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# The Influence of Complement on Cytomegalovirus Neutralization by Antibodies

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With 1 Figure

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## Summary

The influence of complement on the neutralization of cytomegalovirus by antibodies has been studied using human sera and antisera prepared in rabbits with various strains of cytomegalovirus (Ad 169, C 87, Davis and T 27). Examination of patient sera showed that addition of 2 per cent complement had only a weak and non-significant enhancing effect on neutralization, even by sera taken early in the course of infection.

It proved possible to produce specific antisera with high titres in rabbits with the four strains of cytomegalovirus. It was found that addition of 2 per cent complement had an enhancing effect on neutralization of cytomegalovirus by hyperimmune sera. Concerning the Ad 169 and C 87 antisera the effect of complement was essentially limited to early antisera, whereas the effect was especially pronounced on late Davis and T 27 antisera. The late Ad 169 and C 87 sera did not require complement, whereas all late Davis and T 27 antisera were complement-requiring. These differences, which most likely are bound to whether the anti bodies are $\gamma$G or $\gamma$M antibodies make it important to use complement in cross-neutralization experiments with hyperimmune sera.

## 1. Introduction

Several viruses are neutralized more efficiently by antibody after addition of fresh serum to the reacting virus-antibody mixture. This has been established inter alia for *herpesvirus hominis*, simian cytomegalovirus and rubella virus (1, 7, 13).

Guinea pig serum is the most commonly used additive, and it appears (8) that the enhancing factors are the first four of the nine components of complement. Consequently, the fresh serum used is often referred to as complement (C'). In the case of some viruses the effect of C' has been found to be greater on early antibodies ($\gamma$M) than on late ($\gamma$G) (1, 13).

During a serological study aimed at differentiation of human cytomegalovirus (CMV) strains (3) it was found that the addition of 2% C' had an enhancing effect on the neutralization of CMV by hyperimmune serum prepared in rabbits. The enhancing effect of C' on CMV neutralization was especially pronounced on a hyperimmune serum prepared with a local strain (T 27) but not on an Ad 169 hyperimmune serum. This finding led to a study of the role of C' in CMV neutralization by human convalescent sera and by rabbit hyperimmune sera. The results are presented below.

## 2. Materials and Methods

### 2.1. Tissue Culture and Media

Cell cultures in plastic Petri dishes were produced according to a technique previously reported (4).

### 2.2. CMV Strains

Three established laboratory strains were used in the study: Ad 169 (11), C 87 (10), and Davis (12), kindly supplied by Dr. G. Carlström, Stockholm, Dr. M. Benyesh-Melnick, Houston, Texas, and by Dr. U. Krech, St. Gallen, Switzerland.

The following seven newly isolated strains were also used: T 27 and T 49 isolated from the urine of renal allograft recipients with serologic evidence of CMV infection, together with strains JS, PØN, MN, MTN and LW isolated from infants.

### 2.3. Sera

The human sera employed were negative and positive standard sera and sera obtained from four (non-immunosuppressed) patients with acute CMV infection with clinical manifestations.

The hyperimmune sera were produced in rabbits using virus antigens from cell cultures according to a technique reported previously (3, 4). Rabbits Nos. 1—5 received antigens alone, whereas, Nos. 7—22 received $2 \times 0.5$ ml complete Freund's adjuvans in addition together with the first injections. Most rabbits were immunized with virus containing cell-culture medium alone. Rabbits Nos. 9, 10, and 20 were immunized with concentrated antigens achieved by a 100-fold concentration of the virus-containing medium by sediment centrifugation at 100,000 *g* for half an hour. Two rabbits (Nos. 11 and 19) were immunized with antigens of non-infected cells produced by twice freeze-thawing a suspension of cells in PBS ($10^6$ cells/ml). This antigen was freed from cell fragments by low grade centrifugation prior to use. Rabbit No. 1 was immunized with cell-free culture medium.

All sera were heat-inactivated at 56°C for half an hour prior to use. Guinea pig serum used as C' was received in a lyophilized state from the Pasteur Institute in Paris. Different batches were used throughout the study. Each batch was titered in a microtiter system using 8 units of hemolysin. The different batches were of the same strength, containing about 1200 hemolytic units of C'/ml.

### 2.4. Fractionating of Sera

One positive human standard serum and two sera from rabbits immunized with strain Ad 169 and T 27 were fractionated in a Sephadex column at pH 7.2 by Dr. T. Hjort at this institute. Fractionation and determination of the various fractions were performed employing the technique described by HJORT (6).

### 2.5. Neutralization Test

Neutralizing (NT) antibodies were determined in a plaque NT system (10) employing a modification of the technique described in a previous paper (4). In order to study the enhancing effect of C' on CMV neutralization, sera were diluted (2- or 4-fold) in tubes after which an equal amount of virus suspension was added to each tube. Mixtures from the various tubes were then divided into two parts, 2% heat-inactivated guinea pig serum (C' absent) was added to one, 2% fresh guinea pig serum (C' present) to the

other. The mixtures were then incubated in a water-bath at 37°C for one hour and then added to two dish cultures. In each titration positive and negative human sera plus fresh and inactivated C' were included and the titre of a serum was calculated geometrically as the reciprocal value of the serum dilution causing 60 per cent plaque reduction as compared to the titre of the virus suspension to which the negative human standard serum had been added.

In the study of the effect of C' concentration on CMV neutralization, the negative sera were diluted 1:10 and the positive sera 1:10, 1:40 and 1:160. The dilutions were mixed with equal amounts of virus suspension and then divided into rows of tubes with 0.8 ml in each tube. Various amounts of guinea pig serum and diluent were then added in a total volume per tube of 0.2 ml giving a C' concentration ranging from 0 to 16% in each row. After incubation in a water-bath for 1 hour, the mixtures were seeded in 3 cultures for surviving virus.

Some rabbit antisera were tested for non-specific virus neutralizing activity against a local strain of *herpesvirus hominis* in a plaque NT test just like the one described for CMV.

## 3. Results

### 3.1. The Effect of C' Concentration on CMV Neutralization

This was studied in several experiments employing rabbit and human sera. The results of one of these experiments using a negative serum, a hyperimmune C' requiring rabbit serum and the homologous virus strain (T 27) are graphically illustrated in Figure 1. The addition of C' in various amounts had no effect on the

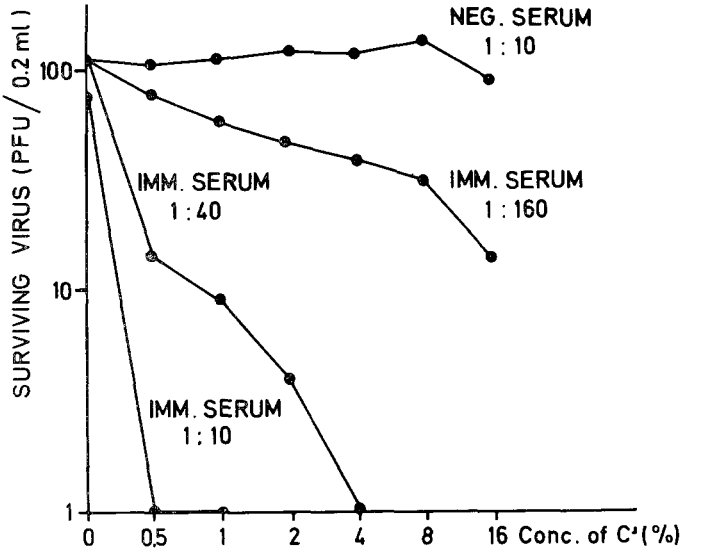


Fig. 1. The effect of C' concentration on CMV neutralization

reaction between virus and negative serum; whereas the addition even of small amounts of C' had a striking effect on the ability of the hyperimmune serum to neutralize virus. The enhancing effect on neutralization of C' was dependent on concentration. In contrast, a similar, marked effect of C' was not seen in experiments using human convalescent sera or CMV hyperimmune sera prepared with strain Ad 169.

Some lots of guinea pig serum were found to have a heat labile inhibitory effect on CMV when used in concentrations higher than 4%. For this reason the rest of the study was carried out using the addition of only 2% fresh or heat inactivated guinea pig serum.

### 3.2. The Effect of C' on the NT Titres of Human and Hyperimmune Sera

In plates seeded with various dilutions of serum plus virus to which fresh C' had been added, fewer plaques were generally observed as compared to mixtures to which inactivated C' had been added.

In experiments with human sera from CMV infected patients, the degree of enhancement was low, but rather constant (Table 1). The effect of C' on early

Table 1. *The Effect of 2 Percent C' on CMV Antibody Titre in Patients with Acute CMV Infections*

| Patients | Serum No. | Months after onset of illness | Antibody titre | | Enhancement factor |
|---|---|---|---|---|---|
| | | | +heated C' | +fresh C' | |
| ABN | 1 | <1 | <2 | 2 | |
| | 2 | 2 | 12 | 20 | 1,7 |
| | 3 | 3 | 20 | 32 | 1,6 |
| | 4 | 4 | 26 | 40 | 1,5 |
| V. J. | 1 | <1 | <4 | <4 | |
| | 2 | 1 | 3 | 5 | 1,6 |
| | 3 | 4 | 16 | 24 | 1,5 |
| I. S. | 1 | 1 | 20 | 30 | 1,5 |
| | 2 | 3 | 200 | 320 | 1,6 |
| | 3 | 11 | 40 | 64 | 1,6 |
| L. Aa. N. | 1 | 1 | <4 | <4 | |
| | 2 | 4 | 40 | 40 | 1 |
| | 2[1] | 4 | 50 | 50 | 1 |
| Human pos. standard | | unknown | 100 | 160 | 1,6 |

[1] Separate determination.

antibodies does not seem to be different from the effect on late antibodies. In two sera from patient L.Aa.N., no enhancement could be registered.

Our experiences with immunization of rabbits and studies of the influence of C' on hyperimmune sera are summarized in Table 2. As can be seen it was possible to produce highly potent, non-C' requiring antisera with strain Ad 169 and strain C 87. This had not been possible with the two other strains. Addition of C' to all of the Ad 169 and C 87 antisera had generally only a weak enhancing effect on CMV neutralization except in early sera; whereas neutralization was enhanced markedly by addition of C' to all late Davis and T 27 antisera.

None of the rabbits immunized with CMV-free antigens yielded antibodies which were able to neutralize CMV and no unspecific neutralizing effect could be registered in CMV hyperimmune sera on *herpesvirus hominis* (Table 3).

Table 2. *Neutralizing Antibodies in Rabbits Immunized with 4 Strains of CMV and Various Control Antigens*

| Strain | Rabbit No. | Serum at weeks of immunization | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 3–5 | 6–8 | 9–11 | >13 |
| Ad 169 | 2 | –[2] (–) | +[3] (++) | ++[4] (++) | | ++ (++) |
| — | 3 | – (–) | – (++) | + (++) | | ++ (++) |
| — | 20[1] | – (–) | | ++ (++) | | ++ (++) |
| C 87 | 16 | – (–) | – (++) | + (++) | | ++ (++) |
| — | 17 | – (–) | – (++) | ++ (++) | ++ (++) | ++ (++) |
| Davis | 21 | – (–) | – (–) | – (++) | – (++) | |
| — | 22 | – (–) | – (–) | – (–) | – (+) | |
| T 27 | 4 | – (–) | – (–) | – (+) | | – (+) |
| — | 5 | – (–) | – (–) | – (–) | | – (+) |
| — | 7 | – (–) | – (–) | – (–) | – (–) | – (++) |
| — | 8 | – (–) | – (–) | – (–) | – (+) | – (++) |
| — | 9[1] | – (–) | – (–) | – (–) | – (+) | – (++) |
| — | 10[1] | – (–) | – (–) | – (–) | – (+) | – (++) |
| Culture medium | 1 | – (–) | – (–) | – (–) | | – (–) |
| Cells | 1 | – (–) | – (–) | – (–) | – (–) | – (–) |
| — | 19 | – (–) | – (–) | – (–) | – (–) | – (–) |

The sera were diluted 1 : 10 and tested against strain Ad 169.

[1] immunized with concentrated antigens

[2] –: Less than 60% plaque reduction.

[3] +: 60–90% plaque reduction.

[4] ++: >90% plaque reduction.

In brackets the degree of neutralization with addition of C'.

Table 3. *Test for Non-Specific Virus Neutralizing Activity of Rabbit Hyperimmuneserum*

| Rabbit No. | Serum | Dilution of Herpesvirus hominis | | |
|---|---|---|---|---|
| | | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ |
| 1 | Preimm. | Conf.[1] | 12,16[2] | 1,0 |
| 1 | 18. week | Conf. | 15,8 | 3,0 |
| 2 | 18. week | Conf. | 10,14 | 1,3 |
| 5 | 18. week | Conf. | 19,20 | 4,0 |

[1] Confluent plaques.

[2] Number of plaques per dish.

### 3.3. The Influence of C' on γM and γG Antibodies

To elucidate the above-mentioned results, one positive human serum and two rabbit hyperimmune sera were fractionated and the NT titres of serum, γM and γG fractions determined in parallel with addition of fresh and inactivated C'. The rabbit sera were 18 weeks immune sera prepared with strains Ad 169 and T 27. The results are shown in Table 4 from which is seen that the antibodies in the human serum and the Ad 169 antiserum were mostly γG antibodies, whereas the T 27 antibodies were mostly γM antibodies. Furthermore, it is to be seen that the CMV neutralization by γM antibodies was much more C' dependent than neutralization by γG antibodies.

Table 4. *The Effect on CMV Neutralization by Serum and Serum-fractions*[1]

| Sera (strain) | Fractions | Antibody titre | | Enhancement |
|---|---|---|---|---|
| | | +inactiv C' | +fresh C' | (-fold) |
| Human pos. | γM | 2 | 2 | 1 |
| reference | γG | 45 | 60 | 1.3 |
| | serum | 90 | 150 | 1.6 |
| Rabbit | γM | ≦2 | 6 | 3 |
| (Ad 169) | γG | 180 | 256 | 1.4 |
| | serum | 230 | 512 | 2.2 |
| Rabbit | γM | ≦2 | 128 | 64 |
| (T 27) | γG | ≦2 | 32 | 16 |
| | serum | ≦2 | 384 | 190 |

[1] The human serum and the Ad 169 serum were tested against strain Ad 169, the T 27 serum were tested against strain T 27.

### 3.4. The Neutralization of Various CMV Strains by Hyperimmune Sera Prepared with Two Strains of CMV

The negative and positive sera used in this study were all diluted 1:4 and 1:10. Inactivated and fresh C' was added prior to mixing with the virus strains. The result of this study is seen in Table 5. Employing Ad 169 antiserum (γG antibodies),

Table 5. *The Neutralizing Effect of Two Hyperimmunsera on 10 Strains of CMV*

| Strain | Chall. dose PFU/0.1 ml | Per cent plaque reduction | | | |
|---|---|---|---|---|---|
| | | Ad 169 serum | | T 27 serum | |
| | | 1:4−C'[1] | 1:10 +C'[2] | 1:4−C' | 1:10 +C' |
| Ad 169 | 268[3] | 99 | 100 | 47 | 95 |
| C 87 | 152 | 100 | 100 | 62 | 97 |
| Davis | 79 | 92 | 100 | 48 | 95 |
| T 27 | 585 | 95 | 99 | 74 | 100 |
| T 49 | 40 | 95 | 100 | 80 | 100 |
| JS | 122 | 100 | 100 | 67 | 97 |
| PØN | 102 | 84 | 97 | 57 | 100 |
| MN | 101 | 90 | 88 | 64 | 85 |
| MTN | 56 | 96 | 100 | 89 | 100 |
| LW | 305 | 99 | 97 | 49 | 93 |

[1] added 2% inactivated C'.
[2] added 2% fresh C'.
[3] mean of two plates.

all strains were neutralized as plaque depression was greater than 84%. Addition of C' enhanced the degree of neutralization. Only six of the strains could be efficiently (≧60% plaque depression) neutralized by T 27 (γM antibodies) antiserum, diluted 1:4 alone; whereas this serum used in dilution 1:10 also neutralized from 85—100% of the various strains when C' was added.

## 4. Discussion

In their study of the rise of NT antibodies against *herpesvirus hominis*, YOSHINO and TANIGUCHI (13) found that during animal immunization C' requiring

NT antibodies appeared early and reached a considerable level before a rise in CF and non-C' requiring NT antibodies could be registered. Examination of patient sera revealed that a similar immune response occurred as a result of herpes virus infection. Normal healthy persons showed no NT antibodies which could be enhanced by C' to a marked extent.

In the case of rubella it is known that the commonly used procedure of heating sera to 56° C for half an hour preceding titration of antibody may reduce the titre of NT antibody to rubella virus. The addition of fresh serum (C') even in small amounts has been shown to restore the NT activity and also to enhance rubella antibody titre (7).

Further ABLASHI *et al.* (1) showed that neutralization of a simian CMV by γM antibodies prepared in rabbits was enhanced significantly in the presence of C'. A similar effect of C' was not seen in neutralization experiments using strain Ad 169 and a rabbit hyperimmune serum (9).

Previous studies (2) in this laboratory have shown that in human CMV infection the rise in CF antibodies precedes the rise in NT antibodies, especially in cases of primary infection. In both primary and reactivated infections the rise in NT antibodies runs a longer course than the rise in CF titre. It might be reasonable to suppose that the slow rise in NT antibodies is preceded by a rise in C' requiring NT antibodies, but the present studies have shown that this seems not to be the case in human CMV infection.

The studies reported here, on the other hand, confirm previous findings (3) to the extent, that it may be important to add C' in cross-NT reactions using antisera produced in rabbits. It has also been shown, that the influence of C' on the various rabbit sera is very different and that the effect seems to be due to whether the antibodies in the hyperimmunesera are γG or γM antibodies. The NT titres of γG antibodies are only slightly enhanced, whereas the titres of γM antibodies may be enhanced much more. It is in accordance with other studies (1, 8) that the enhancing effect of C' is greater on γM than on γG antibodies.

Our experiences with the immunization of rabbits show that it is possible to produce highly potent specific antibodies against CMV strains although with highly variable results. The late antibodies produced against strains Ad 169 and C 87 were non-C' dependent; whereas all late sera produced against strains Davis and T 27 were C' requiring.

It is not possible to give a reasonable explanation of the discrepancy on the basis of the present study. It may be due to the use of relatively smaller amounts of antigen during immunization of the rabbits with strains Davis and T 27 than with the strains Ad 169 and C 87. The findings may, however, also be due to differences between the two groups of strains; an assumption which is supported by the fact that the use of 100-fold concentrated antigen (T 27) for immunization of rabbit 9 and 10 did not result in production of non-C' requiring antibodies.

On the basis of cross-NT experiments using a tube-NT-test, with human convalescent sera and homologous and heterologous strains, WELLER *et al.* (12) in 1960 proposed that the human cytomegaloviruses do not constitute a single serotype. Strains Davis and Ad 169 were proposed as types 1 and 2, respectively. Experiences gained with the NT test in various laboratories do not seem to have provided evidence for this supposition (2, 5). The results of the present study

using CMV hyperimmune sera, established laboratory strains (Ad 169, Davis and C 87) and newly isolated strains and the sensitive plaque NT test indicate that to a wide degree the various strains of CMV share NT antigens. It is, however, certain that minor antigenic differences do exist between them (2, 3, 12).

These findings further provide experimental evidence for the use of only one strain in diagnostic work aimed at detection of NT antibodies in human CMV infection.

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