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Fine Specificity of the Human Immune Response to the Major Neutralization Epitopes Expressed on Cytomegalovirus gp58/116 (gB), as Determined with Human Monoclonal Antibodies

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The humoral immune response to human cytomegalovirus (CMV) membrane glycoprotein gp58/116 (gB) has been studied by establishing cell lines producing specific human monoclonal antibodies. These cell lines were generated from peripheral blood lymphocytes obtained from a healthy carrier. Hybridomas producing gp58/116-specific antibodies were detected by reactivity to procaryotically expressed proteins containing the major neutralizing epitopes of this glycoprotein complex. One antibody, ITC88, which recognized an epitope located between amino acid residues 67 and 86 of gp116, potently neutralized the virus at 1 to 2 μg of immunoglobulin G per ml. Only four of the six human antibodies detecting the major neutralizing domain of gp58 neutralized the virus, and none of them required complement for activity. All antibodies that bound mature, processed gp58 recognized a conformational epitope involving sequences between residues 549 and 635. However, small differences existed between the antibodies in the actual minimal requirement for C- and N-terminal parts of this epitope. By peptide mapping with several of the antibodies, the epitope was shown to consist mainly of residues between amino acids 570 to 579 and 606 to 619. Despite the conformational nature of the epitope, the antibodies recognized both reduced and denatured native antigen. Presence of carbohydrates was not required for antigen binding of these gp58-specific human antibodies, but in at least one case, it greatly enhanced antigen recognition, indicating an importance of carbohydrate structures in some epitopes within the major neutralizing specificity of gp58.

Human cytomegalovirus (CMV) is commonly present in the human population, and many individuals are carriers without expressing clinical symptoms of disease. This virus is, however, also an important human pathogen with an ability to cause disease in immunocompromised subjects (17). Furthermore, infants born with congenital infection are at risk of morbidity and long-term sequelae (1). Consequently, a major interest in the prevention and treatment of clinical disease has developed. Attempts to use immunoglobulin preparations containing high titers of CMV antibodies in the treatment of viral disease have had some effects (33, 51), although the fact that only partial protection has been achieved suggests the importance of a protective T-cell immunity in the normal defense against the virus. However, the use of polyclonal antibodies in passive immunotherapy of CMV-induced disease is possibly complicated by the presence of virus-specific antibodies that actually may prevent the neutralization of the virus (29, 47). After a careful selection of suitable specificities, neutralizing human monoclonal antibodies may present a solution to this problem in the treatment of active disease. Development of vaccines may also present a future way of preventing disease, in particular if one can avoid inducing unwanted specificities in the serological response.

Monoclonal antibodies with neutralizing activity against CMV have been described (21, 31, 40, 47), and antigenic targets are found in surface glycoprotein complexes gp58/116 (gB, gC-I), gp47-52 (gC-II), and gp86 (gH, gC-III) (14), of which gp58/116 has been shown to contain the major neutralization epitope (4). This protein complex is known to be synthesized as a 130-kDa precursor which is cleaved into two covalently linked molecules (gp116 and gp58). The N-terminal fragment (gp116) contains one linear, isolate-specific epitope (32) and one linear, isolate-cross-reactive, neutralization epitope which does not require complement for the antibody-mediated biological activity (31). The gp58 molecule is known to carry one immunodominant, neutralization domain (20, 22, 29, 37, 47). Human monoclonal antibodies recognizing this protein complex have previously been developed (10–12, 30, 46).

In this study, we have generated eight human monoclonal antibody-producing cell lines originating from lymphocytes obtained from a CMV-seropositive donor. The CMV-specific human monoclonal antibodies have been utilized to evaluate the fine specificity of the human immune response to the major neutralization epitopes of gp58/116 and to study the implications of these specificities on antigen recognition and biological function.

MATERIALS AND METHODS

Antigens. The molecular weight-based names of gB are used as previously described (31). Briefly, the N- and C-terminal fragments of gB are called gp116 and gp58, respectively. In addition, the completely processed but uncleaved precursor is referred to as gp130. Molecular weights, determined by Western blot (immunoblot), of these

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forms may differ slightly from the values indicated by these names. Native CMV antigens and fractions enriched for membrane antigens were prepared from CMV (Ad-169)-infected human lung fibroblasts (27). Recombinant CMV gp58 (amino acids [aa] 484 to 650; pMbg58) was produced as a β-galactosidase (aa 1 to 375) fusion protein in *Escherichia coli*. A recombinant protein expressing a part of CMV gp116 (aa 28 to 101; pHM90-5) (31) was also used for screening purposes. Recombinant fusion proteins expressing other, short fragments of CMV gp58 together with β-galactosidase (aa 1 to 375) or tryptophan synthetase were produced as previously described (22).

Monoclonal antibodies. All human monoclonal antibodies studied in this investigation were prepared as culture supernatants in spent tissue culture medium. They were used without further purification or after ammonium sulfate (2 M) precipitation. Human monoclonal antibodies specific for tetanus toxoid (provided by R. Carlsson, BioInvent International AB, Lund, Sweden) and CMV pp65 (35) were used as negative controls, and the mouse anti-CMV gp58 monoclonal antibody 27-287 was used as a positive control. The latter antibody is by itself not neutralizing, but it binds to the major neutralization determinant of gB and is able to block the complement-independent, neutralizing activity of mouse antibody 7-17 upon binding to intact virus (47).

Generation of human monoclonal antibodies. Human lymphoblastoid cell lines producing antibodies recognizing recombinant CMV gp58 (pMbg58) and gp116 (pHM90-5) were established after Epstein-Barr virus (EBV) transformation of L-leucyl-L-leucine methyl ester-treated peripheral blood mononuclear cells, obtained from normal, CMV-seropositive blood donors (Lund University Hospital Blood Bank, Lund, Sweden), by using technology recently reviewed (34). In order to rescue antibody production and to increase antibody productivity, hybridomas were established by fusing the antigen-specific human lymphoblastoid cell lines to human × mouse heteromyeloma K6H6/B5 (6) or CB-F7 (15), by using electrofusion technology or by polyethylene glycol-mediated fusion of bulk cultured EBV-transformed cells (34). Hybridomas were cloned at least twice at limiting dilution on irradiated (30 Gy) feeder cells (human peripheral blood mononuclear cells or mouse peritoneal macrophages) (34).

ELISA. Antigen-specific reactivity of human antibodies was determined by enzyme-linked immunosorbent assay (ELISA). Recombinant CMV antigen, diluted in phosphate-buffered saline (PBS) or in PBS containing 6 M urea, was coated into 96-well microtiter plates. Cell culture supernatants were diluted in sample dilution buffer (10 mM sodium phosphate, 0.5 M NaCl, 0.1% Tween 20 [pH 8.0]) prior to analysis (34), and detection of bound antibody was performed with peroxidase-labelled, γ-chain-specific goat antibodies (Zymed Laboratories, Inc., San Francisco, Calif.). Reactivity to native CMV antigens was determined in a similar assay, as previously described (35). Cross-reactivity to herpes simplex virus type 1 and varicella-zoster virus was evaluated by routine ELISA techniques (28, 44).

To study the participation of carbohydrate structures in the epitopes, native membrane antigen was coated in PBS onto ELISA plates. Following a wash to remove nonbound antigen, carbohydrate structures containing vicinal hydroxyl groups were oxidized by using sodium periodate and then reduced by sodium borohydride (52). The effect of sialic acid substitution on carbohydrates was studied by treating coated antigen with neuraminidase from *Clostridium perfringens* (Sigma, St. Louis, Mo.; 0.025 U/ml of PBS, 2 h at 37°C). The enzymatic treatment was carried out under conditions which completely removed epitopes recognized by antibody B72.3 (18). Remaining antibody reactivity was detected by ELISA, as described above.

Blocking of monoclonal antibody reactivity by human and mouse monoclonal antibodies or polyclonal sera was performed by preincubating antigen-coated microtiter plates with blocking antibody, prior to addition of the test antibody. If the species of origin of the blocking and test antibody was the same, biotinylated test antibodies (35) were used. Binding of the test antibody was determined by incubation with peroxidase-labelled streptavidin, rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) or goat anti-human immunoglobulin G (IgG) (Zymed Laboratories, Inc.), as required by the analytical setup. *o*-Phenylenediamine was used as the enzyme chromogen.

Determination of antibody light chain and subclass was performed by ELISA, as previously described (35). Affinity constants were determined by a noncompetitive ELISA with native extracted membrane antigens or recombinant antigen, as previously outlined (2).

Epitope mapping with synthetic peptides. Overlapping peptides, of 20 aa in length (overlapping by 10 to 14 aa), covering the entire sequence (8) of pMbg58, were prepared by solid-phase synthesis (41). Peptides covering residues 67 to 86 of gp116 were similarly prepared. These peptides were diluted to 10 μg/ml in 50 mM sodium carbonate buffer and coated directly to the wells of 96-well microtiter plates. After incubation with human monoclonal antibodies, specific reactivity was detected (42). Soluble peptide T7-13 (residues 67 to 86 of gp116) was also used to inhibit the binding of antibodies to recombinant gp116 (pHM90-5). A peptide derived from human immunodeficiency virus type 1 IIIB gp120 (aa 320 to 339) was used as a negative control.

SDS-PAGE and Western blot. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of native CMV membrane antigens was performed under reducing conditions by the method of Laemmli (23). Molecular weights were assessed in comparison to molecular weight standards (Bio-Rad, Richmond, Calif.). Electrophoretically separated proteins were transferred to nitrocellulose by using a semidry electrotransfer technique (16). The nitrocellulose was blocked with 1% bovine serum albumin (BSA) in TBS (50 mM Tris, 100 mM NaCl [pH 7.4]) (TBS-BSA). After incubation of human monoclonal antibodies for 2 h at 0.5 to 1 μg of IgG per ml of TBS-BSA (containing 0.05% Tween 20), bound antibody was detected with peroxidase-labelled goat anti-human IgG (Zymed) and by the enhanced chemiluminescence technology (Amersham International, Amersham, Buckinghamshire, United Kingdom). All washing steps were performed in PBS containing 0.1% Tween 20. Reactivity was finally detected by exposing Kodak XAR-5 diagnostic film (Eastman Kodak Company, Rochester, N.Y.) to the nitrocellulose filter. In one set of experiments, N-linked carbohydrates were removed by incubation of reduced and denatured antigen for 20 h with *N*-glycosidase F (Boehringer Mannheim GmbH, Mannheim, Germany; 10 U/ml) prior to electrophoresis.

Recombinant proteins expressing parts of gp58 were separated by SDS-PAGE and blotted to nitrocellulose prior to incubation with antibody, as previously described (49).

Virological tests. Immunofluorescence staining of CMV-infected human lung fibroblasts was performed, as previously described (50). Cross-reactivity with human herpesvirus 6 and EBV was also evaluated by immunofluorescence studies (24, 25). Neutralization of CMV was performed by a microneutralization modification of the technique described

TABLE 1. Summary of characteristics of anti-CMV gp58/116-specific human monoclonal antibodies

Clone	Fusion partner	Immunoglobulin chains	Affinity constant (M^{-1})			Immunofluorescence[a]	Virus neutralization[b]	Western blot reactivities (kDa)
			pHM90-5	pMbg58	Native antigen			
ITC33	K6H6/B5	γ1/λ	ND[c]	3×10^7	2×10^9	C	No	58, 130
ITC34	K6H6/B5	γ1/κ	ND	2×10^9	ND	C + N	No	83, 110, 150
ITC39	CB-F7	γ1/λ	ND	7×10^7	1×10^9	C	No	58, 130
ITC48	K6H6/B5	γ1/κ	ND	2×10^8	9×10^9	C	Yes	58
ITC52	K6H6/B5	γ1/κ	ND	2×10^8	3×10^{10}	C	Yes	58, 130
ITC63B	CB-F7	γ1/λ	ND	2×10^8	3×10^9	C	Yes	58, 130
ITC63C	CB-F7	γ1/λ	ND	1×10^8	3×10^9	C	Yes	ND
ITC88	K6H6/B5	γ1/κ	2×10^9	ND	3×10^8	C	Yes	92, 130

[a] C and N indicate a cytoplasmic and nuclear staining pattern, respectively.
[b] Identical results were obtained in the absence and presence of complement.
[c] ND, not determined.

by Gilljam and Wahren in the presence or absence of complement (13).

RESULTS

Generation of human monoclonal antibodies. EBV transformation of human peripheral blood mononuclear cells, following the removal of lysosome-rich cell populations by treatment with L-leucyl-L-leucine methyl ester, provided a number of nonclonal cell lines producing antibodies recognizing recombinant antigens derived from CMV gB. The frequency of specific cell lines established from each lymphocyte donor did not correlate with the serum titer against the recombinant antigens (data not shown). To improve the stability of antibody production, cell lines producing specific antibodies were pooled and fused by the electric pulse-mediated technique to a human × mouse heteromyeloma. Seven hybridoma cell lines producing human IgG1 antibodies recognizing pMbg58 (recombinant gB [aa 484 to 650]) were obtained from human lymphoblastoid cell lines established from one donor, following electrofusion (Table 1). One hybridoma secreting antibody specific for pHM90-5 was established by polyethylene glycol-mediated cell fusion of EBV-transformed B cells, grown in bulk culture for 5 days prior to fusion. The lymphocytes used in this latter experiment were obtained from the donor presented above 8 months after the collection of lymphocytes, giving rise to the pMbg58-specific hybridomas.

Recognition of recombinant and native antigen and synthetic peptides. The specific human monoclonal antibodies recognizing the major neutralization determinant of gp58, as expressed by pMbg58, could be separated by differences in their fine specificities. Six of these antibodies (ITC33, ITC39, ITC48, ITC52, ITC63B, and ITC63C) all recognized native antigen with a higher affinity constant (14 to 150 times) compared with that of recombinant antigen (Table 1). The epitopes recognized by five of these antibodies were overlapping and corresponded closely with the determinant of gp58 recognized by mouse monoclonal antibody 27-287, as determined by blocking experiments (Fig. 1). Antibody ITC48, however, was able to block only partially the reactivity of the mouse antibody, despite its relatively high affinity for the native antigen. To further evaluate the fine specificity of these antibodies, their recognition of short recombinant fragments of gp58 was determined. It was clear that they all recognized overlapping sequences, although their requirement for particular amino acids to create a recognizable epitope differed. The minimal epitopes could be found to be located between residues 549 and 635 (ITC33), 552 and 635 (ITC39, ITC52, ITC63B, and ITC63C), or 552 and 630 (ITC48) (Table 2), as determined by immunoblotting with these fragments. The reactivity of four of these antibodies was, furthermore, found to be associated with sequences located between residues 570 and 579 as well as between residues 606 and 619 (Fig. 2).

Antibodies recognizing the major neutralization determinant of gp58 all recognized denatured antigen after SDS-PAGE and Western blotting, although with different intensities (Fig. 3). Both the high-molecular-weight precursor (gp130) and the C-terminal fragment (gp58) bound ITC33, ITC39, ITC52, ITC63B, and ITC63C, while antibody ITC48 recognized only the latter fragment. In contrast to the above-mentioned antibodies, ITC34 showed a high affinity for the recombinant antigen (Table 1), although the total antibody binding capacity of a given amount of antigen was substantially lower compared with those of other pMbg58-specific antibodies (data not shown). The epitope recognized by this antibody was, furthermore, poorly represented in membrane antigen preparations, although ITC34 recognized infected cells as determined by ELISA and flow cytometry (data not shown). On the basis of blocking experiments and recognition of gp58-related recombinant fragments, it was clear that this antibody recognizes an epitope different from the major neutralization determinant recognized by the other antibodies.

Antibody ITC88 was originally selected for its reactivity with a recombinant fragment of gp116 (pHM90-5), which was recognized with a high affinity (Table 1). As expected from this specificity, it recognized both the high-molecular-weight precursor as well as the mature N-terminal fragment of gB (Fig. 3). Its fine specificity was more exactly determined to reside between amino acids 71 and 78 of gB (Fig. 4).

Carbohydrate involvement in neutralization determinants. The neutralization epitope expressed by gp116 seemed not to require carbohydrates to mediate high-affinity binding of ITC88. Nonglycosylated recombinant antigen was recognized with a high affinity (Table 1), and treatment of native antigen with neuraminidase or periodate did not reduce antibody binding (Fig. 5). In addition, treatment with *N*-glycosidase F did not affect antibody binding following SDS-PAGE and Western blotting (Fig. 5). In contrast, some of the antibodies recognizing the major determinant of gp58-bound recombinant, nonglycosylated antigen with a low affinity (Table 1). Their recognition of native antigen was substantially affected by prior treatment to remove carbohydrate structures. Although neuraminidase treatment did not substantially affect antibody binding, periodate treatment re-

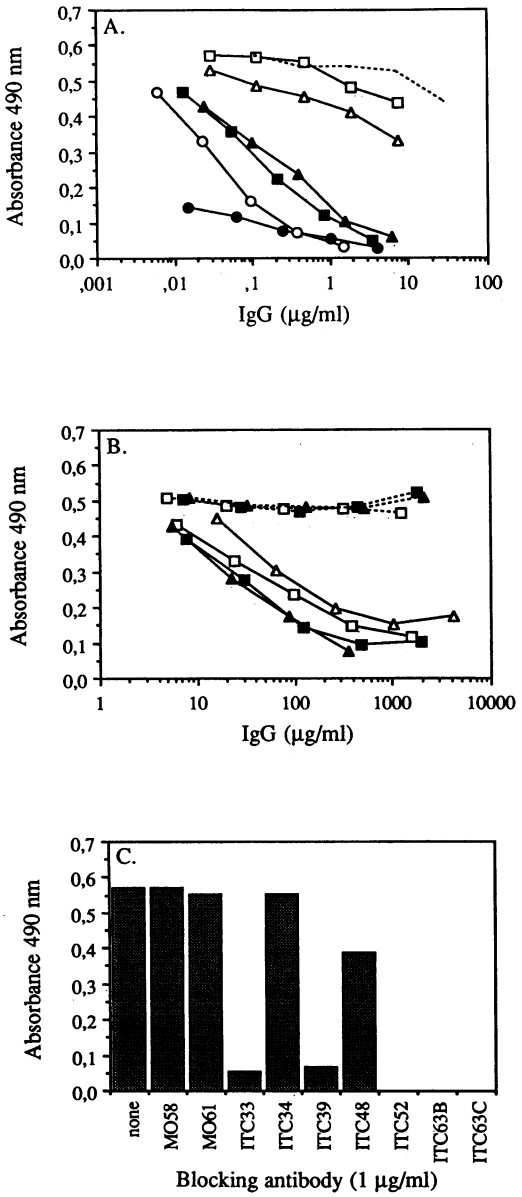

FIG. 1. Blocking of the epitope recognized by biotinylated ITC52 in membrane preparations of CMV-infected human fibroblasts by human monoclonal antibodies ITC33 (■), ITC34 (□), ITC39 (▲), ITC48 (△), ITC52 (●), ITC63B (○), and MO61 (broken line) (A) and by human polyclonal sera (B). Four of the serum donors were seropositive (solid lines; titer [A_{490} = 1.0], >1/100) as determined by ELISA against the antigen preparation, while three donors were seronegative (broken lines) by the same criteria. (C) Blocking of the epitope recognized by the mouse monoclonal antibody 27-287 with human monoclonal antibodies. MO58 and MO61, which served as negative controls, are human antibodies (IgG1) recognizing CMV pp65 (35).

duced the binding of the gp58-specific antibodies, without reducing the binding of the carbohydrate-independent antibody ITC88 (Fig. 5). The reduction in binding under these experimental conditions was in the range of 20 to 60%, and it differed between the antibodies. The most periodate-sensitive specificity (recognized by ITC33) was to a large degree also eliminated by *N*-glycosidase F treatment, while the overlapping epitope recognized by antibody ITC52 was more refractory to this treatment (Fig. 5).

Neutralizing activity of anti-gB specific monoclonal antibodies. The ITC88 antibody, which is specific for gp116, and four (ITC48, ITC52, ITC63B, and ITC63C) of the seven antibodies against gp58-related sequences neutralized the virus, both in the absence and presence of complement (Table 1). Among these antibodies, ITC88 was the most potently neutralizing antibody, showing 50% neutralization at 1 to 2 μg of IgG per ml.

Recognition of clinical isolates of CMV and other herpesviruses. Immunofluorescence studies of human lung cells infected with clinical isolates of CMV showed that the human antibodies described here against CMV gB recognized five of five tested specimens. In contrast, none of these antibodies recognized antigens expressed by other herpesviruses (EBV, human herpesvirus 6, herpes simplex virus type 1, and varicella-zoster virus).

Presence of antibodies in sera with specificities similar to those of the human monoclonal antibodies. It was evident that specificities closely related to the specificities of the monoclonal antibodies occurred frequently in seropositive serum, as determined by the ability of human sera from CMV-seropositive lymphocyte donors to inhibit the binding of gp58- and gp116-specific human monoclonal antibodies (Fig. 1 and 4). Furthermore, it was clear that a major part of the seroreactivity against the N-terminal part of gp116 (Ad-169) was overlapping with the specificity of ITC88, as shown by the ability of a synthetic peptide to efficiently block the reactivity of serum antibodies to the recombinant protein pHM90-5.

DISCUSSION

In this investigation, the immune response to CMV gB at the clonal level is extended from the immunized mouse system to the naturally infected human host. Previously, data on the reactivity of human polyclonal antibodies against the gB antigen have been collected (3, 5, 19, 22, 26, 38, 48), and it has been shown that 40 to 70% of serum CMV-neutralizing activity is specific for gB (4). Three to seven epitopes have been described for the major neutralizing determinant of gp58, on the basis of studies of the murine immune response against gB (20, 29, 37). On the basis of the human monoclonal antibodies described here, at least six different reactivities against aa 484 to 650 of gB have been observed. This assignment is derived from mutual blocking activity as well as peptide and recombinant protein recognition. In addition, other epitopes not present in the recombinant antigen used for initial screening may be located within this sequence.

Even though five of the six gp58-specific antibodies recognize the antigen after SDS-PAGE and Western blotting, it seems that several or all of these antibodies recognize discontinuous epitopes. The individual epitope integrity is, however, more or less sensitive to denaturing and reducing conditions. Carbohydrate structures appear to be important for maintaining the correct configuration of the major epitopes of gp58, as shown by a reduction in antibody reactivity after periodate oxidation or enzymatic deglycosylation of the antigen. This may partly explain the lower affinity of these antibodies for procaryotically expressed gp58 fragments. A definition of the unique epitope recognized by ITC34 could not be made. The different compartmentation of the antigen containing this epitope, as detected by immunofluorescence, may indicate that ITC34 recognized some precursor of gB (39).

The major neutralization epitope present on gp58 was

TABLE 2. Binding of the human monoclonal antibodies to recombinant fragments of gp58 as determined by SDS-PAGE and Western blotting

Peptide	Sequence (aa)	Binding of following antibody preparation[a]									
		Mouse antibody	Human antibody[b]	Human sera[b]	ITC33	ITC34	ITC39	ITC48	ITC52	ITC63B	ITC63C
pMbg58	484–650	+	+	+	+	+	+	+	+	+	+
58-Nci	496–621	−	−	−	−	−	−	−	−	−	−
gig58-8	484–616	−	−	−	−	−	−	−	−	−	−
58-Hin	593–644	−	−	−	−	−	−	−	−	−	−
58-Dde	607–638	−	−	−	−	−	−	−	−	−	−
gig58-7	484–588	−	−	−	−	−	−	−	−	−	−
gig58-2	549–645	+	+	+	+	−	+	+	+++	+	+
gig58-2N[c]		−	−	−	−	−	−	−	−	−	−
Exo58-315	549–635	+	+	+	+	−	+	+	+++	+	+
Exo58-36	549–630	−	+	+	−	−	−	+++	−	(+)	−
Exo58-35	549–628	−	−	−	−	−	−	−	−	−	−
Exo58-5A15	552–635	+	+	+	−	−	+	+	+	+	+
Exo58-5E39	563–635	−	−	−	−	−	−	−	−	−	−

[a] −, no detectable reactivity; (+), barely detectable reactivity; +, reactivity; +++, very strong reactivity.
[b] Provided for comparison (based on data from Wagner et al. [49]).
[c] This protein is identical to gig58-2 (aa 549 to 645) except that it carries a 4-aa (IAMA) insert at position 616.

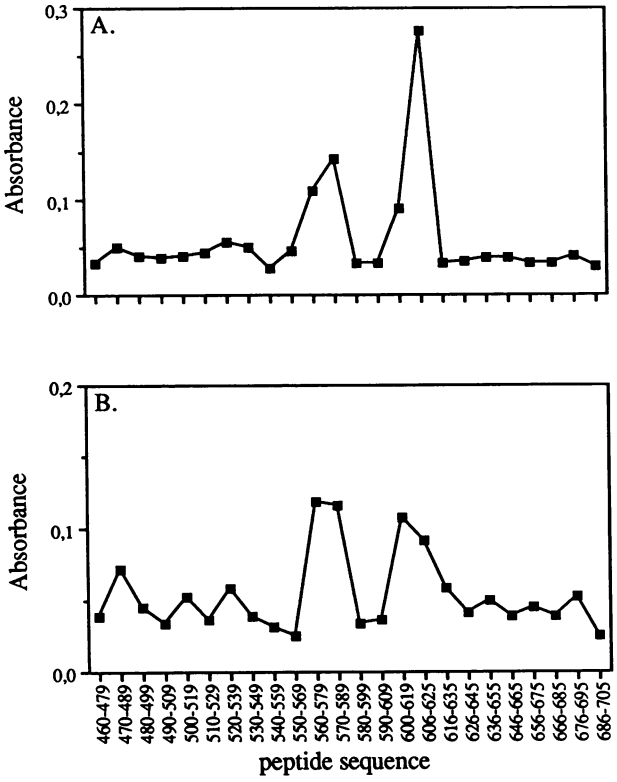

FIG. 2. Binding of human monoclonal antibodies ITC33 (A) and ITC63C (B) to solid-phase bound, overlapping peptides covering a large part of the external domain of CMV gp58 and including the entire sequence of pMbg58 (aa 484 to 650). The binding pattern obtained for ITC63B was identical to the one shown for ITC63C, while ITC48 and ITC52 showed results similar to those of ITC33. The specificity of binding of ITC48 to peptide sequence 470 to 489 (not shown) is controversial, since some control antibodies also react with this peptide. Neither ITC34 and ITC39 nor control antibody MO58 (35) showed any specific binding in this analysis.

originally believed to be a linear sequence close to residue 616 (47). Recent evidence obtained by using mouse monoclonal antibodies and human sera points, however, towards a more complex epitope structure (20, 49). This has now been confirmed at the clonal level in the human system by this study, since residues far apart in the primary sequence appear to be important for proper antigen binding. The ability of some gp58-specific antibodies to bind short solid-phase immobilized peptides was significant, although the signal obtained was low, as would be expected of antibody binding to peptides expressing only a part of the relevant epitope. Similar antibody recognition of single, linear sequences, which in the native antigen together form a complete epitope, have previously been reported for antibodies recognizing the VP1 protein of foot-and-mouth disease virus (36). The dependence on a three-dimensional conformation was further underlined by the lower affinity of the human antibodies for recombinant compared with native antigen.

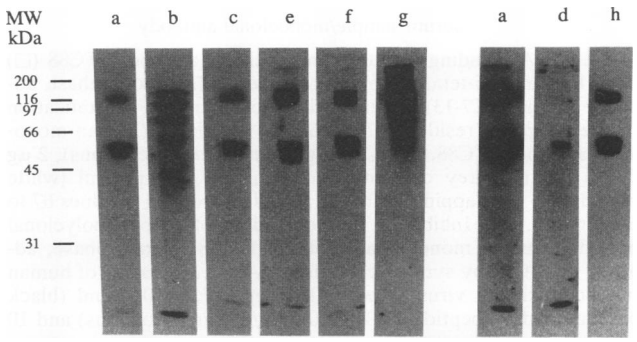

FIG. 3. Determination of the molecular weights of native antigen recognized by monoclonal antibodies ITC33 (lanes a), ITC34 (lane b), ITC39 (lane c), ITC48 (lane d), ITC52 (lane e), ITC63B (lane f), ITC88 (lane g), and mouse anti-gp58 antibody 27-287 (lane h) by SDS-PAGE and Western blot. Molecular mass standards are indicated to the left. The amount of antigen used in lane c, for detection of binding of ITC34, is three times higher compared with that in the other lanes. In order to detect the reactivity of ITC48, the exposure time for the three lanes to the far right is four times longer compared with that of the other lanes.

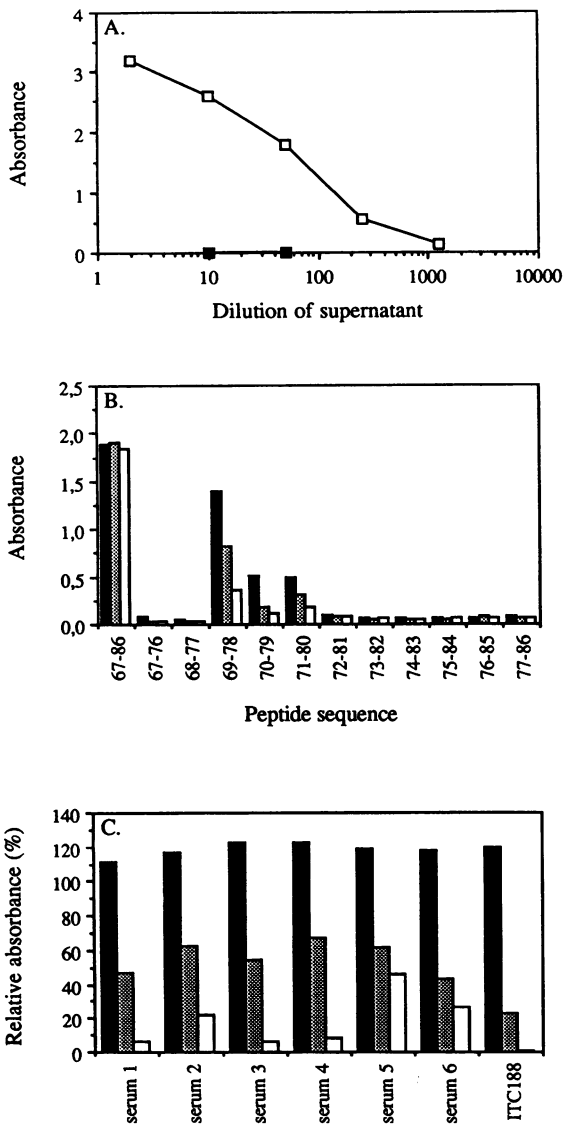

FIG. 4. (A) Binding of human monoclonal antibody ITC88 (□) and a human anti-tetanus toxoid antibody (■) to solid-phase adsorbed peptide (T7-13) covering the cross-reactive neutralization epitope of gp116 (residues 67 to 86). (B) Binding of human monoclonal antibody ITC88, at 4 μg of IgG per ml (black columns), 2 μg of IgG per ml (grey columns), and 1 μg of IgG per ml (white columns), to overlapping synthetic peptides covering residues 67 to 86 of gp116. (C) Inhibition of the binding of human polyclonal antibodies and of monoclonal antibody ITC88 to solid-phase, adsorbed pHM90-5 by synthetic peptide A5-4 [aa 320 to 339 of human immunodeficiency virus type 1 (IIIB) gp120] at 10 μg/ml (black columns) and by peptide T7-13 at 0.5 μg/ml (grey columns) and 10 μg/ml (white columns).

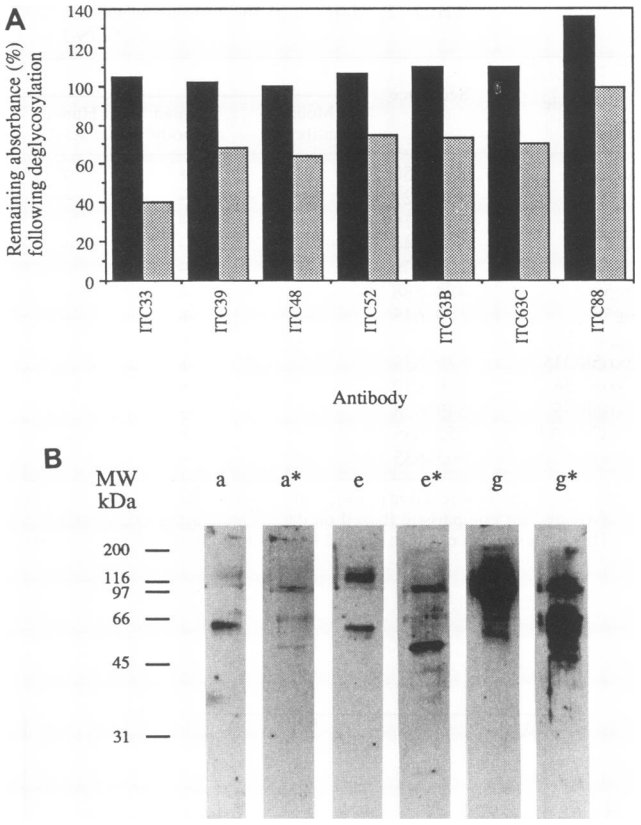

FIG. 5. (A) Determination of carbohydrate involvement in gB epitopes recognized by human monoclonal antibodies, after neuraminidase (black columns) and periodate-borohydride (grey columns) treatment. Reactivity to treated and untreated native membrane antigen was evaluated by ELISA and plotted as a quotient. Data for antibody ITC34 are not shown, because of its poor binding in this assay format. (B) Effects on the molecular mass of the antigen and on the intensity of binding by ITC33 (lanes a), ITC52 (lanes e), or ITC88 (lanes g) to antigen following enzymatic removal of N-linked carbohydrates. *, antigen had been enzymatically deglycosylated prior to SDS-PAGE and Western blot.

Considering the fact that these antibodies were originally selected in vivo with the properly glycosylated and folded native antigen after infection with the virus, such differences in affinity constant are not surprising.

On the basis of enzymatic degradation studies of gp58, it was shown that methionines located at positions 541, 564, and 635 (Ad-169 sequence [8]) may be involved in the formation of the epitope recognized by the gp58-specific mouse antibodies (20). By using recombinant fragments expressing different parts of gp58, it was evident that the minimal requirement for reactivity of the human antibodies in most cases included Met-564 as well as amino acid residues close to Met-635. This supports the results obtained by using mouse monoclonal antibodies. Furthermore, peptide mapping indicated that sequences between these methionine residues are important for antibody binding (Fig. 2). One antibody, ITC48, had a less extensive sequence requirement in the C-terminal part of the epitope, making it similar in reactivity to a previously described neutralizing human antibody (Table 2) (49).

In contrast to the major neutralization epitope on gp58, the epitope on gp116 detected by ITC88 seemed to be less dependent on intact protein structures. The antibody bound well to solid-phase immobilized peptide (residues 67 to 86), and its reactivity to immobilized recombinant antigen was effectively inhibited by the soluble peptide. Furthermore, the affinity for the *E. coli*-derived recombinant fragment pHM90-5 derived from gp116 was higher compared with that

for native antigen, indicating little dependence on a labile tertiary structure. The peptide reactivity pattern maps the epitope recognized by ITC88 to a sequence close to the epitopes recognized by other neutralizing gp116-specific antibodies (19, 31, 32). In addition, this epitope seems to coincide with the epitope that is detected by most serum antibodies recognizing the N-terminal part of CMV Ad-169 gp116, since a substantial part (55 to 95%) of the seroreactivity to the recombinant protein pHM90-5 could be inhibited by this peptide.

To evaluate the possible biological effects of these human monoclonal antibodies, their neutralizing activity against CMV Ad-169 was studied by two different assays. The gp116-specific antibody potently neutralized the virus in the absence of complement, in agreement with the behavior of other human and mouse antibodies recognizing closely related epitopes (19, 31). In contrast, pMbg58/gp58-specific antibodies were less effective in neutralizing the virus, and ITC33, ITC34, and ITC39 were unable to do so even in the presence of complement (Table 1). The complement-independent neutralizing activity of antibodies binding to *E. coli*-derived gp58 is in agreement with previous studies. Britt et al. (5) showed that procaryotically derived gB would mainly induce a complement-independent polyclonal, neutralizing response in mice, while eucaryotically expressed gB mainly induced a complement-dependent neutralizing response.

Neutralizing antibodies recognizing CMV determinants may be useful for therapeutic applications in immunosuppressed individuals. In this study it has been shown that some neutralizing gp58-specific human antibodies have characteristics differentiating them from nonneutralizing antibodies. In particular, recognition of a high epitope density in recombinant gp58 (pMbg58) seems to correlate with neutralizing activity. Some characteristics may vary within the group of neutralizing antibodies, but at least two of them (one from reference 49 and antibody ITC48) that bind to an epitope located between amino acid residues 552 and 630 have been found to be neutralizing, while three antibodies that did not neutralize CMV also did not recognize this particular sequence. This sequence may, thus, serve as a useful target to quantify part of the neutralizing response. The usefulness of a monoclonal antibody for in vivo therapy depends on its recognition of several different isolates of the pathogen. It is known that the sequences recognized by the gp58-specific antibodies are highly conserved among several CMV strains and clinical isolates (7, 9) and that neutralizing antibodies against gp58 are frequently strain cross-reactive (46). Only 3 of 33 isolates carried any amino acid substitutions between residues 609 and 626 of gB in comparison to the laboratory strain CMV Ad-169 (9), and very few substitutions can be identified in the relevant part of gp58 (7). As suggested by the conserved nature of the epitope, all pMbg58-specific antibodies recognized the clinical CMV isolates tested. The gp116 protein carries a variable N-terminal sequence (8, 43), and isolate-specific antibodies, some of which may neutralize CMV in a complement-dependent manner, have recently been identified and mapped to amino acid residues 50 to 54 (32). The neutralization epitope recognized by ITC88 is, however, located in a more conserved part of gp116. This epitope is most likely an important target for cross-reacting antibodies with therapeutic potential (31, 32, 45).

For utilization of the major immunodominant domain of human CMV in future vaccines it is important to select immunogen candidates which induce neutralizing and not neutralization-blocking antibodies. Evaluation of fine specificities may prove to be a valuable tool when examining the responses achieved with various vaccine candidates, in particular if certain fine specificities can be linked to the presence or absence of biological function. We have shown that the immune response to CMV gp58 in humans involves several, overlapping fine specificities, some of which may be beneficial targets for protective antibodies, while other specificities have to be avoided since they may block biological functions mediated by neutralizing antibodies.

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