# Preparation of Specific Antisera to Cytomegaloviruses in Goats[1] (36003)

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(Introduced by J. L. Melnick)

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Evidence which suggests antigenic differences among human cytomegaloviruses (CMV) has come from studies in which native human and monkey sera were tested against selected CMV strains in the complement-fixation and neutralization tests (1–4). Although some antigenic heterogenicity was shown in these studies, sera from monkeys and humans who often had latent CMV infections, lack known specificity and have not generally proven useful for a detailed antigenic analysis of CMV strains from different sources. Neutralizing antisera to human CMV have been prepared in monkeys captured in the wild (5), rabbits (6–9), or in guinea pigs (10); and antisera to monkey CMV have been prepared in rabbits (11). However, these antisera were either of low potency (5, 6, 8–11) or cytotoxic (7), thus rendering them difficult to use in precise antigenic analysis of different strains.

A recent study in our laboratory (12) has demonstrated that specific high-titered antisera to known human (C87 and AD169) and simian (GR2757 and GR2598) cytomegaloviruses can be produced in baboons or monkeys, hand-reared in captivity and free of preexisting antibody. The antisera to both strains of human CMV contained mainly complement-requiring neutralizing (CRN) antibody whereas the antisera to the simian viruses contained complement-independent neutralizing (CIN) antibody (12). Cross-neutralization tests indicated a two-way cross between the two human viruses and a two-way cross between the two monkey viruses with negligible interspecies reactivity (12).

Since the type of primates used in the above study (12) are not readily available, attempts were made to determine whether goats (free of preexisting antibody to either monkey or human CMV) could be used. This paper describes the successful preparation of specific, high-potency antisera to human and simian cytomegaloviruses in goats and the further characterization of CMV by goat antisera.

*Materials and Methods. Viruses.* Cell-free stocks of human CMV strains C87 (13), AD169 (14), and Davis (15), and simian CMV strain GR2757 (1) were prepared in human embryonic lung (HEL) fibroblasts as previously described (1, 16, 17) and kept at —90° in the presence of 35% sorbitol (16). Sorbitolized stocks were used for immunization of goats and for neutralization tests. Virus infectivity was determined by the modified microplaque technique (16). Virus particle counts were performed by Dr. R. McCombs in this laboratory by the droplet-pseudoreplication method (18) using an Hitachi 11B electron microscope.

*Immunization procedure.* Twenty to 35 kg goats, free of preexisting antibody to CMV, were immunized with each of the three human strains (C37, AD169, and Davis) and with simian strain GR2757. The immunization schedule consisted of four weekly inoculations followed by a booster 2 weeks later. Each inoculum consisted of approximately 1

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TABLE I. Development of Neutralizing Antibodies to Human and Monkey CMV in Goats.

| Serum taken at day | Neutralizing antibody titers[a] to the homologous virus in animals immunized with: Human CMV: C 87 (Goat 2300) −C'[b] | C 87 (Goat 2300) +C' | AD 169 (Goat 2303) −C' | AD 169 (Goat 2303) +C' | Davis (Goat 2305) −C' | Davis (Goat 2305) +C' | Monkey CMV: GR 2757 (Goat 2301) −C' | GR 2757 (Goat 2301) +C' |
|---|---|---|---|---|---|---|---|---|
| 0 | <16 | <16 | <16 | <16 | <16 | <16 | <16 | <16 |
| 21 | 50 | 2400 | <16 | 50 | 30 | 500 | 1000 | 1000 |
| 35 | 80 | 1100 | 120 | 1200 | 200 | 1300 | 900 | 800 |
| 49 | 170 | 1600 | 200 | 1800 | 100 | 900 | 1800 | 1400 |

[a] Reciprocal of highest final serum dilution giving 60% plaque reduction; average values of 3 to 4 tests given.

[b] −C' = test in the absence of complement; +C' = test in the presence of 5 to 10 hemolytic units of complement.

$\times 10^6$ plaque-forming units (about $1 \times 10^9$ particles) of cell-free virus in 5 ml, given ip (3 ml), iv (1 ml) and im (1 ml). All five im inoculations were given with Freund's complete adjuvant. Three trial bleedings (days 0, 21, and 35) were taken during the immunization period and the animals were bled out after the final inoculation (day 49). All sera were stored at −90°.

*Neutralization test.* The plaque reduction neutralization test for CMV (1, 5) was modified to allow for the incorporation of fresh complement (C') in the reaction mixture (12). Unheated guinea pig serum, free of nonspecific viral inhibitors, was employed as a source of C' and kept at −90° until used. Each serum sample was tested in parallel for the presence of C'-requiring neutralizing (CRN) antibody and of C'-independent neutralizing (CIN) antibody. The test employed 4-fold dilutions of heat-inactivated (56°, 30 min) serum (first dilution of 1:8), C' diluted to contain 5 to 10 hemolytic units (usually a dilution of 1:8 or 1:16) and virus diluted to contain 1200 plaque-forming units (PFU) per 0.2 ml. Eagle's medium, free of $NaHCO_3$ and supplemented with 5% heat-inactivated (56°, 30 min) fetal bovine serum, was used as diluent for all reagents. Serum, C', and virus (for CRN tests), or serum, diluent, and virus (for CIN tests) were mixed at a ratio of 2:1:1. For virus controls, diluent was used to substitute for either serum (control for the CRN test) or serum and C' (control for the CIN test). After incubation for 1 hr at 37°, residual infectivity was measured as described previously (1, 16). Serum titers were expressed as the final serum dilution producing 60% plaque reduction (1).

*Results.* The course of development of CIN and CRN antibodies in the inoculated goats was followed using sera taken after the third, fourth, and fifth inoculation (days 21, 35 and 49, respectively). The preinoculation sera of all animals were free of neutralizing antibody to the immunizing viruses (Table I). A comparison of the antibody titers from all bleedings indicated that, in goats, the three human CMV strains (C87, AD169, and Davis) elicited mainly CRN antibody (maximal homologous titers of 1300 to 2400) while the monkey strain GR2757 elicited only CIN antibody (maximal titer of 1800). In contrast to previous findings with monkeys and baboons (12), goats produced a moderate amount of CIN antibody (titers of up to 200) to the human CMV strains (Table I). Since the goats were bled out at day 49, it was possible that peak antibody titers had not been attained by that time. Therefore, another goat was immunized with human strain C87, using an extended schedule of 9 inoculations over a 12-week period, to determine if even higher titers, particularly of CIN antibody, could be obtained. An-

TABLE II. Cross-Neutralization Tests with Goat Antisera to Human and Monkey CMV.

| | Neutralizing antibody titers[a] to: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Human CMV | | | | | | Monkey CMV | |
| | C 87 | | AD 169 | | Davis | | GR 2757 | |
| Antiserum[b] (goat no.) | −C'[c] | +C' | −C' | +C' | −C' | +C' | −C' | +C' |
| Anti-C 87 (2300) | 170 | 1600 | 110 | 540 | 80 | 830 | <16 | <16 |
| Anti-AD 169 (2303) | 240 | 2400 | 200 | 1800 | 260 | 2300 | <16 | <16 |
| Anti-Davis (2305) | <16 | 900 | <16 | 700 | 100 | 900 | <16 | <16 |
| Anti-GR 2757 (2301) | <16 | <16 | <16 | <16 | <16 | <16 | 1800 | 1400 |

[a] Reciprocal of highest final serum dilution giving 60% plaque reduction; average values of 3 to 4 tests given.

[b] Taken at day 49, 2 weeks after the fifth inoculation.

[c] −C' = test in the absence of complement; +C' = test in the absence of 5 to 10 hemolytic units of complement.

alysis of multiple bleedings from this animal indicated that there was no increase in either CIN or CRN antibody titers after the fifth inoculation.

The extent of antigenic relatedness between the three human strains and the monkey strain of CMV was examined in CRN and CIN cross-neutralization tests using antisera taken from the final bleeding at day 49 (Table II). Whether the tests were carried out in the absence or in the presence of C', the antisera to all three human strains failed to neutralize the monkey virus and vice versa, indicating a lack of interspecies cross. On the other hand, a reciprocal cross was observed between the human strains, the antiserum against AD169 showing the broadest reactivity. In the presence of C', the anti-AD169 and anti-Davis sera neutralized equally well all three human viruses; whereas the anti-C87 serum revealed a 2- to 3-fold higher titer to the homologous virus. The homologous CIN antibody titers were generally too low for meaningful interpretation of cross-reactions. However, it appears that in the absence of C' the anti-Davis serum (titer of 100) failed to neutralize the other two viruses.

*Discussion.* The results of this study indicate that goats free of preexisting antibody are suitable for the preparation in relatively large volumes of specific high-titered antisera to human and monkey cytomegaloviruses. Following the immunization schedule described, maximal antibody titers to both types of CMV could be attained after 4 to 5 inoculations. Confirmatory to our earlier observations with primates (12), human CMV elicited mainly CRN antibodies in goats (titers up to 2400), whereas monkey CMV elicited CIN antibodies. However, in the goat antisera, the CIN antibody titers to the human strains were somewhat higher (homologous titers of 100 to 200) than those found in primates (titers of <16 to 90) immunized with human strains C87 and AD169 (12). In both studies however there was no increase in CIN antibody titer upon prolonged immunization. Thus, the predominance of CRN antibodies in hyperimmune primate and goat sera appears to be a property unique for human CMV. A recent preliminary report (9) indicated that C' did not significantly enhance the neutralization titers of rabbit antisera to human CMV strain AD169. However, the antibody titers attained (9) were usually low (128 to 256) and until more detailed data are published no strict comparisons can be made with our study.

The CRN antibody elicited by human CMV was specific; it did not neutralize the monkey strain of CMV; and vice versa, antiserum to monkey CMV failed to neutralize the human strains both in the absence and in the presence of C'. Again, analogous to the results with antisera produced in primates (12) the goat antisera failed to reveal interspecies cross between human and monkey

CMV. On the other hand, reciprocal neutralization tests revealed an extensive cross between the neutralizing antigens of human strains C87, AD169, and Davis. The antiserum to strain AD169 had the broadest cross-reactivity. It remains to be seen whether small antigenic differences exist among these viruses.

The 3-fold difference in CRN antibody titer between strain C87 and AD169 revealed by the anti-C87 serum suggests such a difference. These two viruses have been reported earlier to differ also in their complement-fixing antigens, as measured by reactivity with monkey sera following natural infection (1). However, at present no clear distinction can be made between the three human strains used. It has been suggested that late 19S antibodies can distinguish between different strains of simian CMV (19). Similar experiments with the goat antisera described above may shed further light as to possible differences between the neutralizing antigens of these as well as other human CMV strains.

*Summary.* Goats were immunized with human cytomegaloviruses (CMV), strains C87, AD169, and Davis and with simian CMV strain GR2757. The human strains elicited chiefly complement-requiring neutralizing (CRN) antibodies and smaller amounts of complement-independent neutralizing (CIN) antibodies, while the simian strain elicited only CIN antibody. Reciprocal crossing between the neutralizing antigens of C87, AD169, and Davis viruses was found. There was no interspecies crossing between the human and simian viruses.

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