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Complement-Requiring Neutralizing Antibody in Guinea Pigs with Primary and Recurrent Genital Herpes (41952)

Z. M. ZHENG[1] AND G. D. HSIUNG*,†

*Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut 06510, and †Virology Laboratory, Veterans Administration Medical Center, West Haven, Connecticut 06516

Abstract. Guinea pigs inoculated intravaginally with herpes simplex virus type 2 (HSV-2) strain 1868 produced a serum complement-requiring neutralizing (CRN) antibody during primary acute infection, i.e., 10 days postinoculation. The CRN antibody titers in the guinea pig sera decreased to less than 1:10 after heating at 56°C for 30 min. It was found that 32 units of complement were necessary to obtain a satisfactory HSV-2 neutralizing antibody titer. Nonheated sera significantly reduced virus infectivity titers when mixed with 3.5 $\log_{10}$ PFU of HSV-2 and incubated at 37°C for 20 to 60 min ($P < 0.001$), whereas the same sera after heating at 56°C for 30 min showed no inhibitory effect. Only 27.3% of infected guinea pigs had low serum non-CRN antibody titers ranging from 1:20 to 1:40. In addition, no evidence of increase in CRN antibody titers was noted during spontaneous recurrent genital herpes infection.

In the routine procedure for virus neutralization tests, it is the common practice to heat sera at 56°C for 30 min, since most virus neutralization tests do not require the presence of complement. However, in certain instances including tests for arbovirus (1), Newcastle disease virus (2), and human cytomegalovirus (3), complement is required for neutralization. The need for complement in neutralization tests for HSV has been recognized in rabbits (4, 5), guinea pigs (6) and humans (6), but has not been routinely used in the detection of HSV antibody by the neutralization test; thus, low antibody titers following HSV infection have been reported (7, 8).

Genital herpes in the guinea pig model has been used in studies of latent infection (9) and the effect of antiviral agents (10–12) in our laboratory during the past several years. In an early study (9), an attempt was made to study antibody response in the guinea pigs following genital infection. It was recorded that complement was added to the guinea pig sera in the neutralization test in order to demonstrate the neutralizing antibody titer to HSV-2. However, the specific function of the complement in the neutralization test for HSV was not clear. In order to investigate the mechanism and specific function of complement in HSV neutralization tests, a systematic study was undertaken using serial serum samples taken from guinea pigs following primary genital infection with HSV-2. The results are included in the present report.

Materials and Methods. *Virus stocks.* HSV-2 strain 1868 was used throughout the study. This virus strain was originally isolated from genital lesions and typed by cell culture selection, 5-bromovinyl-2-deoxyuridine (BVDU) sensitivity and plaque reduction neutralization tests (13). Virus stocks were prepared in guinea pig embryo (GPE) cell cultures. When infected cell monolayers showed advanced CPE, they were frozen and thawed and virus suspensions were clarified by centrifugation. The virus-containing supernatant was frozen in aliquots at −70°C with 10% DMSO.

Cell cultures and virus assay. Primary GPE cells were prepared from guinea pig embryos at 30 days of gestation as previously described (9). GPE cells were grown in Eagle's minimum essential medium (MEM) containing Hanks' balanced salt solution (HBSS), and 10% heat-inactivated newborn donor calf serum (NDCS). Confluent cell monolayers were maintained in MEM containing Earle's

[1] Z. M. Zheng is a visiting research scientist from the Virus Research Institute, Hubei Medical College, Wuhan, Hubei, People's Republic of China.

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balanced salt solution (EBSS) and 2% NDCS. The HSV-2 infectivity titers were determined by plaque formation in GPE cells growing in plastic plates overlaid with 0.5% methylcellulose in MEM–EBSS and 10% NDCS. The infected cells were fixed and stained as previously described (13).

Complement. Guinea pig complement was purchased commercially from Gibco (Grand Island, N.Y. 14072). Complement units were determined by hemolysis assay (14). No specific HSV-2 antibody in the complement could be determined using the neutralization test.

Guinea pig inoculation, virus isolation from the genital tract. Young female Hartley guinea pigs weighing 450–500 g were used in this study. Animals were inoculated intravaginally under ether anesthesia with 0.1 ml of HSV-2 strain 1868 containing 10^5 PFU as previously described (12). The severity of clinical illness was scored daily. Virus excretion from the genital tract was determined by assaying vaginal swabs taken on days 3, 5, 7, 10, and 13 using GPE cell monolayers. Some animals were monitored daily for development of recurrent genital lesions after 30 days postinoculation.

Serum sampling. Guinea pigs infected with HSV-2 were bled by cardiac puncture under ether anesthesia. The guinea pig blood was kept at 4°C overnight and clarified by centrifugation at 1000 rpm for 15 min. Sera obtained were stored at −70°C until use.

Plaque reduction neutralization. Serial twofold dilutions (1:10 to 1:320) of sera were made with PBS. A 0.25 ml serum dilution was mixed with 0.25 ml of HSV-2 strain 1868 suspension containing 50–60 PFU. Then, the virus–serum mixtures and virus controls were incubated at 37°C for 1 hr. After incubation, 0.2 ml of each virus–serum mixture was inoculated in duplicates onto confluent GPE cells in 24-well plates. After 1 hr of adsorption at 35°C, monolayers were overlaid with medium containing 0.5% methylcellulose. Three days later, the medium was removed, the cell monolayers were fixed and stained, and the plaque forming units were enumerated. The neutralizing antibody titer was taken as the highest dilution of the serum which inhibited 90% or more of plaque formation.

Results. *Determination of complement requirement of HSV-2 serum neutralization test.* Five serum samples were used for determination of the complement units that were necessary for the HSV-2 serum neutralization test (Fig. 1). Sera of guinea pigs 1, 2, 3, and 4 were taken 67 days after genital inoculation with HSV-2 and serum of guinea pig 393 was taken at 24 days postinfection. All sera were heated at 56°C for 30 min, then different concentrations of complement were added. As shown in Fig. 1, antibody titers of all sera were below 1:10 (lowest serum dilution tested) after heat inactivation at 56°C for 30 min. When 32 units of complement were added to the heated serum, the antibody titers of animals 1, 3, 4, and 393 reached a maximum stable level except animal 2, whose requirement for complement was 64 units. The addition of 128 units of complement to the serum of animal 2 did not increase the serum antibody titers. In order to further determine the requirement of complement in the HSV neutralization test, 12 additional serum samples taken from another 12 guinea pigs infected with the same strain of HSV-2 were used (Fig. 2). Sera 1, 2, 3, and 4 were obtained 17 days postinfection, sera 5, 6, 7, and 8 at 21 days, and sera 9, 10, 11, and 12 at 28 days. For comparison, the antibody titers of each serum, i.e., nonheated, heated, and heated plus complement were assayed. In the presence of 32 units of complement,

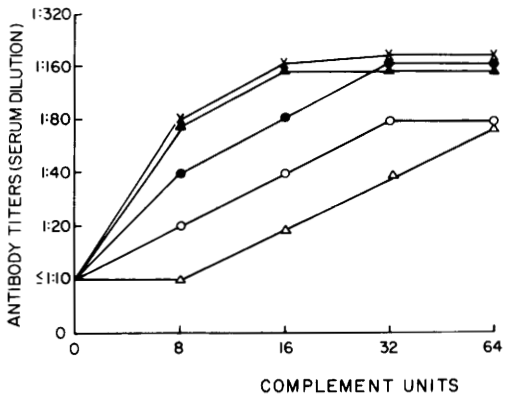

FIG. 1. Requirement of complement units for serum neutralization test. At 0, all sera were heated at 56°C for 30 min. Then, different concentrations of complement were added. Animals 1 (○), 2 (△), 3 (×), 4 (▲) and 393 (●).

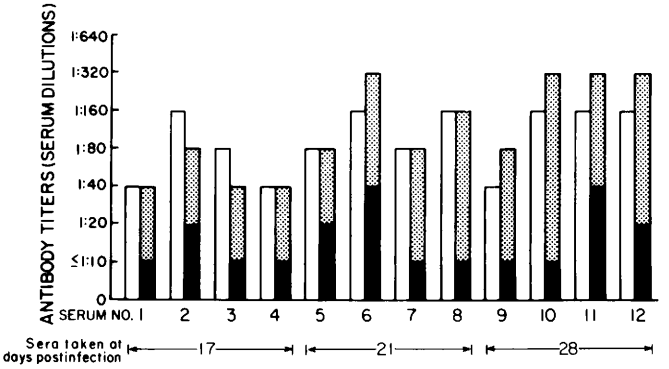

FIG. 2. Comparison of CRN antibody titers in nonheated, heated, and heated-plus-complement serum. (□) nonheated, (■) heated (56°C for 30 min), (□) heated-plus-complement (32 units), ≤1:10 considered to be negative, the lowest serum dilution tested.

five heated sera (Nos. 1, 4, 5, 7, and 8) showed the same titers as nonheated sera, two (Nos. 2 and 3) had twofold lower and five (Nos. 6, 9, 10, 11, and 12) showed twofold higher titers than the nonheated sera taken from the same animals.

Comparison of neutralization kinetics between heated and nonheated sera. Five sera of guinea pigs were used in this experiment. Sera 3, 4, and 8 were obtained at 28 days after HSV-2 inoculation and sera 362 and 365 were collected 21 days postinfection. All sera were diluted with PBS at 1:10 and 0.25 ml of the diluted serum were mixed with 0.25 ml of HSV-2 virus stock containing $10^{3.5}$ PFU/0.1 ml. These mixtures were incubated at 37°C for 20, 40, and 60 min. Samples were removed from each mixture and assayed for virus infectivity titers using GPE monolayers. As shown in Fig. 3, nonheated sera significantly reduced virus infectivity titers on an average of 1.4 $\log_{10}$ at 20 min ($P < 0.001$), 2.0 $\log_{10}$ at 40 min ($P < 0.001$), and 1.86 $\log_{10}$ at 60 min ($P < 0.001$). Three of the five sera which had been heated showed no neutralizing capacity even after 40 and 60 min incubation with the virus at 37°C. It was also noted that some sera, for example, sera 362 and 365, after heat inactivation, were still able to neutralize the virus ($P < 0.05$) after 40 and 60 min incubation although the neutralization capability was lower than without heat inactivation.

CRN antibody response in guinea pigs following intravaginal inoculation with HSV-2. Eight guinea pigs infected intravaginally with HSV-2 strain 1868 were observed for CRN antibody response. In addition, genital lesions and vaginal virus shedding were checked. Figure 4 shows that serum CRN antibody first appeared in low titer, 1:32, 10 days postinfection at which day the maximum lesion score (3.1) was observed but minimum virus shedding in the vagina was detected (0.45 $\log_{10}$ PFU/0.1 ml). Serum CRN antibody progressively increased to a mean titer of 1:120 at 21 days, reached a maximum

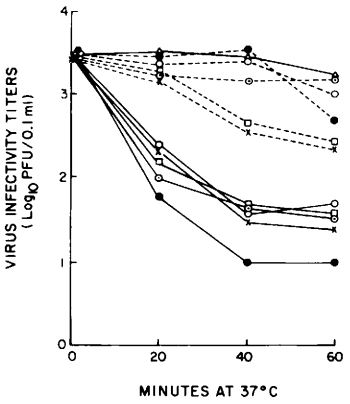

FIG. 3. Neutralization kinetics of nonheated and heated sera. Heated (- - -), nonheated (——), virus control (△), sera 3 (⊙), 4 (○), 8 (●), 362 (×), and 365 (□). $P < 0.001$: heated vs nonheated sera at 20, 40, 60 min. $P < 0.01$: nonheated sera at 20 min vs nonheated sera at 40 min. $P < 0.05$: heated sera of 362 and 365 vs virus control at 40 and 60 min. Statistical analyses were performed using student's *t* test (two-tailed).

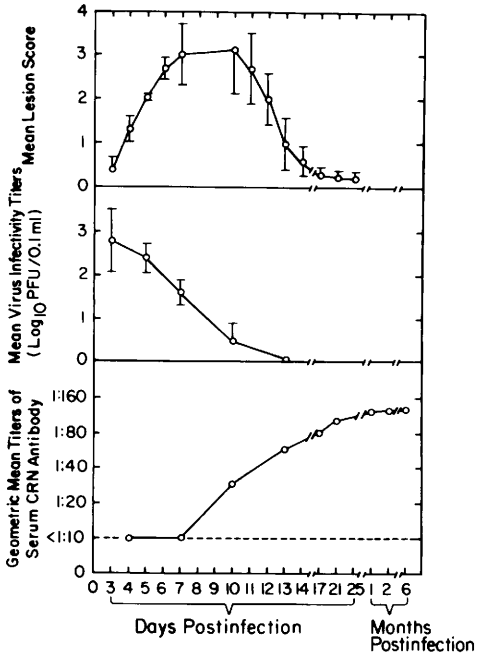

FIG. 4. Genital herpes infection in guinea pigs: Lesion score, virus shedding, and serum CRN antibody response. Lesion score: 0, no symptoms; 1, swelling, erythema of the vaginal mucosa, or 1–2 herpetic lesions; 2, 3–10 lesions; 3, 11–20 lesions; 4, >21 lesion or confluent lesion; 5, loss of bladder control or paralysis; −1, healing begins (drying crusting of lesions).

level 1:130 at 1 month and remained at that level at least 6 months.

Assays of serum CRN antibody in guinea pigs before and after recurrent genital herpes. A total of 44 infected guinea pigs were tested for both CRN antibody and non-CRN antibody titers 1 month after infection. All of the sera had CRN antibody titers ranging from 1:40 to 1:320. Twelve of them (27.3%) showed serum non-CRN antibody, but titers were two- to eightfold lower than CRN antibody titers. Among them, only three animals had 1:40 non-CRN antibody titers. Of the six guinea pigs with recurrent genital lesions (Table I). only one, 267, had a fourfold increase in CRN antibody titer after genital lesions recurred; the other five showed no significant increase in CRN antibody titers after recurrence.

Discussion. Serological tests have been widely utilized in diagnosing viral disease and studying viral pathogenesis. HSV serum antibody levels have been employed in evaluating the relationship between HSV antibody responses and latency in experimental animals (15, 16). Due to the requirement of "complement" in the neutralization test for HSV, the presence of HSV antibodies was often missed when heat inactivated sera were used in the neutralization test (7, 8).

Our data showed that guinea pigs following genital infection with HSV-2 had high titers of serum CRN antibodies. Most heat inactivated sera lost their capability to neutralize HSV-2 and antibody titers dropped to undetectable levels. The amount of "complement" required in the serum neutralization test varied from 16 to 64 units. Sera containing 32 units of "complement" showed the most satisfactory results in the present study. In a previous report from our laboratory (9), only 8 units of complement were used; thus a significantly lower level of serum antibody titers was recorded in that study. Although certain guinea pigs with genital herpes do

TABLE I. EXAMPLES OF SERUM CRN ANTIBODY IN GUINEA PIGS WITH RECURRENT GENITAL HERPES

	Before recurrence		Recurrence		After recurrence	
Guinea pig	Serum obtained (days postinfection)	Serum CRN antibody titers	Recurrent lesions observed (days postinfection)	Total days lesions observed	Serum obtained (days postrecurrence)	Serum CRN antibody titers
99	176	1:80	198	1	28	1:160
267	75	1:40	95	2	29	1:160
362	30	1:160	50	1	26	1:80
375	30	1:80	42	1	38	1:80
394	23	1:80	53	1	21	1:80
397	31	1:160	44	1	22	1:80

Note. Guinea pigs were intravaginally inoculated with 0.1 ml of HSV-2 strain 1868 containing 5.0 $\log_{10}$ PFU.

produce non-CRN antibody in low titers, the percentage of guinea pigs producing non-CRN is only 27.3% in the present study.

The appearance of serum CRN antibody, the excretion of virus in the vagina, and clinical lesions were inversely related. The increase in serum CRN antibody titers was consistent with the reduction in virus infectivity titers from the vagina and the healing of genital lesions during primary infection. However, the presence of CRN antibody in the guinea pig did not prevent genital lesion recurrence. In addition, spontaneous genital herpes recurrence did not result in an increase in serum CRN antibody titers in most of the guinea pigs. Thus, serum CRN antibody is not a useful marker for detection of a recent episode of recurrent herpes.

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