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# Virus-Specific Antibody Activity of Different Subclasses of Immunoglobulins G and A in Cytomegalovirus Infections

G. ANNIKA LINDE,[1]* LENNART HAMMARSTRÖM,[2] MATS A. A. PERSSON,[2] C. I. EDVARD SMITH,[2] VIVI-ANNE SUNDQVIST,[1] AND BRITTA WAHREN[1]

*Department of Virology, National Bacteriological Laboratory, and Department of Virology, Karolinska Institute, S-105 21 Stockholm[1]; Department of Clinical Immunology, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge[2]; and Department of Immunobiology, Karolinska Institute, S-104 05 Stockholm,[2] Sweden*

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Healthy donors and patients with primary or reactivated cytomegalovirus infections were examined for antiviral antibodies of the immunoglobulin G (IgG) and IgA subclasses. For this purpose, monoclonal antibodies against the four IgG and the two IgA subclasses were used in enzyme-linked immunosorbent assays with cytomegalovirus antigen. IgG1 and IgG3 were the principal anti-cytomegalovirus subclasses in serum samples from healthy donors and patients. IgG1 was higher in reactivated than in primary disease. Cytomegalovirus-specific IgG2 was detected only in serum samples with high total cytomegalovirus IgG. A few patients with high total serum IgG4 also had cytomegalovirus-specific IgG4 antibodies. During convalescence, there was no significant increase in total IgG of any subclass. Both IgA1 and IgA2 antibodies with cytomegalovirus specificity were detected in patients who had cytomegalovirus antibodies of the IgA class.

Human immunoglobulin G (IgG) comprises four subclasses, IgG1, 2, 3 and 4 (4, 17). The IgG molecules of the different subclasses are distinguished by their structure and biological activities. Complement fixation, binding to phagocytic cells, binding to heterologous skin, and reactivity with staphylococcal protein A vary among the subclasses (14). In serum from young healthy adults, IgG1 constitutes 60.9% of the total IgG, IgG2 constitutes 29.6%, IgG3 5.3%, and IgG4 4.2% (8). There are, however, both age-related and genetically determined differences in subclass concentrations in individual sera (7, 8). The distribution of antigen-reactive antibodies among subclasses may differ from that of total IgG. Antibodies to bacterial polysaccharides were found to be mainly of the IgG2 subclass (12). Restricted subclass responses were found to several bacterial antigens (9, 11, 18).

Only a few authors have studied the subclass distribution of antiviral antibodies. Neutralizing antibodies to poliovirus and herpes simplex virus and hemagglutination-inhibiting antibodies to rubella virus were claimed to be mainly of subclass IgG3 (2). IgG1, on the other hand, was found to be the dominating subclass reacting with measles antigen in serum and cerebrospinal fluid from patients with subacute panencephalitis and multiple sclerosis (16). In Epstein-Barr virus infection, a 50% increase of total IgG1 was seen (5), but the virus specificity of this IgG was not established.

No information is available regarding the virus-specific activities of different IgG subclasses in acute and convalescent stages of a viral infection. Studies on the antigen reactivities of the different subclasses used to require separation procedures (2, 16), which ruled out comparative investigations. Monoclonal antibodies to all four subclasses are now available (6), and by using these we have established enzyme-linked immunosorbent assays (ELISAs) to measure both the total amount and the antigen reactivity of each IgG subclass. This system can probably be applied to any virus antigen. Here we have examined the subclass distribution of cytomegalovirus (CMV)-specific IgG in healthy persons. We have also studied the changes of total and virus-specific subclass IgG and IgA that appear with time in primary and reactivated CMV infections.

## MATERIALS AND METHODS

**Antisera.** Ascitic fluids with monoclonal antibodies against IgG1-4 (6) were purchased from Seward Laboratories, London, United Kingdom. Clones BAM 08, BAM 09, BAM 10, and BAM 11 were used. Purified monoclonal antibodies against IgA1 and IgA2 were obtained from Becton & Dickinson, Mechelen, Belgium. Rabbit anti-human IgG and IgA came from DAKO Patts A/S, Copenhagen, Denmark, and were

conjugated to alkaline phosphatase. Goat anti-rabbit IgG-alkaline phosphatase was from Sigma Chemical Co., St. Louis, Mo. Unlabeled and peroxidase-labeled rabbit anti-mouse immunoglobulins were purchased from DAKO Patts. Polyclonal sheep anti-human IgG1-4 was purchased from Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

**Patient serum and immunoglobulin preparations.** For standardization of the reagents in the virus-specific assays, Gammaglobulin Kabi 16.5% (Kabi, Stockholm, Sweden) and varicella-zoster immunoglobulin (Zig) 16% (National Bacteriological Laboratory, Stockholm, Sweden) were used. World Health Organization standard serum 67/97 was the standard in the ELISA for total subclass IgG and IgA. Fourteen serum samples from healthy blood donors (seven CMV antibody positive and seven CMV antibody negative) were examined. In addition, 31 serum samples from 11 patients with primary CMV infections and 19 serum samples from 6 patients with reactivated CMV infections were selected from our diagnostic virus laboratory. None of the patients had any known concurrent disease. All serum samples had been assayed previously by ELISA for total CMV IgG and IgM antibodies (15). From the results, it was estimated whether the infection was primary or reactivated. IgG seroconversion from a titer of <50 or the presence of high IgM titers (≥1,000) was regarded as a sign of primary infection. Serum samples with significant IgG titer rises but with no or low IgM titers (<1,000) were assumed to be from patients with reactivated infections. Serum from a patient with a known IgA2 deficiency was included as a control.

**ELISA for virus-specific IgG subclasses.** Microplates (M29AR, Dynatech, Zug, Switzerland, or Nunc I, Copenhagen, Denmark) were used. Reagent solutions were added to the wells in 100-μl portions in all assays. All incubations were performed for 105 min at 37°C unless otherwise mentioned. The plates were washed four times between the different steps in the assays. All buffers have been described previously, as well as the ELISA for total CMV IgG and IgM (15).

**Standardization of dilutions of monoclonal antibodies and conjugate.** Gammaglobulin Kabi or Zig immunoglobulin diluted 10-fold from $10^{-3}$ to $10^{-8}$ was used for coating M29AR plates. After incubation at room temperature over night, the plates were treated for 2 h with 2% bovine serum albumin in phosphate-buffered saline. Mouse monoclonal antibodies directed towards IgG1-4 and diluted in ELISA buffer in twofold dilutions from 1:100 to 1:6,400 were then added. Peroxidase-labeled rabbit anti-mouse IgG absorbed with human IgG coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was diluted twofold from 1:100 to 1:1,600 and added to the plates in a chessboard manner. Orthophenyldiamine was used as a substrate. The absorbance was read at 450 nm in a Titertek Multiscan (Flow Laboratories, Irvine, Scotland). The lowest concentrations of monoclonal antibodies and anti-mouse IgG that gave maximal absorbances in combination with the lowest background were chosen. The dilutions for the clones to be used in the virus-specific ELISA were anti-IgG1, 1:100; anti-IgG2, 1:400; anti-IgG3, 1:1,600; and anti-IgG4, 1:800. The conjugate was used in a dilution of 1:800. Optimal dilutions of anti-IgA1 and IgA2 were 1:100.

**Antigen-specific subclass ELISA.** Plates (Nunc I) were coated with a CMV nuclear antigen (15). Human serum samples to be examined were diluted 10-fold from 1:10 to 1:1,000 and were added in quadruplicate to the plates. The 1:10 dilution was added five times. After incubation and washing, dilutions of monoclonal anti-IgGs were added so that each serum dilution was examined for all subclasses. The monoclonal antibodies were also added to CMV-coated but serum-free wells as a control. After incubation and rinsing, anti-mouse conjugate was added to all wells, including those without monoclonal antisera but with the 1:10 human serum dilutions. Peroxidase conjugate and substrate were added and reading was performed as described above. All serum samples were examined on two different occasions. The coefficient of variation was calculated from the results of double analyses of the 1:10 serum dilutions. The background level was calculated as mean absorbance at 450 nm ($A_{450}$) + 2 standard deviations and was assayed three times with seven CMV antibody-negative serum samples diluted 1:10. The background values for $A_{450}$ were 0.30 (IgG1) and 0.25 (IgG2-4). The coefficient of variation within each test was assayed with CMV antibody-containing serum samples and was 19% (IgG1), 21% (IgG2), 11% (IgG3), and 13% (IgG4).

Subclasses of the CMV-specific IgA were assayed in the same way as the subclasses of the CMV-specific IgG described above, with minor modifications: serum samples were incubated overnight at room temperature. Incubation time with monoclonal antibodies was 3 h. The rabbit antimouse immunoglobulin absorbed with human IgG was added at a dilution of $10^{-3}$. After 3 h of incubation, alkaline phosphatase-conjugated goat anti-rabbit IgG was added and incubated at room temperature overnight before adding *para*-nitrophenyl phosphate (Sigma Chemical Co.) as a substrate. The background absorbances were $A_{450}$ = 0.10 (IgA1 and 2).

**Quantification of subclasses in serum.** Total amounts of IgG1-4 and IgA1-2 were determined in a sandwich ELISA (3, 10). Microtiter plates M129B (Dynatech) were coated with purified monoclonal antibodies against the different subclasses diluted 1:100 (1:1,000 for anti-IgG3) in sodium carbonate buffer, pH 9.6. After incubation overnight, the antibody solution was emptied from the wells, and 1% bovine serum albumin in the same type of coating buffer was added. The plates were incubated at room temperature for 3 h and were used immediately or stored in the cold for up to a week. The samples to be assayed were diluted 1:500 to 1:2,000 in phosphate-buffered saline with 0.5% Tween and 0.5% bovine serum albumin. The plates were washed, and samples and reference samples (WHO 67/97 1:100 to 1:32,000) were added. After 5 h, alkaline phosphatase-conjugated (1) rabbit anti-human IgG or IgA was added, and incubation overnight followed. The next day *para*-nitrophenyl phosphate was added, and $A_{405}$ was read. The absorbances obtained for the test samples were related to the titration curve of the reference serum. The subclass concentrations of the WHO 67/97 serum pool have been determined previously (8).

The concentrations of the IgG subclasses for some

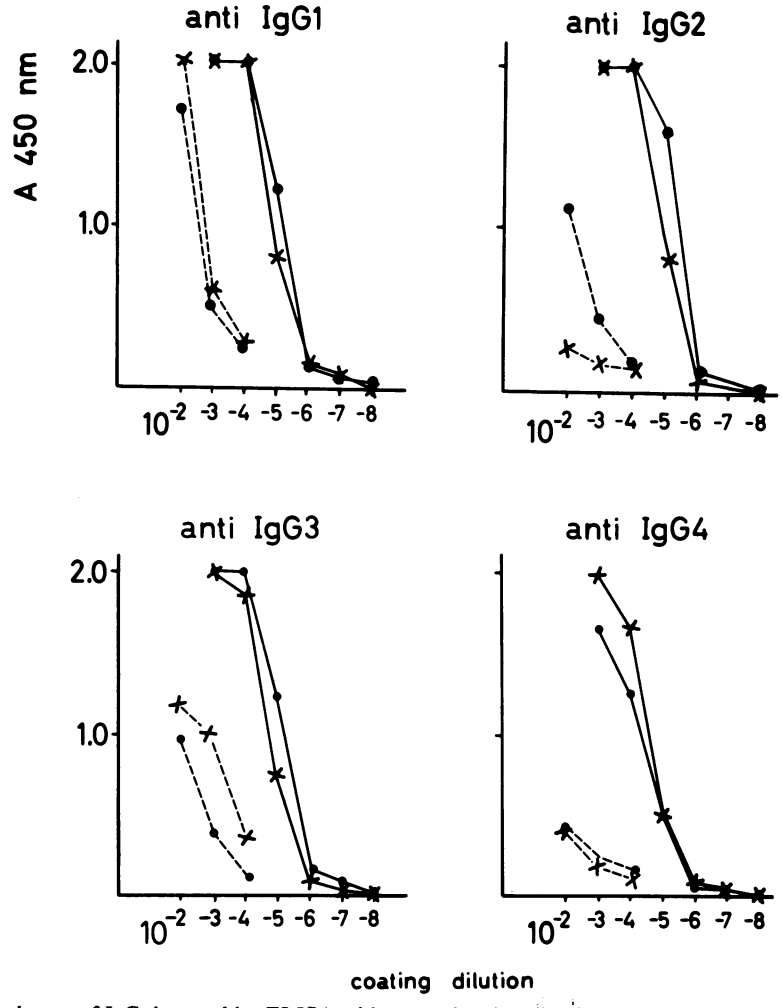


FIG. 1. Subclasses of IgG detected by ELISA with monoclonal antibodies to human IgG1 through 4. Plates were coated with various dilutions of Gammaglobulin Kabi (●–●) or zoster immunoglobulin (×–×) to demonstrate the anti-IgG activity of each monoclonal antibody at its optimal dilution (see text). The same dilutions of monoclonal antibody were used to demonstrate the anti-CMV IgG1-4 activity of Gammaglobulin Kabi (●----●) and Zig (×----×) on CMV antigen-coated plates.

serum samples were determined in parallel in ELISA and in single radial immunodiffusion by using polyclonal antisera according to the instructions of the manufacturer (Netherlands Red Cross Blood Transfusion Service). A complete description of the ELISA for quantification of subclasses in serum will be presented elsewhere (M. A. A. Persson et al., manuscript in preparation).

**Total IgG and IgA.** Immunoglobulin in whole serum was measured with Tripartigen plates (Behringwerke, Marburg, West Germany).

**Presentation of results.** Total IgG and IgA are given as grams per liter. Total subclass IgG is given as grams per liter, and total subclass IgA is given as ratios of absorbance values. For the CMV-specific ELISA, titration curves are shown for some serum samples. For the material as a whole, the results are presented as mean absorbances in at least two assays of the 1:10 serum dilution with background absorbances subtracted.

## RESULTS

**Standardization of reagents.** The two immunoglobulins used for standardization of reagents gave similar $A_{450}$ values for total IgG subclasses (Fig. 1). At a dilution of $10^{-5}$ of both immunoglobulins, the absorbance decreased steeply with all monoclonal antibodies. At this dilution, the $A_{450}$ values for Kabi IgG were 1.25, 1.50, 1.15 and 0.60 for IgG1 through IgG4, respectively. This should be compared with the known distribution of respective subclass of 77, 22, 3, and 0.6% in this gammaglobulin (13). With the monoclonal antibodies used, the assay is thus

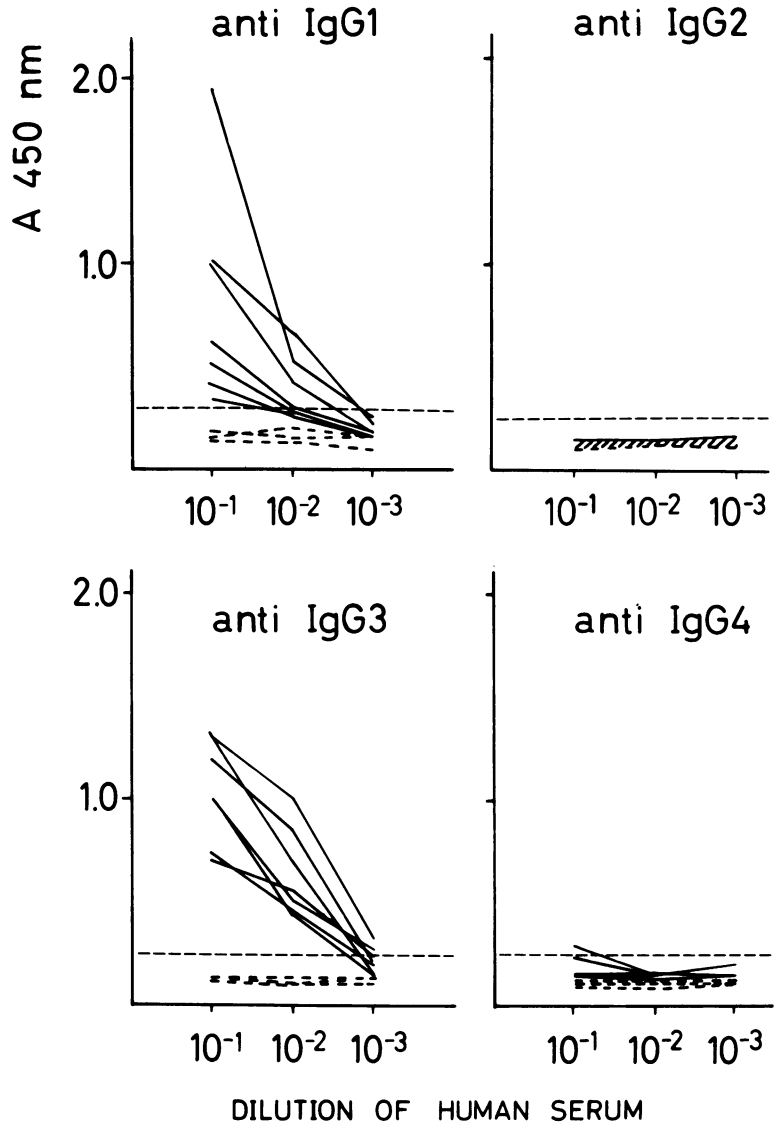


FIG. 2. CMV-specific IgG detected in subclasses IgG1 through 4 in serum samples of seven healthy seropositive donors (—) and three seronegative donors (---). The background value for each clone is shown (horizontal broken line).

least sensitive for IgG1, intermediate in sensitivity for IgG2, and most sensitive for IgG3 and IgG4. The assays are thus qualitative rather than quantitative. This is true also for the virus-specific assays.

**Background absorbances and reproducibility of the CMV-specific ELISA.** The backgrounds in the virus-specific ELISA ranged between 0.25 and 0.30, and the coefficients of variation ranged between 11 and 21% (see above). When monoclonal sera were added directly to CMV-coated plates, the $A_{450}$ values were the same as or slightly higher than when antibody-negative sera had been added, indicating a low nonspecific binding between antigen, plates, or both and monoclonal sera. Additional coating with bovine serum albumin did not reduce this background. The $A_{450}$ value for human serum and conjugate was always below 0.1.

**CMV-specific IgG subclass reactivity in immunoglobulins and serum samples from healthy donors.** The titration curves for anti-CMV activity of the immunoglobulins in the CMV-specific IgG ELISAs are shown in Fig. 1. For both immunoglobulins, high absorbance values were found with IgG1 and IgG3. IgG4 anti-CMV activity

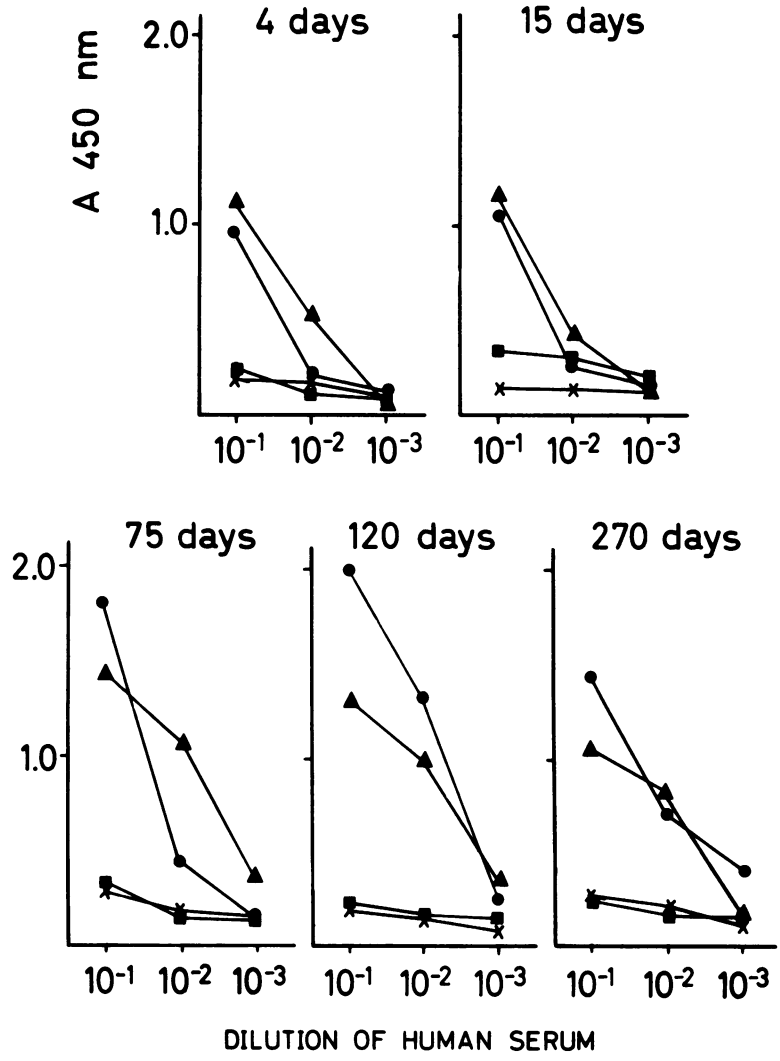


FIG. 3. Appearance of CMV-specific IgG subclasses 4 to 270 days after initial symptoms in a patient with reactivated CMV infection. Anti-IgG1 (●), IgG2 (×), IgG3 (▲), and IgG4 (□) monoclonal antibodies were used for CMV subclass IgG detection.

was also detectable. IgG2 was almost lacking in the Zig but was detectable in the Kabi gammaglobulin.

Titration curves for healthy blood donors are shown in Fig. 2. As with the immunoglobulins, the CMV antibody-positive serum samples had CMV specific IgG1 and IgG3 antibodies. Two of the healthy seropositive donors had traces of anti-CMV IgG4. IgG2 CMV antibodies were not found in CMV antibody-positive healthy persons. The CMV antibody-negative donors had $A_{450}$ values well below the background for all subclasses.

**Subclass IgG CMV reactivity in serum samples from patients with CMV infection.** Eight acute-phase serum samples negative in the CMV IgG ELISA were also negative in the subclass ELISAs. The subclass pattern of CMV-positive serum samples from patients with CMV disease was similar to that of serum samples from healthy donors, but the variation between the absorbances of subclasses 1 and 3 was larger. Fig. 3 shows the CMV-specific subclass pattern for five consecutive serum samples from one patient. This was a 55-year-old man with severe pneumonia, a titer rise in CMV IgG ELISA from 2,000 to 23,000, and traces of CMV IgM antibodies in serum samples taken from days 15 to 120. In the first two serum samples, $A_{450}$ for IgG3 was slightly higher than for IgG1. In a serum sample taken 120 days after the onset of the disease, there was instead a dominance of IgG1.

TABLE 1. Frequency of CMV IgG subclass positive sera from patients with current CMV infections

| IgG subclass | Primary CMV infection | | Reactivated CMV infection | |
|---|---|---|---|---|
| | Frequency of positive sera | Corrected mean $A_{450}$[a] | Frequency of positive sera | Corrected mean $A_{450}$ |
| 1 | 23/23 | 0.38 | 19/19 | 1.24 |
| 2 | 19/23 | 0.04 | 18/19 | 0.17 |
| 3 | 23/23 | 1.03 | 19/19 | 1.37 |
| 4 | 7/23 | 0.12 | 8/19 | 0.06 |

[a] The mean values were calculated on positive serum samples only and after background values (see text) had been subtracted.

After 270 days, when the patient had recovered and his total CMV IgG titer had decreased, the absorbances for IgG1 and 3 were also lowered. During the acute phase of the disease, traces of IgG4 CMV antibodies were found.

The number of subclass CMV-positive serum samples and the mean $A_{450}$ levels corrected for background absorbance of 42 serum samples from 17 patients with current CMV infection are presented in Table 1. All serum samples had IgG1 and IgG3 CMV antibodies. The mean $A_{450}$ values for IgG1 were higher in reactivated than in primary infection. IgG2 anti-CMV could be detected in most of the serum samples and was related to the total IgG anti-CMV titer. Corrected $A_{450}$ values for IgG2 were somewhat lower in primary than in reactivated infections. In both primary and reactivated infections, a few of the serum samples had clearly measurable IgG4 antibodies.

To illustrate the difference in CMV IgG1 between primary and reactivated disease, we calculated the ratio between corrected mean $A_{450}$ values for IgG1 and IgG3 at different times after the onset of disease (Table 2). IgG1 was higher in reactivated than in primary disease.

**Total subclass IgG.** Total IgG of all serum samples was 12.8 ± 4.8 g/liter. The distribution of IgG subclasses in whole serum is presented in Table 3. There were no significant changes in subclass distributions between CMV acute-phase and convalescent-phase serum samples. Eight patients with detectable CMV-specific IgG4 in both acute-phase and convalescent-phase serum had a higher total IgG4 level, their mean content of total IgG4 being 0.72 ± 0.5 g/liter, whereas the mean for patients without detectable CMV IgG4 was 0.40 ± 0.3 g/liter ($P < 0.05$, Student $t$ test).

**CMV-specific and total subclass IgA.** Total serum IgA of the patients with CMV was 2.5 ± 1.2 g/liter. Ten of the patients in the study had a measurable content of total CMV IgA. Eighteen

TABLE 2. Ratio of CMV IgG1:CMV IgG3 in patients with current CMV infections

| Days after onset of symptoms | Primary CMV infection | | Reactivated CMV infection | |
|---|---|---|---|---|
| | No. of sera | Ratio[a] | No. of sera | Ratio |
| 0–29 | 12 | 0.33 | 6 | 0.72 |
| ≥30 | 11 | 0.33 | 13 | 1.01 |

[a] Ratio = ($\bar{x}A_{450}$ of IgG1 − 0.3)/($\bar{x}A_{450}$ of IgG3 − 0.25).

serum samples from these patients were available for study. The subclasses of CMV-specific and total IgA were determined (Table 4). Antibodies of both subclass IgA1 and IgA2 were found to react with CMV antigen. There was no difference between acute-phase and convalescent-phase serum samples. The mean absorbance ratio for all samples of total IgA1 to total IgA2 was 1.5; the ratio for virus-specific IgA1 to IgA2 was 2.1. This indicates that anti-CMV antibodies of the IgA1 subclass are proportionally increased as compared with total serum IgA1 levels. ($P < 0.1$, $t$ test between ratios of total compared with virus-specific subclasses).

## DISCUSSION

CMV-specific IgG appeared to be mainly of subclasses IgG1 and IgG3. In primary disease, IgG3 was detected first and usually gave the highest absorbance values after 30 days of primary disease. In reactivated CMV, IgG1 and IgG3 were detected in both the acute and the convalescent phases. Both subclasses were also detectable in seropositive individuals.

IgG2 antibodies were measurable only when total CMV IgG was high. IgG2 to CMV was lacking in the Zig immunoglobulin. The reason for this is not known, but it may depend on the difference in fractionation methods between the gammaglobulins Kabi and Zig or on a difference in the populations who donated the serum samples. Presence or absence of CMV-specific IgG4 seemed to be related to the donor. IgG4 content in serum is known to vary more than the other subclasses (7), and IgG4 antibodies to herpes

TABLE 3. Total IgG subclass contents of serum

| Serum | Total IgG of subclass (g/liter ± SD): | | | |
|---|---|---|---|---|
| | IgG1 | IgG2 | IgG3 | IgG4 |
| Acute phase of CMV | 6.9 ± 2.8 | 3.9 ± 1.5 | 1.2 ± 0.6 | 0.5 ± 0.5 |
| Convalescent phase of CMV | 8.3 ± 3.0 | 4.4 ± 1.9 | 1.6 ± 0.7 | 0.5 ± 0.5 |
| Ten healthy persons | 5.7 ± 3.2 | 2.4 ± 0.9 | 1.2 ± 1.1 | 0.5 ± 0.4 |

TABLE 4. IgA subclasses in patients with current CMV disease

| Serum | No. of samples | CMV-specific IgA | | | Total IgA |
|---|---|---|---|---|---|
| | | $A_{450}$ | | Ratio IgA1:IgA2 | Ratio IgA1:IgA2 |
| | | IgA1 | IgA2 | | |
| Acute phase of CMV | 8 | 0.7 ± 0.3 | 0.3 ± 0.2 | 2.2 ± 1.4 | 1.8 ± 0.7 |
| Convalescent phase of CMV | 10 | 0.7 ± 0.3 | 0.4 ± 0.2 | 2.0 ± 0.6 | 1.3 ± 0.6 |
| IgA2 deficiency | 1 | 1.4 | 0.01 | | |
| Healthy seronegative persons (control) | 3 | 0.1 | 0.1 | | 1.1 |

simplex and varicella viruses were found in certain individuals only (our unpublished results). Patients with higher than average levels of total IgG4 also had detectable anti-CMV of subclass IgG4. Thus, persons with high total IgG4 may also have virus-specific IgG of this subclass. In patient serum samples in which CMV IgA was detectable, it appeared to be of both the A1 and A2 subclasses, with a mean A1:A2 ratio of 2.1, whereas the mean ratio of total IgA1 to IgA2 was 1.5. The monoclonal anti-IgA1 and anti-IgA2 antisera can be assumed to measure total subclass IgA as efficiently as virus-specific subclass IgA. It appears, therefore, that IgA1 has a higher reactivity to the CMV antigen. The explanation for the dominance of IgG1 and IgG3 as CMV-specific antibodies is not known. Functionally, these subclasses bind to phagocytic cells, and they are also the most efficient in complement fixation (14). Those two mechanisms are probably important in the control of virus disease.

Owing to different efficiencies of the monoclonal antibodies used for measuring subclass antibody content, our assays are not quantitative. The changes in relation of detectable subclass antibodies were therefore expressed as absorbance ratios.

The subclass specificity of monoclonal antibodies has been described by Lowe et al. (6). The small cross-reactivity observed between subclasses might depend upon contamination of myeloma proteins used for such specificity control. The finding of distinct CMV-reactive subclasses during primary and reactivated disease also favors the concept that the monoclonal antibodies are subclass specific. The avidity may, however, vary among clones. It is well known that high-avidity polyclonal antisera to IgG1 and IgG2 are difficult to produce, and the same seems to be true for monoclonal antibodies. As judged by the reactivity with immunoglobulin preparations for which the subclass content is known (9) and the World Health Organization reference serum, IgG1 is the least reactive of the four monoclonal antibodies used.

It would have been possible to correct the dilution of the other monoclonal antibodies in the assays so that the amount of subclass IgG would have been about the same for all subclasses. In that case, we would not have detected any of the virus-specific IgG2 or IgG4 antibodies and some of the IgG3 antibodies would also have remained undetected. In this work, therefore, we concentrated on the presence or absence of CMV-specific subclass antibodies with the highest sensitivity obtainable.

The monoclonal antibodies to subclasses of IgG and IgA provide an opportunity to examine the antigen reactivity of different subclasses as well as the total amount of subclass IgG and IgA. Our findings are of general importance for viral serology. In view of the high amount of antiviral reactivity of IgG3, the use of protein A, which does not react with human IgG3, for precipitation in radioimmunoassays and for preparation of immune IgG is questionable. Another issue of importance is whether commercial anti-human IgG antisera for diagnostic use react equally well with all IgG subclasses. With the aid of antiviral subclass studies one might be able to account for discrepancies among different serological methods for the same antiserum.

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