the virus infection. This generalization however, is not correct, since the patient who had the most severe reaction to Egypt virus infection produced antibodies in a titer of 1:64, and several other patients who produced no detectable antibodies had no clinical reaction to the virus infection and a less than average duration of viremia. It is difficult to abandon the concept that antibody forming ability bears some relation to the capacity of the host to fight off an invading micro-organism, but it seems clear from these data that antibody forming ability is not the major factor controlling the reaction of the host to infection with these viruses.

A comparison of antibody production by patients with lymphomatous disease, and patients with other types of neoplastic disease is of interest because of our previously reported observation that patients with lymphomatous disease tended to have longer persistence of viremia and a more severe clinical reaction to infection with Egypt 101 virus than did patients with other types of neoplastic diseases. Among the 30 patients on whom adequate sera were studied after infection, there were only 2 patients with lymphomatous diseases (one lymphosarcoma and 1 reticula cell sarcoma). While this number is insufficient to permit generalization, it is of interest that these 2 lymphoma patients failed to show any detectable anti-Egypt antibody, whereas this was true of only 4 (14%) of patients with other kinds of cancer.

In no patient did detectable antibodies appear in the serum while virus was still detectable in the blood. The interval between the cessation of viremia and the first appearance of serum antibodies was as short as 1 week in a few patients, but in most patients was 2 or 3 weeks.

**Miscellaneous Observations**

Five patients were inoculated with Ilhéus virus and 4 with the original strain of West Nile virus while receiving intensive therapy with cortisone. This was done in hopes of increasing the percentage of infections which could be established with these viruses, and was based upon the observations that treatment of mice with cortisone increased their susceptibility to lethal infection with these viruses (26) and increased slightly the rate of virus propagation (27). The dose of cortisone was usually 200 mg by mouth every 6 hours for 3 days before and 7 days after virus inoculation. The percentage of infections established in these cortisone-treated patients, and the duration of viremia, was not impressively greater than in patients receiving the same viruses but not treated with cortisone. Neither did antibody formation differ significantly in the patients inoculated with West Nile virus (both treated and untreated patients are included in the data presented in Figures 1 to 4). However, mean antibody titers and rate of antibody production in the cortisone-treated patients who received Ilhéus virus were suggestively greater than among non-cortisone treated patients.
ILHEUS ANTIBODY FORMATION WITH AND WITHOUT CORTISONE TREATMENT

Figure 17. Mean antibody responses following Ilhéus virus inoculation during intensive cortisone treatment, and in non-cortisone treated patients.

Because of this difference the 5 cortisone-treated patients have not been included in the data concerning Ilhéus virus in Figures 9 to 11, in Table I, or in the previous discussions. The number of cases in these groups is insufficient to bear statistical analysis, but the data suggest that the maximum doses of cortisone which can be used in the treatment of man are not sufficient to interfere with his antibody forming ability.

Heterophile antibodies were studied in serial serum specimens from 6 patients following inoculation of viruses (West Nile and/or Ilhéus) prepared as mouse brain suspensions. Three of these patients had heterophile antibody titers not in excess of 1:56 prior to virus inoculation and no increase in heterophile antibody titer thereafter. Another patient had a pre-inoculation titer of 1:224, but had no further increase following virus inoculation. The remaining 2 patients, however, had initial titers of 1:12 and showed marked increases in heterophile antibodies, reaching maximum titers of 1:3584 in 1 patient and 1:1792 in the other patient. These extremely high titers persisted for 6 and 8 weeks respectively and then began to fall toward normal. Absorption studies showed that this heterophile antibody could be removed by absorption with guinea pig kidney or with normal mouse brain tissue. In 1 of these patients studies of antinormal mouse brain antibodies were performed by complement fixation technique and it was found that there was a progressive increase in antibodies against normal mouse brain from negative (less than 1:20) prior to virus inoculation to a maximum of 1:80 coincident with the highest heterophile titers. Sera were not available for study of anti-normal mouse brain antibodies in the second patient who showed high heterophile antibodies. These results suggest the presence of heterophile antigens in mouse brain, and suggest that virus vaccines prepared from mouse brain are a potential cause of elevated heterophile antibodies (true Forssman type) in man.

Tissues obtained from one patient at autopsy (9 weeks after West Nile virus infection) were studied for the presence of tissue-fixed antibodies. These tests were performed by routine neutralization techniques in mice by using a 10 per cent suspension of tissue in place of serum. Serum collected from this same patient at autopsy neutralized 2.6 logs of West Nile virus. Less than 2.0 logs of virus were neutralized by 11 specimens of nervous tissue, lymph nodes, and striated muscle. Between 2.0 and 2.9 logs of virus were neutralized by metastatic tumor from lung, normal liver, and a supraclavicular lymph node. Tumor tissue from liver neutralized 3.4 logs of virus. Since all of these tissue specimens neutralized at least 1 log of virus, it appears that at least part of this effect was due to some non-specific neutralizing component of tissue or merely contaminating serum antibody, rather than specific tissue-fixed antibody. However, the fact that 4 of the tissues neutralized more virus than did serum suggests that in these 4 tissues at least there may have been true tissue-fixed antibody. Limitation of our tissue studies to this single patient, and failure to study these tissues against other viruses, make the results difficult to interpret.

SUMMARY AND CONCLUSIONS

Serial specimens of human serum were studied for the presence of anti-virus antibodies following the intentional inoculation of viruses as part of a clinical experimental study of the effects of these
virus infections upon advanced cancer. Included in the study are sera from 20 patients following the inoculation of Smithburn's isolate of West Nile virus, and 50 patients following inoculation of the Egypt 101 isolate of West Nile virus; 20 patients following Ilhéus virus, 10 patients following Newcastle disease virus, and 1 or more patients following inoculation of Bunyamwera, Semliki Forest, Br I, Rabies, and R viruses, and the Egypt 19 and Egypt 21 isolates of West Nile virus. Almost all sera were studied by complement fixation technique, and many were studied by neutralization techniques. Sera from 3 patients were also studied for anti-hemagglutinating antibodies against West Nile virus.

All of the techniques included in this study appeared to be approximately equivalent in their capacity to detect and quantitate antibody formation. In general, these viruses caused production of detectable antibodies in a majority of patients between 3 and 4 weeks after virus inoculation. Thereafter neutralizing antibody titers remained generally stable for the duration of this study, which rarely exceeded 6 months, but complement-fixation antibody titers often fell after 3 to 4 months. Newcastle disease virus differed from the other viruses studied in that the onset of complement-fixing antibody formation was more rapid and the titer of serum antibodies was greater than seen with the other viruses studied. This difference, however, was not observed when the neutralization technique was used.

These data are in agreement with the conclusion that Egypt 101 virus and West Nile virus are antigenically identical, and data from a single patient is compatible with the view that Egypt 21 virus is also identical with West Nile virus. No satisfactory data were available for any conclusion concerning the Egypt 19 virus. There was no major cross-reaction between the West Nile viruses and Ilhéus virus. Antibodies against Japanese encephalitis virus were demonstrated in 2 patients following inoculation of Egypt 101 and Ilhéus virus. These same sera did not react with Western equine encephalitis virus.

There were no data which suggested that any of the patients included in this study had had any previous exposure to West Nile or any antigenically similar virus. A few patients showed suggestive, but not conclusive, evidence of previous contact with antigenic material similar to Ilhéus and Newcastle disease viruses.

Antibody formation occurred following inoculation of virus whether the virus propagated or not. Among patients inoculated with a single virus, there was no apparent correlation between the amount of virus propagation and the magnitude of the antibody response. However, when comparing different viruses, it appeared that those viruses which were more infective for man also stimulated greater antibody production. There were suggestive data to indicate that antibody formation in patients with lymphomatous neoplasms is poorer than in patients with other types of neoplastic disease. There was no apparent relationship between antibody forming ability and various types of cancer other than lymphoma.

REFERENCES
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