Use of Recombinant Human Antibody Fragments for Detection of Cytomegalovirus Antigenemia

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The determination and quantitation of peripheral blood leukocytes (PBLs) expressing human cytomegalovirus (HCMV) antigens is widely employed in clinical virology for rapid diagnosis of HCMV-related infections. We describe how CMV antigenemia may be accurately detected by means of human recombinant monoclonal Fab fragments rescued from a combinatorial phage display library prepared from an HCMV-infected donor. Fourteen recombinant Fabs were tested against HCMV-positive PBLs from a patient with ongoing HCMV infection. Three clones were found to react specifically with the nuclei of these cells. These three recombinant Fabs were subsequently tested, individually and pooled together, against 60 PBL samples taken from immunosuppressed patients. The reactivity observed was comparable to that obtained with mouse monoclonal antibodies commercially available for this purpose. The three recombinant Fabs were shown to react specifically with the 65-kDa viral tegument phosphoprotein encoded by UL83 (pUL83), which is the most abundant viral antigen in HCMV-infected PBLs.

The development of a simple diagnostic assay which allows the direct identification and quantitation of peripheral blood leukocytes (PBLs) expressing human cytomegalovirus (HCMV) pUL83 has been a major advance in the diagnosis of clinically relevant HCMV infection (3, 6, 8, 10–13). The test uses HCMV pUL83-specific antibody in either immunoperoxidase (13) or immunofluorescence (IF) (6, 10) assays. pUL83 is the major viral antigen present in HCMV-infected PBLs, even though in some cells a low level of an intermediate-early gene product of 72 kDa coded by UL123 has been reported. Two pools of anti-pUL83 mouse monoclonal antibodies have been shown to react strongly with HCMV-infected PBLs and are commercially available. Their use has clearly demonstrated the utility of the antigenemia test for immunocompromised hosts, including transplant recipients and AIDS patients (11).

The development of antibody libraries displayed on the surface of filamentous phage has recently provided a more efficient approach for the production of human monoclonal antibodies than that offered by earlier technologies, such as cell fusion and Epstein-Barr virus transformation (5). This technique provides a means of capturing, and specifically selecting from, the breadth of the antibody response produced by a given individual. Of particular note, antibody libraries constructed from the bone marrow of immune donors have led to the generation of panels of novel human antibodies specific for a diverse group of human viral pathogens, including HCMV (5, 14). In this study we examine the ability of HCMV-specific human recombinant Fab fragments derived from antibody-phage libraries to detect virus antigenemia in PBLs.

MATERIALS AND METHODS

Library construction, phage selection, and preparation of soluble Fab. A human antibody Fab library, displayed on the surface of M13 filamentous phage, was prepared from an HCMV-positive donor as described previously (1, 2, 14).

For this study an immunoglobulin G1(κ) [IgG1(κ)] library of 3×10^6 members was constructed by using bone marrow lymphocyte RNA. HCMV-specific antibodies in the patient's serum were detected by a standard diagnostic test (Behring, Marburg, Germany). The library was probed by using lysates of virus-infected cells to coat enzyme-linked immunosorbent assay (ELISA) plates in a panning procedure described previously (2). Following panning and enrichment for CMV-binding antibody-phage, soluble Fab fragments were prepared as bacterial supernatants by a freeze-thawing procedure (2). To assess the specificity of the recombinant Fab, supernatants were tested in an ELISA by using the antigen against which they were panned and in IF assays with virus-infected PBLs. In order to identify the antigenic targets, selected Fab clones were grown in quantity and purified from bacterial supernatants over an anti-human IgG Fab affinity column (14).

Indirect IF assay. In preparation for IF studies, cytopins and fixation of PBLs were carried out as described by Gerna et al. (6). Briefly, dextran-enriched PBL preparations were obtained by centrifugation of 2×10^5 cells onto glass slides at $900 \times g$ for 3 min at room temperature with a cytocentrifuge (Cytospin 2; Shandon Southern Products, Runcorn, United Kingdom). Slides were air dried and fixed with 5% paraformaldehyde–2% sucrose in phosphate-buffered saline (PBS) for 10 min at room temperature. Following three washes, the cells were permeabilized with 0.5% Nonidet P-40–10% sucrose–1% fetal calf serum for 5 min at room temperature and then washed three times. After being air dried, the slides were incubated with a commercially available pool (C10/C11) of mouse monoclonal antibodies (Clonab; Biotest, Dreieich, Germany) or with recombinant Fab supernatants. Specific binding was revealed by employing a fluorescein isothiocyanate-conjugated polyclonal antiserum specific for the antibody (or antibody fragment) used in the first step. After three final washes, slides were air dried, mounted, and examined with a Zeiss epifluorescence microscope.

Determination of antibody specificity. Purified murine monoclonal antibody 1131, specific for pUL83 of HCMV (ViroStat, Portland, Maine), was adsorbed onto ELISA plates overnight at 4°C in PBS (200 ng/well). As a control, additional ELISA wells were coated with purified mouse monoclonal antibody 1103, recognizing glycoprotein D of herpes simplex virus types 1 and 2 (HSV-1 and -2) (Goodwin Institute, Plantation, Fla.). The ELISA wells were then blocked for 1 h at 37°C with 3% bovine serum albumin (BSA) in PBS. Total cell extracts from CMV-infected human foreskin fibroblasts in 1× radioimmunoprecipitation assay (RIPA) buffer (1% Nonidet P-40–1% sodium deoxycholate in PBS) were subsequently applied to half of the anti-pUL83-coated wells for 30 min at room temperature. HSV-1 extracts were applied to half of the control wells coated with the anti-HSV antibody. The remaining wells received only 1× RIPA buffer. The plates were then washed with PBS containing 0.05% Tween 20, and the recombinant human anti-HCMV Fabs (0.5 µg/well) were then applied in 1% BSA–5% normal goat serum in PBS and incubated for 1 h at room temperature. AC-8, a human recombinant Fab to HSV glycoprotein D (4), was employed as a control. After extensive washing, a goat anti-human IgG F(ab')₂ conjugated to alkaline phosphatase (Pierce) was applied at a dilution of 1:1,000 in PBS containing 1% BSA–5% normal goat serum. After 1 h of further incubation, the plates were

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FIG. 1. IF reactivities of anti-pUL83 mouse monoclonal antibodies (C10/C11) (A and D) and human recombinant Fab GL15 (B and C) with HCMV-positive PBLs (obtained from a kidney transplant recipient with an ongoing HCMV infection) (A and B) and with human fibroblasts (MRC5) infected with the Towne strain of HCMV at a low MOI and fixed 72 (C) or 96 (D) h p.i.

TABLE 1. Comparison between C10/C11 mouse monoclonal antibodies and human recombinant Fabs in the recognition of HCMV-positive PBLs in 57 blood samples from immunosuppressed patients

No. of positive PBLs/100,000 cells	No. of samples found by:				
	C10 + C11	GL8	GL15	GL18	GL8 + GL15 + GL18
>2,000	1	0	0	1	1
1,000–2,000	3	4	3	3	4
301–1,000	3	3	4	3	2
50–300	4	4	4	4	4
10–49	7	7	7	9	9
1–9	9	8	8	7	7
0	30	31	31	30	30

incubated with *p*-nitrophenyl phosphate (1 mg/ml) in a 0.1 M NaHCO₃ buffer (pH 8.6) to reveal alkaline phosphatase activity and were read at 405 nm.

Nucleic acid sequencing. Nucleic acid sequencing was carried out on a 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif.) with a *Taq* fluorescent dideoxynucleotide terminator sequencing kit (ABI). Primers for the elucidation of antibody light- and heavy-chain sequences are given in the report of Williamson et al. (14).

RESULTS AND DISCUSSION

Fourteen recombinant Fabs were shown to react positively with HCMV-infected cells by both ELISA and IF. These clones were tested in parallel on PBLs from three patients shown to have more than 10^3 HCMV-positive cells among a population of 10^5 PBLs. Three recombinant Fabs (GL8, -15, and -18) produced positive nuclear staining in all three PBL preparations, with the intensity of the reaction following the rank order GL18>GL8>GL15. These three antibody clones were sequenced and found to possess three distinct heavy-chain amino acid sequences (data not shown). An image of a positive IF reaction 4 days postinfection (p.i.) is shown in Fig. 1A. For comparison, staining with C10/C11 is illustrated in Fig. 1B.

GL8, GL18, and GL15 were then examined, separately and in combination, for IF reactivity with 57 PBL preparations from 53 immunocompromised patients (33 AIDS patients and 10 kidney, 6 bone marrow, and 4 liver transplant recipients) in parallel with the commercially available murine monoclonal antibody cocktail C10/C11. The results obtained are shown in Table 1. GL18 gave results essentially identical to those obtained with C10/C11 mouse monoclonal antibodies, while GL8 gave results similar to those obtained with C10/C11 when the number of pUL83-positive PBLs was high (more than 50 positive cells among 2×10^5 cells) but failed to recognize one positive sample. The same sample was not recognized by GL15 but had two positive cells detected by C10/C11. A pool of the three recombinant Fabs reacted with all samples identified as CMV positive by the C10/C11 cocktail. C10/C11-negative PBL preparations were similarly negative when tested with recombinant Fabs, either individually or in combination.

GL8, -15, and -18 were further assessed by IF with HCMV-infected fibroblasts fixed at different times p.i. The Towne strain of CMV was used in these experiments, and the cells were infected at a low multiplicity of infection (MOI) (0.2 PFU/cell). Infected cells started to react positively with each of the Fab clones at 48 h p.i. The positivity was mainly cytoplasmic and localized in small granules (very likely representing viral dense bodies). Cytoplasmic granular positivity was more intense at later times (72 h p.i.), as shown in Fig. 1C for GL15. At this time p.i., cells that were strongly positive in the cyto-

FIG. 2. pUL83 capture ELISA. Purified mouse monoclonal antibody to pUL83 (ViroStat) was adsorbed to the wells of an ELISA plate (columns 1 and 2). Total cell extracts from CMV-infected cells were then applied to the wells represented in column 1, while the wells in column 2 served as controls and received no cell extract. Additional ELISA wells were coated with a mouse antibody to HSV glycoprotein D and were then either incubated with total cell extracts from HSV-1-infected cells (column 3) or left in PBS (column 4). The three human recombinant Fabs GL8, -15, and -18 all displayed strong immunoreactivity with immunoadsorbed pUL83 (column 1). In contrast, the Fabs did not display significant binding to either the anti-pUL83 or anti-HSV glycoprotein D mouse monoclonal antibodies alone (columns 2 and 4, respectively) or to the immunoadsorbed HSV-1 glycoprotein D (column 3). Human recombinant Fab AC-8, specific for HSV glycoprotein D, was used as a control and displayed specific reactivity exclusively for immunoadsorbed HSV-1 glycoprotein D (column 3, bottom right panel).

plasm were surrounded by a number of cells showing nuclear positivity (Fig. 1C). Equivalent results were obtained when C10/C11 was used (Fig. 1D). The data suggest that viruses produced by the cytoplasm-positive cell enter the surrounding cells at a high MOI. This possibility was confirmed by infecting cells at a high MOI. A pool of three Fabs, as well as C10/C11, reacted positively with the nuclei of HCMV-infected fibroblasts at 24 h p.i.

GL8, -15, and -18 were affinity purified and assessed for their binding in a capture ELISA assay. Figure 2 illustrates the reactivities of GL8, -15, and -18 with pUL83 and the HSV envelope glycoprotein D, immunologically captured from cells infected with HCMV and HSV-1, respectively. All three HCMV-specific Fabs reacted specifically with pUL83 and did not react with either the capturing antibodies alone or HSV-1 glycoprotein D.

The detection of HCMV antigenemia has been an important breakthrough in recent years in the diagnosis of clinically relevant HCMV infection in the immunocompromised host (11). However, due largely to the high cost of the immunological reagents used, the test is not universally employed in clinics. This study has examined the efficacy of using recombinant human antibody Fabs, which may potentially be available at low cost either as a commercially available kit or by production in the clinical laboratory, in the HCMV antigenemia assay. The data indicated that 3 of 14 HCMV-specific recombinant Fabs examined (GL8, -15, and -18) reacted specifically with the nuclei of PBLs derived from patients with ongoing HCMV infection. Furthermore, in ELISA studies, all three recombinant Fabs were shown to react with the 65-kDa tegument

protein of HCMV, which is known to be the antigen present in greatest abundance in PBLs during active HCMV infection.

The results suggest that a cocktail composed of these recombinant Fabs in the form of bacterial supernatants may be as sensitive as commercially available mouse antibodies in the early diagnosis of clinically relevant HCMV infection. In addition, the use of affinity-purified Fab fragments in PBL staining would avoid problems associated with a background signal resulting from the interaction of the Fc portion of mouse IgG with Fc receptor molecules (9). Moreover, the recombinant antibodies may be genetically linked to marker molecules, such as the green fluorescent protein of the bioluminescent jellyfish *Aequorea victoria* (7), facilitating PBL labeling in a single step.

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