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MIXED INFECTIONS OF VENEZUELAN
EQUINE ENCEPHALOMYELITIS
AND Q FEVER IN
MACACA MULATTA MONKEYS

DECEMBER 1962

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK
The work reported here was performed under Project 4B11-02-068, Aerobiological Research, Task -01, Stability and Virulence of BW Aerosols. The expenditure order was 2201301.

Nicholas L. Pollok, III

Aerobiology Division
DIRECTOR OF BIOLOGICAL RESEARCH

Project 4B11-02-068

December 1962
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**ANIMAL RESEARCH**

In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society of Medical Research.
The author wishes to thank Mr. William C. Patrick, III, Process Development Division, for providing the viral and rickettsial suspensions used in this study, and Mr. Nicholas Hahon, Aerobiology Division, for his many helpful suggestions in preparing this paper. I wish to acknowledge the technical assistance of Mr. Lee Carey, Mr. Meredith Blank, and Mr. Lawrence Shaw.

ABSTRACT

Preliminary studies suggest that the Macaca mulatta monkey, when exposed simultaneously to aerosols of the Trinidad strain of Venezuelan equine encephalomyelitis virus (500 to 700 respiratory infectious doses) and the AD strain of Coxiella burnetii (10 to 30 respiratory infectious doses), exhibits a concurrent mixed infection with VEE and Q fever. The course of the dual infection in the monkey is characterized by an abrupt early onset of viremia and febrile response (48 to 72 hours) to VEE that lasts approximately eight days. The onset of Q fever, as characterized by a rickettsemia, occurs in the final stages of VEE infection (seventh to eleventh days); recovery of rickettsiae from monkey blood persists beyond the fifteenth day after aerosol exposure. The presence of VEE virus-neutralizing antibody and Q fever complement-fixing antibody in 28-day convalescent serum supports these findings.
DIGEST

The results of this preliminary study on mixed infections of Venezuelan equine encephalomyelitis and Q fever in Macaca mulatta monkeys suggest the following:

(a) Respiratory exposure of M. mulatta monkeys to aerosols of VEE virus leads to a VEE infection in these animals. Infection was based on evidence of viremia, and the existence of significant levels of virus-neutralizing antibody after 28 days.

(b) Respiratory exposure of M. mulatta monkeys to aerosols of C. burnetii leads to a Q fever infection. Recovery of the rickettsiae from the blood and the existence of significant levels of CF antibody after 28 days substantiate this finding.

(c) Respiratory exposure of M. mulatta monkeys to separate aerosols of VEE virus and of C. burnetii rickettsiae leads to a concurrent mixed infection of VEE and Q fever. Both infections are confirmed by the recovery of the respective etiologic viral and rickettsial agents from the blood and by the existence of specific antibodies against these infectious agents after 28 days.

(d) No evidence of cross-infection was found in either the VEE virus control or the Q fever control M. mulatta monkeys.
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I. INTRODUCTION

The concept that the course of disease may be modified by the activity of another infectious agent in the same host was conceived over a century and a half ago. Suggestive evidence to support this hypothesis was proffered by Jenner who, in 1804, observed that the clinical course of vaccinial infection may vary with concurrent herpes simplex.¹

Schlesinger² states that, when two pathogens attack the same host, one of four things may happen:

(a) The two pathogens may develop independently and produce characteristic manifestations of each infection (dual infection). Such independent growth has been recorded in animal tissues by Syverton and Berry³ with vaccinia, herpes, and B virus.

(b) The pathogen may undergo genetic interaction and produce progeny with new properties (recombination). Genetic recombination has been demonstrated unequivocally with influenza virus by Burnet⁴ and with vaccinia by Fenner and Comben.⁵

(c) One pathogen may so modify cells or the tissues of a host that a second one cannot multiply normally or produce characteristic injury (interference). Hoskins⁶ first reported a generalized interference phenomenon with neurotropic and viscerotropic strains of yellow fever virus. Findlay and MacCallum⁷ showed, in their studies with combined inoculations of Rift Valley fever and yellow fever viruses, that interference occurred between viruses that are not antigenically related. Mika et al⁸ further explored interference on the basis of influence of immunological factors in combined Brucella suis and Coxiella burnetii infections. Henle's review⁹ of the interference phenomena between animal viruses describes numerous examples.

(d) When two pathogens are inoculated into the same host, one may enhance the virulence of the other (synergism). Horsfall and Lennette,¹⁰ in studies of concurrent infection of ferrets with human influenza and canine distemper viruses, showed that the combined infection with both viruses produced but small amounts of influenza-neutralizing antibody, and influenza virus persisted in undiminished concentration in the ferret's lung throughout the course of the infection. In contrast, ferrets infected with influenza virus alone rapidly produced considerable quantities of neutralizing antibody, and after the sixth day virus was not demonstrable in their lungs. Francis and DeTerregrosa,¹¹ in their studies of combined intranasal infection of mice with Hemophilus influenzae suis and influenza virus, showed that superimposing bacteria that ordinarily do not survive in the respiratory tract upon an established infection with influenza virus promotes the capacity of the organism to establish itself, and adds to the severity of the disease. The evidence demonstrates as well that, under certain conditions, the virulence of organisms may be clearly enhanced by such a procedure.
The above phenomena show clearly that any studies involving mixed infections must consist of highly specialized conditions that include the selection of suitable pathogens, factors of dosage, timing, and the opportunity to infect the same host concurrently. Correspondingly, when attempting to establish a mixed infection in a host, one must consider the possibility of an interference between the two infectious agents. However, it is by no means a universal occurrence. With certain unrelated agents, interference never has been demonstrated. In most instances, mixed infection has been established by adequate tests for the presence and increase of both infectious agents.

Experimental results in this study will be determined in the light of these basic tests.

This study was initiated to determine whether the *Macaca mulatta* monkey can develop a mixed infection with Venezuelan equine encephalomyelitis (VEE) virus and the rickettsia of Q fever (*Coxiella burnetii*) when exposed to aerosols of these agents via the respiratory route.
II. MATERIALS AND METHODS

A. VIRUS

1. Evaluation of Pathogenicity - Trinidad Strain

The strain of VEE virus used in this study was isolated originally from a donkey by Randall\textsuperscript{1,2} in Trinidad and had undergone approximately 14 passages in embryonated chicken eggs.

For preliminary evaluation of the pathogenicity of the virus, serial tenfold dilutions of purified whole embryo material were made in Difco heart infusion broth (HIB), pH 7.1, and inoculated intracerebrally (IC) into each of ten 10- to 14-gram white mice (Swiss strain) in amounts of 0.03 milliliter. The titer ($3.36 \times 10^{10}$) was calculated according to the method of Reed and Muench\textsuperscript{1,3} and was expressed in terms of mouse intracerebral 50 per cent lethal doses per milliliter (MICLD\textsubscript{50} per milliliter). The aerosol spray culture was a $10^{-3}$ dilution of purified whole embryo material in HIB.

2. Neutralization Test

For virus neutralization tests, serial tenfold dilutions of a mouse brain-adapted strain of the same virus were mixed in equal parts with undiluted serum from the exposed animals, and inoculated intraperitoneally (IP) into each of five 10- to 14-gram mice in final amounts of 0.05 milliliter. Mice were observed for 14 days. The virus neutralization index was expressed as the antilogarithmic difference between the exponential titers of the immune and nonimmune sera, as determined by the Reed and Muench method.

B. RICKETTSIA

1. Evaluation of Pathogenicity - AD Strain

The AD strain of \textit{Coxiella burnetii} used in this study was originally isolated from cattle by Huebner \textit{et al.}\textsuperscript{4} in southern California and had undergone three passages in guinea pigs and approximately ten passages in embryonated chicken eggs.

The pathogenicity of \textit{C. burnetii} was based on the complement-fixation (CF) response of convalescent guinea pigs. The inoculum was suspended in HIB, pH 7.1, and it was inoculated IP in serial tenfold dilutions into each of five 250- to 350-gram Hartley strain guinea pigs in amounts of one milliliter. The titer of the rickettsiae ($3.17 \times 10^{10}$) was calculated according to the method of Reed and Muench, and was expressed in terms of a 50 per cent infectivity response in guinea pigs (GPIIPD\textsubscript{50} per milliliter). The aerosol spray culture was a $10^{-3.5}$ dilution of purified yolk-sac material in HIB.
2. Complement-Fixation Test

A formalin-inactivated antigen of C. burnetii (Nine Mile strain, Lederle Laboratories, New York, N.Y.) was used to detect the existence of complement-fixing antibodies in the sera of those animals exposed to C. burnetii. The diagnostic test is a micro-method modification of the Kolmer-Boerner test, which utilizes the overnight "icebox" incubation period for the fixation of complement.

C. AEROBIOLOGICAL PROCEDURES

Separate aerosols of VEE virus and C. burnetii were established in a 1500-liter rotating drum (3.5 rpm) by spraying cultures for five minutes with a modified Vaponefrin nebulizer. This nebulizer has been described in detail by Day. Aerosols were maintained at 50 per cent relative humidity and ambient temperatures of 21° to 26°C.

Prior to VEE virus aerosol exposure, 12 Macaca mulatta monkeys weighing an average of six pounds each were given intramuscular injections of a tranquilizer (Sernyl, 1.3 milliliters with two milligrams per milliliter). Because previous use of this drug showed no apparent respiratory depression, this procedure was a convenient adjunct to safe handling of the animals in the exposure unit.

Eight monkeys were exposed to an aerosol of VEE virus. Four of these were used as VEE virus controls; the remaining four monkeys were held for later exposure to Q fever. After VEE virus exposures, the rotating drum was simultaneously air washed and sterilized with ultraviolet light for one hour. Previous studies with this technique have shown that no VEE virus has been recovered from the drum after treatment with ultraviolet light for 50 minutes. Subsequent to drum sterilization, an aerosol of C. burnetii was sprayed into the drum. The remaining four VEE virus-exposed monkeys along with four normal monkeys (Q fever controls) were exposed to the rickettsial aerosol. Animals were exposed to aerosols for one minute.

During each animal exposure, aerosol samples were collected in Shipe impingers filled with HIB and olive oil antifoam. Aerosol samples were pooled and then assayed, depending on the specific pathogen, in an appropriate host. Assay procedures are discussed in Sections A and B.
D. CLINICAL STUDIES

Pre-exposure (base-line) blood serum samples were obtained from all 12 monkeys, and all sera were tested for any pre-existing antibody levels against the viral or rickettsial agents used in this study. Following aerosol exposures, animal temperatures were taken twice daily, and heparinized blood samples were drawn from each animal for 15 days. Viremia (2, 4, 6, 8, 10, 12, and 14 days) and rickettsemia (3, 5, 7, 9, 11, 13, and 15 days) studies were done. For the viremia studies, 0.2 milliliter of a 1:2 dilution of monkey blood and HIB was inoculated IP into each of five mice to detect VEE virus. For rickettsemia studies, 0.5 milliliter of a 1:2 dilution of monkey blood and HIB was inoculated IP into guinea pigs (three each) to detect infection with C. burnetii. Further studies included: total erythrocyte, leucocyte, and leucocyte differential cell counts; erythrocyte sedimentation rates (ESR). A final blood serum sample was obtained from each animal 28 days post-exposure for serological testing for specific antibody response to infection with VEE virus, C. burnetii, or both pathogens.

During this 28-day holding period, the monkeys were caged individually; however, all cages were held in the same cabinet system. To facilitate handling, all monkeys were tranquilized with Sernyl each morning before bleedings. The effect of this drug lasted approximately 30 minutes.
III. RESULTS AND DISCUSSION

The results and discussion of this work are reported as a mean response from each of the three groups of monkeys exposed to the various infectious agents, and any reference to individual members of a specific exposure group will be identified as such.

Serological tests on pre-exposure serum samples from the 12 monkeys showed that these animals had no antibodies against VEE virus or C. burnetii. Titrations of pooled impinger samples from each agent aerosol showed that the inhaled dose of VEE virus ranged from 5.63 to 6.92 x 10^3 MICLD<sub>50</sub> per monkey. The inhaled dose of C. burnetii ranged from 100 to 300 GPIID<sub>50</sub> per monkey. In this species of monkey, this represents approximately 500 to 700 median respiratory infectious doses of the Trinidad strain of VEE virus and 10 to 30 median respiratory infectious doses of the AD strain of C. burnetii, respectively.

At the earliest time of testing for viremia, forty-eight hours after aerosol exposures, VEE virus was isolated from the blood of both the dual-infection (VEE virus and C. burnetii) and the VEE virus control monkeys. Viremias persisted in both test groups at varying levels until the tenth day (Table I). Febrile responses (104°F) were observed in some of the monkeys in both groups on the third day after exposure, and by the fourth day all the monkeys in both groups were febrile. Coincident with a fall in animal temperatures on the ninth day, isolation of VEE virus was not possible beyond the tenth day. The course of VEE infection in the dual-infection monkeys did not appear to differ from that shown in the VEE virus control animals. Table II shows the course of viremia in all test groups, and the fever curves for these groups can be seen in Figures 1, 2, and 3.

| TABLE I. VEE VIRUS RECOVERIES FROM MACACA MULATTA MONKEY BLOOD<sup>a</sup>/ | Days After Virus Exposure | Mouse Mortality, Accumulated Dead/Total Inoculated |
| Test Group | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 28 |
| VEE CONTROLS | | | | | | | | |
| Monkey 1 | 5/5<sup>b</sup> | 5/5 | 2/5 | 0/5 | 1/5 | 0/5 | 0/5 | 0/5 |
| 2 | 5/5 | 5/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| 3 | 5/5 | 5/5 | 0/5 | 0/5 | 3/5 | 0/5 | 0/5 | 0/5 |
| 4 | 5/5 | 5/5 | 0/5 | 0/5 | 3/5 | 0/5 | 0/5 | 0/5 |
| VEE AND Q FEVER | | | | | | | | |
| Monkey 5 | 5/5 | 5/5 | 0/5 | 1/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| 6 | 5/5 | 5/5 | 0/5 | 0/5 | 4/5 | 0/5 | 0/5 | 0/5 |
| 7 | 5/5 | 5/5 | 5/5 | 0/5 | 3/5 | 0/5 | 0/5 | 0/5 |
| 8 | 2/5 | 5/5 | 4/5 | 0/5 | 1/5 | 0/5 | 0/5 | 0/5 |

<sup>a</sup> Blood diluted 1:2 in NIH and 0.2 milliliters inoculated IP in mice (10- to 16-gram).

<sup>b</sup> Brains from dead mice were ground and inoculated IC in mice (10 to 14 grams) to determine if death was due to VEE infection.
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a. Data reported on basis of four monkeys per test group.
b. Serum Neutralizing Index (SN1) > 4.83 log of VEE virus neutralized per milliliter of serum (67,700 MPLD30 VEE virus neutralized).
c. Complement-Fixation Test.
d. Death in guinea pigs due to VEE.
Figure 1. Mixed Infections Study with VEE Virus and *C. burnetii* in *Macaca mulatta* Monkeys.
Figure 2. Mixed Infections Study with VEE Virus and *Coxiella burnetii* in *Macaca mulatta* Monkeys.
Figure 3. Mixed Infections Study with VEE Virus and *C. burnetii* in *Macaca mulatta* Monkeys.
C. burnetii was isolated from the blood of two monkeys in the dual-infection group on the seventh and ninth days, respectively, and rickettsemias were evident in all the animals of this group by the eleventh day. (Table III). The Q fever control animals were not rickettsemic until the eleventh day. Rickettsemias persisted in both the dual-infection and the Q fever control groups after the fifteenth day (Table III). Febrile responses to C. burnetii infection in both test groups were inconclusive.

**TABLE III. COXIELLA BURNETII RECOVERIES FROM MACACA MULATTA BLOOD**

<table>
<thead>
<tr>
<th>Test Group</th>
<th>Days After Rickettsial Exposure</th>
<th>Guinea Pig Infectivity</th>
<th>Accumulated Infected/Total Inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>VEE AND Q FEVER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey 5</td>
<td>NS</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Monkey 6</td>
<td>NS</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Monkey 7</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Monkey 8</td>
<td>NS</td>
<td>0/1</td>
<td>1/3</td>
</tr>
<tr>
<td>Q FEVER CONTROLS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monkey 9</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Monkey 10</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Monkey 11</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Monkey 12</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

a. Blood diluted 1:2 in HIB and 0.5 milliliters inoculated IP in guinea pigs (250 to 350 grams).
b. Infectivity determined by presence of complement fixing antibodies in guinea pig serum.
c. No Sample. Guinea pigs died as a result of VEE infection.

Additional clinical studies used in this work were not too helpful in determining animal response to a dual infection, and as a result the information was fragmentary. However, erythrocyte sedimentation rates were on the whole more elevated, and for a longer period of time, in the dual-infection test animals than in either of the two control groups. Figure 4 illustrates the course of sedimentation rates. Total leucocyte counts show that a leucopenia (moderate) developed shortly (fifth day) after the onset of VEE virus infection in the dual-infection and the VEE control groups. This condition did not persist, for on the next day leucocyte counts in both groups of animals were on the rise. The Q fever controls gave a rather erratic leucocytic response during this 15-day bleeding period. Leucocyte differentials and leucocyte (total) counts are shown in Figures 5, 6, and 7.
Figure 4. Blood Sedimentation Rates for *Macaca mulatta* Monkeys Infected with VEE Virus and *Coxiella burnetii*.
Total WBC

Segmented Polymorphonuclear Leucocytes

Lymphocytes

Monocytes (Scale on Right Side of Graph)

Figure 5. Total Leucocyte and Leucocyte Differential Counts for VEE Control Monkeys.
Figure 6. Total Leucocyte and Leucocyte Differential Counts for VEE and C. burnetii Exposed Monkeys
Figure 7. Total Leucocyte and Leucocyte Differential Counts for C. burnetii Control Monkeys.
Twenty-eight-day blood samples were negative for VEE virus and *C. burnetii* in all 12 monkeys. Serological tests of blood serum samples indicated that eight monkeys had been infected with VEE virus. These animals were from the dual-infection and the VEE virus control groups. A serum neutralization index in excess of 67,000 was demonstrated for each animal exposed to VEE virus. Virus-neutralizing antibodies against VEE virus were not detected in the Q fever control monkeys. A serodiagnosis of Q fever by complement-fixation test was obtained for the dual-infection and the Q fever control groups of monkeys. The VEE virus control monkeys demonstrated no CF antibodies against *C. burnetii* after 28 days of observation. Table II lists the individual animal serologic responses to specific infectious agents.

In view of the isolations of VEE virus and *C. burnetii* rickettsiae plus the formation of significant levels of specific antibody against these two pathogens in the blood of the dual-infection group monkeys, these data support the assumption that a concurrent mixed infection existed in these animals.

This, following a simultaneous respiratory challenge to both infectious agents, the abrupt onset of VEE in the monkey was characterized by viremia and fever. The incubation period in each instance was short, limited to several days. Viremia and fever occurred early (48 to 72 hours), and, depending on the animal, persisted for approximately eight days, with varying levels of virus being recovered. As mentioned previously, one monkey was rickettsemic on the seventh day and another monkey on the ninth day. However, rickettsiae were detected in all the monkeys on the eleventh day, and rickettsemias persisted past the fifteenth day. In the absence of a febrile response in these animals, there were no outward signs that infection with *C. burnetii* was in progress. The diagnosis of Q fever was later determined by serologic tests (CF).

In general, the course of VEE virus infection in the dual-infection monkeys did not seem to differ from that in the VEE virus control animals. Both groups were febrile, viremic, and formed significant levels of virus neutralizing antibody against VEE virus. And the antibody levels in the dual-infection group appeared not to be suppressed as a result of the infection with *C. burnetii*. The course of Q fever in the dual-infection animals again appeared to parallel the response seen by the Q fever controls. Whether the earlier onset of Q fever in some of the dual-infection animals was caused by the already existing VEE virus infection or was the variation in the response of individual animals to Q fever infection is not known. In any case, both groups were rickettsemic, and CF antibodies against *C. burnetii* were detected in significant titers.
Future studies of mixed infections, and in particular those involving VEE and Q fever, will explore a possible early onset of one disease as affected by the existence of another disease in the same host. Another factor relative to this study is the site of viral and rickettsial multiplication in the host's cells, and if such multiplication takes place in the same cell, what portion of the cell is utilized as a multiplication site by the virus or the rickettsia. It is hoped that the use of fluorescent antibody techniques will give some of the answers to these questions. Likewise, to be determined in future studies, is the duration and extent of rickettsemia in the monkey, since C. burnetii was still present in the blood on the fifteenth day. And finally, the matters of agent-exposure sequence and factors of dosage variance are to be investigated to determine what roles these factors play in mixed infections of VEE and Q fever in monkeys (Macaca mulatta).
LITERATURE CITED


15. BWL Technical Memorandum 9-15, "Studies on the Ability of Pasteurella tularensis to Survive in Large and Small Particles at Low Relative Humidity," Aerobiology Division, Fort Detrick, Frederick, Maryland, June 1960. UNCLASSIFIED.
MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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