neonates and immunocompromised patients. Varicella can be prevented by Oka varicella vaccine and varicella-zoster immune globulin (VZIG) [Takahashi et al., 1974; Zaia et al., 1983; Asano et al., 1994]. While the relative roles of humoral and cellular immunity in preventing VZV-derived illness have not yet been clearly determined, the clinical effectiveness of VZIG and maternal antibody in preventing or modifying varicella indicates the human monoclonal antibody capable of neutralizing VZV may function therapeutically [Grose et al., 2000].

The VZV genome contains around 70 open reading frames [Davison and Scott, 1986]. Among them, four major glycoprotein complexes, gB, gC, gE:gI, and gH:gL are located on the surface of the virus particle [Grose, 1990]. Analyses of isolated monoclonal antibodies against these complexes indicated that the gH:gL complex forms a major neutralization epitope [Grose et al., 1983; Forghani et al., 1984; Keller et al., 1984; Montalvo and Grose, 1986; Sugano et al., 1991]. Although monoclonal antibodies against the gE:gI complex can neutralize VZV, it requires involvement of the complement system [Forghani et al., 1984; Keller et al., 1984]. Because the gH:gL complex plays an important role in the entry of the virus particle into cells [Rodriguez et al., 1993; Duss et al., 1995; Shiraki et al., 1997; Pasieka et al., 2004], the monoclonal antibody against this complex inhibits viral entry and cell-to-cell spread of the virus. In this study, therefore, the gH:gL complex as an antigen (Ag) was used.

Two different methods have been developed to isolate the human monoclonal antibody [Winter et al., 1994; Mendez et al., 1997]. One is utilization of transgenic animals that produce human monoclonal antibody [Mendez et al., 1997]. The second technology is construction of antibody libraries using a phage-display system [Winter et al., 1994]. In the present study, an antibody library called AIMS4 constructed from B lymphocyte-rich tissues of several dozen people was used for screening [Higo-Moriguchi et al., 2004]. The repertoire of antibody in this library should reflect the repertoires in the tissues of the persons who donated B lymphocytes for preparation of mRNAs. Anti-VZV-gH antibody was isolated and analyzed the role of the light (L) chain of these antibodies by replacing the L chain of clone 94 by L chains from the other neutralizing clones in this useful AIMS4 system. Because infection with VZV is very common worldwide, the repertoire of antibodies in AIMS4 would cover a variety of VZV-specific antibodies acquired through natural exposure to the virus. This study describes the successful isolation of anti-gH monoclonal antibodies with strong neutralizing activities toward VZV.

MATERIALS AND METHODS

Viruses and Cells

MRC-5 and human embryonic lung (HEL) cells were grown in minimum essential medium supplemented with 10% and 2% fetal bovine serum, respectively. The Oka vaccine strain of VZV [Takahashi et al., 1994] was propagated in MRC-5 cells. Four fresh clinical isolates of zoster and HEL cells were used for the neutralization test. Thirty wild strains of VZV isolated from varicella or zoster were analyzed in the immunoperoxidase staining experiment. Cell-free virus was prepared as described previously [Shiraki et al., 1982, 1991; Shiraki and Takahashi, 1982]. Chinese hamster ovary (CHO) cells were used for the production of human monoclonal antibody.

Purification of Viral Glycoproteins

The glycoproteins of VZV were purified by affinity chromatography with monoclonal antibodies as described previously [Okuno et al., 1983; Shiraki et al., 1997; Yokoyama et al., 2001]. Briefly, Oka varicella vaccine-infected cells were lysed in the lysing buffer (20 mM Tris–HCl, 1% Triton X-100) and centrifuged at 27,000 rpm for 1 hr. The supernatant was applied to affinity columns for gH and gE, and purified glycoproteins were eluted.

Antibody Library

The human antibody library AIMS4 was used as the source of antibodies. It was constructed by using lymphocyte-rich tissues from several dozen volunteers and included tonsils, umbilical cord blood, bone marrow, and peripheral blood. The Fab form of an antibody fused to a truncated cp3 (Fab-cp3) was expressed on the phage surface. The size of the library is estimated to be around 10^{11} independent clones [Iba et al., 1997; Higo-Moriguchi et al., 2004].

Screening of the Library

Phages exhibiting gH-binding activity were selected by a panning method described previously [Marks et al., 1991; Iba et al., 1997]. The phages recovered by the third round of panning were infected into *E. coli* without helper phages and spread on the plate. Colonies were picked up and grown in a liquid culture. The size of the plasmid DNA was examined to monitor the occurrence of deletions. Expression of the Fab-cp3 molecule was analyzed by Western blotting of the supernatant probed with anti-cp3 antibody. Finally, the clones that had given positive results in these two analyzes were sequenced. The sequencing was performed as described previously [Higo-Moriguchi et al., 2004]. The Ag-binding activity was examined by enzyme-linked immunosorbent assay (ELISA). The conditions of ELISA were essentially the same as described previously [Iba et al., 1997; Higo-Moriguchi et al., 2004].

Preparation of Various Forms of Antibodies

Three forms of antibodies were prepared in the experiments: Fab-cp3, Fab-PP (P indicates an Fc-binding domain of protein A), and IgG_1. The Fab-cp3

molecules initially accumulated in the periplasm of *E. coli* and then gradually were secreted and/or released into the culture medium (crude Fab-cp3). They were purified with anti-cp3 monoclonal antibody-conjugated Sepharose beads. The conversion from Fab-cp3 to Fab-PP was described previously [Ito and Kurosawa, 1993]. Fab-PP molecules can be purified with an IgG-conjugated column. Conversion from a Fab to a human IgG_1 was performed as described previously [Higo-Moriguchi et al., 2004].

Effect of Replacement of the L Chain of Clone 94 Fab-pps by the Other L Chains on Neutralizing Activity

Contribution of the L chain on the neutralizing activity was examined in the highly active neutralizing clone 94 by replacing its L chain by the other L chains. DNA fragment of clones 10, 24, 36, 60, 120, 192, and 431 were digested with NcoI and AscI and inserted into clone 94 expression vector. By this replacement, chimera clones with authentic 94 H chain and L chain of other clones were constructed. The chimeric clones were expressed as Fab-pp form and examined for their neutralization activity.

Virus Neutralizing Test

VZV-neutralizing activity was examined by a plaque reduction assay [Shiraki et al., 1982, 1991; Yokoyama et al., 2001]. Briefly, 300 plaque-forming units (pfu) of cell-free virus in 0.3 ml of virus solution were mixed with 0.3 ml of various concentration of antibodies, and the mixtures were incubated at 37°C for 1 hr. Then, 200 µl of the mixture was inoculated into HEL cells in 60 mm Petri dishes and incubated for 5 days. The neutralizing activity was expressed as the concentration necessary to reduce the number of plaques by 50% (ED_{50}).

Immunostaining of VZV-Infected Cells with Isolated Antibodies

VZV-infected HEL cells were treated with trypsin and suspended in PBS. Aliquots of the cell suspension were spotted on 8-well slides, dried, and fixed in a mixture of 50% methanol and 50% acetone at −20°C for 5 min. Fixed cells were blocked with 5% normal rabbit serum in PBS for 1 hr at room temperature. Antibodies (clones 94 or 24) at the concentration of 5 µg/ml in PBS were added and incubated at room temperature for 1 hr. The slide was stained with FITC-conjugated goat anti-human IgG diluted to 1:200 with PBS. After incubation at room temperature for 1 hr, the slide was further stained with 4,6-diamidino-2-phenylindole dilactate (DAPI) at the concentration of 5 ng/ml at room temperature for 5 min. The slide was observed using fluorescence microscopy.

As the second series of experiments, HEL cells were infected with 30 wild VZV strains and fixed by the same procedure as above. The clone 94 IgG and peroxidase-conjugated anti-human IgG were used as the first and second antibodies, respectively [Shiraki et al., 2003]. Detection was performed according to the manufacturer's instructions using a DAKO liquid DAB substrate chromagen detection system (Dako, Glostrup, Denmark).

Immunoprecipitation

Viral proteins recognized by isolated antibodies were identified by immunoprecipitation [Shiraki and Hyman, 1987; Shiraki et al., 1982, 1991]. Briefly, Oka varicella vaccine-infected and uninfected cells were separately labeled with [^{35}S] methionine and cysteine (37 Tbq/mmol, Amersham Biosciences, Piscataway, NJ) for 6 hr at 37°C, and the labeled cells were lysed and subjected to immunoprecipitation with human IgG form antibodies [Shiraki et al., 1982, 1991; Shiraki and Hyman, 1987]. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The molecular weight markers used were the SDS-PAGE Molecular Weight Standards (BIO-RAD, Hercules, CA).

Effects of Antibody on Plaque Size

HEL cells in 60 mm dishes were infected with 10–20 pfu of cell-free Oka varicella vaccine for 1 hr and then the cells were washed with medium to remove unabsorbed virus. The infected cultures were incubated for 4 days in the presence of various concentrations of clone 94 IgG or human γ-globulin (GAMMAGARD, Baxter, Deerfield, IL). The cells were fixed with a solution of 50% acetone and 50% methanol at −20°C, and the plaques were stained by 3,3′-diaminobenzidine (DAB) in chromogen solution (Dako, Carpinteria, CA), after reaction with anti-VZV immune human serum and anti-human IgG, conjugated with peroxidase (Dako). The infected cells were stained brown and all cells were counter-stained with hematoxylin. Then the plaque size was measured using the NIH Image program (version 1.62, National Institutes of Health, Bethesda, MD). The plaque size for each treatment was determined as the mean of more than 10 plaques.

RESULTS

Isolation of Monoclonal Antibodies that Bind to gH:gL Complex

The gH:gL complex was used to screen the AIMS4 library by the panning method. After three rounds of panning, 100 colonies were picked up and analyzed as described in Materials and Methods. Fifteen different clones were obtained and their Ag-binding activities were examined by ELISA. The results are indicated in Figure 1a. Clones were classified into three groups, those with (1) strong binding activity to gH (clone 11), (2) weak binding activity to gH but higher than to gE (clones 10, 24, 60, 64, 77, 94, and 102), and (3) similar binding activity to gH and gE (clones 9, 13, 36, 49, 86, 89, and 100).

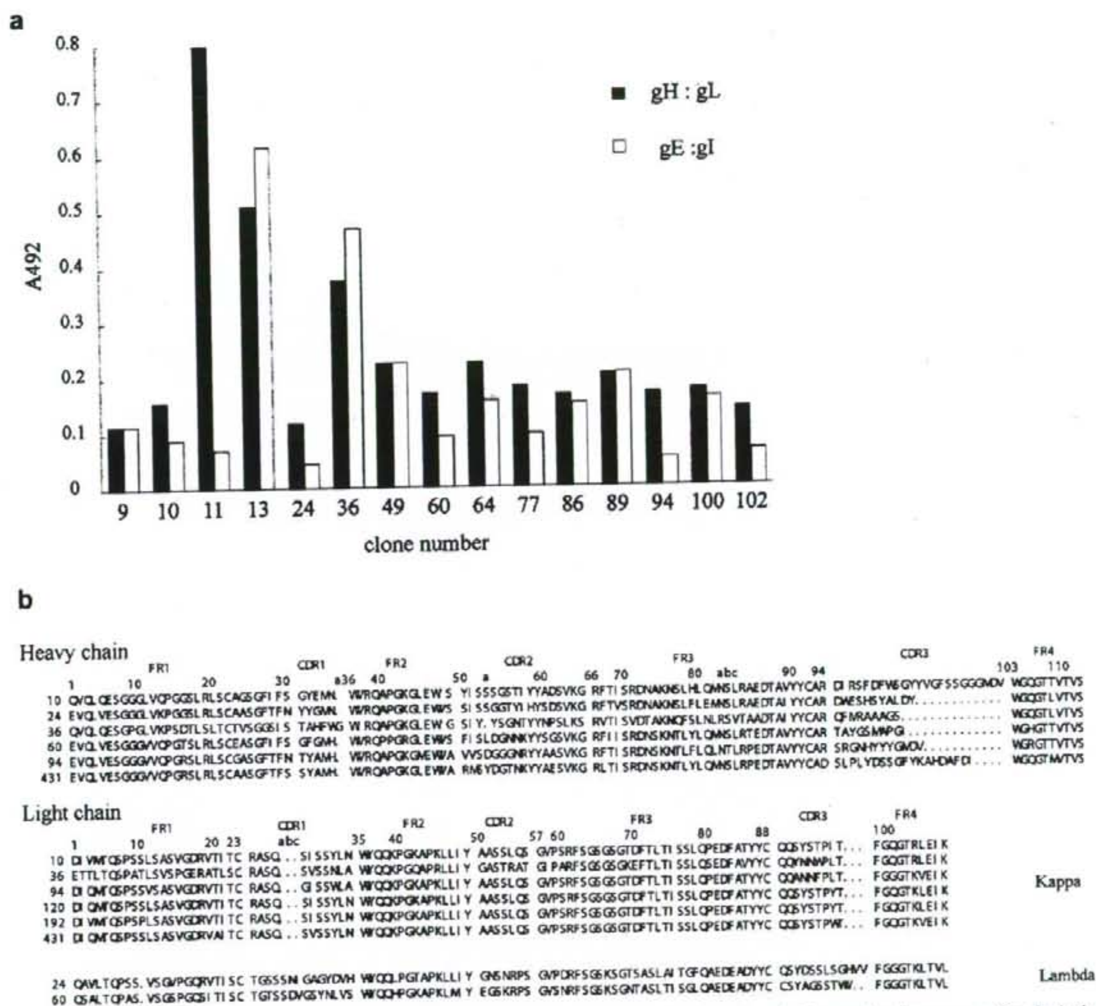


Fig. 1. **a**: ELISA of isolated clones. The gH-binding activities of the 15 clones isolated were analyzed by ELISA. The antibodies used in this experiment were crude Fab-cp3. The gH:gL and gE:gI complexes were used as antigens bound to the plate. Because the sugar concentration of gE is much higher than that of gH:gL, it is possible to speculate that the clones bound to both gL and gE recognize the sugar portion. **b**: Amino acid sequences of VZV-neutralizing antibody clones. The sequences of the H chains of clones 120 and 192 were the same as that of clone 10. Numbering of amino acid residues was according to Kabat's definition [Kabat et al., 1991].

Isolation of Clones with Neutralizing Activity Against VZV

Supernatants of *E. coli* cultures (crude Fab-cp3) were prepared, and the neutralizing activity was tested by a plaque reduction assay. Among 15 clones tested, three clones, 10, 24, and 94, showed high neutralizing activity, and clones 36, 60, and 102 exhibited relatively low neutralizing activity. Clone 11 did not show a distinct neutralizing activity. In order to obtain more clones showing neutralizing activities, around 200 clones were further picked up without sequencing, and their neutralizing activities were examined directly. Six clones, 34, 120, 186, 192, 263, and 431, showed neutralizing activities. Sequence analysis of these clones indicated that clones 10 and 34 were the same, clones 24, 186 and 263 were identical, and the heavy (H) chains of clones 10, 120, and 192 were identical to each other but that their light chains were slightly different. Only one clone, 431, turned out to be different from the already isolated clones. Thus, eight different clones were isolated as VZV-neutralizing antibodies; their amino acid sequences are shown in Figure 1b.

Quantitative Analysis of VZV-Neutralizing Activities

In order to quantify the neutralizing activity of each clone, Fab-PP forms of antibodies were prepared. Both Fab-cp3 and Fab-PP can be easily prepared as secreted molecules using *E. coli* as the host. Empirically, Fab-PP antibodies are more stable than Fab-cp3 antibodies. Conversion from Fab-cp3 to Fab-PP can be achieved by digestion with a restriction enzyme, followed by self-ligation [Ito and Kurosawa, 1993]. Figure 2 shows the relationship between antibody concentration and neutralizing activity. The ED_{50} concentrations among these eight clones showed a wide range, from 0.12 to 400 nM. Among them, clone 94 gave the strongest neutralizing activity.

Isolated Clones Neutralize Wild VZV Without Strain Specificity

The Fab-PP forms of five antibodies, clones 10, 24, 94, 120, and 431, showed neutralization activities against four wild VZV strains and the Oka vaccine strain at a similar level (data not shown).

IgG Form of Antibodies Showed the Neutralizing Activities

Among the eight clones showing neutralizing activities, five representative clones, 10, 24, 36, 60 and 94, were selected to prepare authentic human IgG_1 antibodies. As shown in Figure 3, all five clones in the IgG_1 form exhibited neutralizing activities. While IgG antibodies are divalent unlike the monovalent Fab antibodies, the neutralizing activity of clone 94 was weaker than that of Fab-PP; that is, the ED_{50} of the Fab-PP was 0.12 nM and that of the IgG was 0.65 nM. However, the IgG molecules of the other clones showed slightly stronger neutralizing activities, and, moreover, both forms of the five clones, $94 > 24 > 60 > 10 > 36$, showed the same order of strength. Therefore, this indicated that the three-dimensional (3D) structure of the respective Ag-binding sites was retained after conversion from Fab to IgG. Because clones 94 and 24 had stronger neutralizing activities than the other clones, the following analyses were performed using the IgG form of these two clones.

Neutralizing Antibodies Preferentially Bind to Molecules Present on the Cell Surface

While clones 94 and 24 exhibited much stronger neutralizing activities than the other clones, they gave only weak signals in ELISA, as shown in Figure 1a. In order to clarify this apparent discrepancy, their ability to bind to molecules naturally expressed on VZV-infected cells was examined. As indicated in Figure 4a, both clones 94 and 24 clearly gave the staining patterns that have been shown to reflect gH:gL complex expression [Forghani et al., 1994; Duss et al., 1995].

In the second series of experiments, HEL cells infected with 30 wild VZV strains were immunostained using clone 94. Although a different detection system from the first experiment was used, essentially the same staining pattern was observed in these 30 samples (data not shown).

Both Clones 94 and 24 Bind to gH:gL Complex

In order to identify the molecules recognized by the neutralizing antibodies, labeled viral proteins were immunoprecipitated with clones 94 and 24. As indicated in Figure 4b, both gH and gL molecules were clearly seen in the precipitates of both clones.

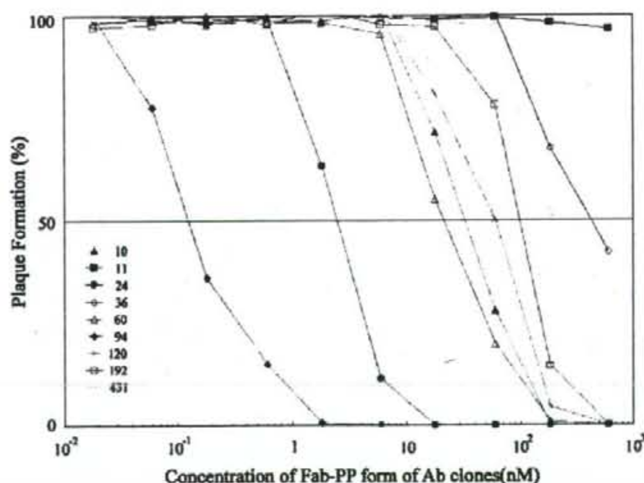


Fig. 2. Quantification of VZV-neutralizing activities by purified Fab-PP forms of clones 10, 11, 24, 36, 60, 94, 120, 192, and 431. Neutralizing activity (ED_{50}) concentrations ranged from 0.12 nM (clone 94) to 60 nM (clone 36) and higher (clone 11).

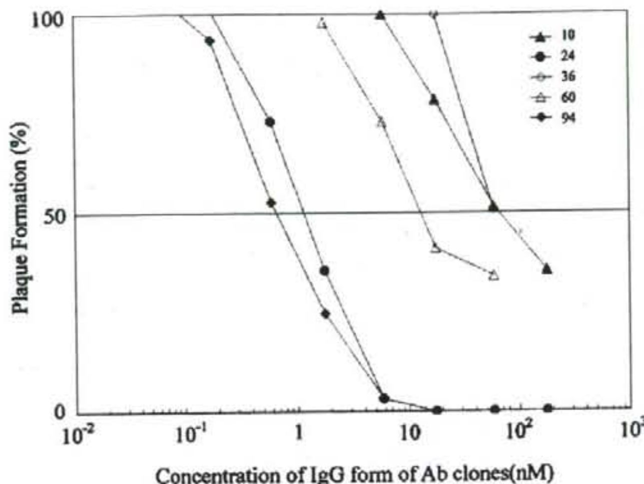


Fig. 3. Quantification of VZV-neutralizing activities by IgG forms of clones 10, 24, 36, 60, and 94. Neutralizing activity (ED_{50}) concentrations ranged from 0.6 nM (clone 94) to 60 nM (clone 36).

Clone 94 IgG Molecule Alone Prevents Virus from Spreading

A plaque is produced by cell-to-cell infection. In the case of VZV, little infectious cell-free virus is released from infected cultured cells [Shiraki and Takahashi, 1982]. Rather, infection is transferred by fusion of contiguous cells to form syncytia [Rodriguez et al., 1993; Duss et al., 1995; Pasieka et al., 2004]. If antibodies prevent the process of infection at any step, even after occurrence of the initial infection, the size of the plaque will be decreased. Figure 5 shows the effects of clone 94 IgG at various concentrations on the size of plaques formed by virus-infected cells. The presence of the IgG molecules alone, without any effector molecules such as complements or effector cells such as NK cells, reduced the size of plaques in a dose-dependent manner. In the presence of 180 nM IgG (clone 94), isolated single cells were stained with immunoperoxidase without infection spreading to neighboring cells (Fig. 5b) indicating complete inhibition of virus spread without inhibition of cell-free virus infection and killing the infected cells by anti-gH monoclonal antibody.

Effect of the Sequence of L Chains on Neutralizing Activity

Figure 6 illustrates the effect of the L chains of clones 10, 120, and 192 on the neutralizing activity. The H chains of clones 10, 120, and 192 were identical and their L chains were kappa-L chains. There were two amino acid variations in framework region (FR)1, one in complementarity determining region (CDR)3, and one in FR4 among L chains of these three clones. The neutralizing activity of these three clones was in the order of clones 10, 120, and 192. When the variation and neutralizing activity of three clones were compared, the variation of isoleucine in clone 10 to tryrosine in clones 120 and 192 at the position 96 of CDR3 increased the neutralizing activity more than the other variations in FR1 and 4, indicating that a variation in CDR3 was more effective in neutralizing the activity than those in FR1 and 4. Considering the importance of the CDR3 region of L chains in creating the structure of the binding site, the neutralizing activity of these three clones was primarily determined by the structure created by the CDR3 region. In comparison to CDR3, the contribution of the FR1 and FR4 regions in generating the structure for binding to gH was secondary.

Effect of Replacing the L Chain of Clone 94 by Those of the Other Neutralizing Clones

Because clone 94 showed strong neutralizing activity, the L chain of clone 94 was substituted with those of clones 10, 24, 36, and 60. The neutralizing activity of the recombined antibodies and their L chain sequences are shown in Figure 7. The neutralizing activity of the authentic clones was in the order of 94, 24, 60, 10, and 36, and the contribution of the L chain to neutralizing activity of the recombined antibodies was in the order of 94, 10, 24, 36, and 60. The L chains of clones 10, 36, 94 were kappa chains and those of clones 24 and 60 were lambda chains. The lambda-L chain of clone 24 preserved the neutralizing activity of the kappa-L chain of the authentic clone 94 despite the difference in the class of L chain, while the neutralizing activity of the lambda-L chain of clone 60 did not match the authentic clone 94 and, in fact, was decreased by more than 10,000 times. Compared to the clone 24 lambda-L chain, the clone 60 lambda-L chain did not retain neutralizing activity. As

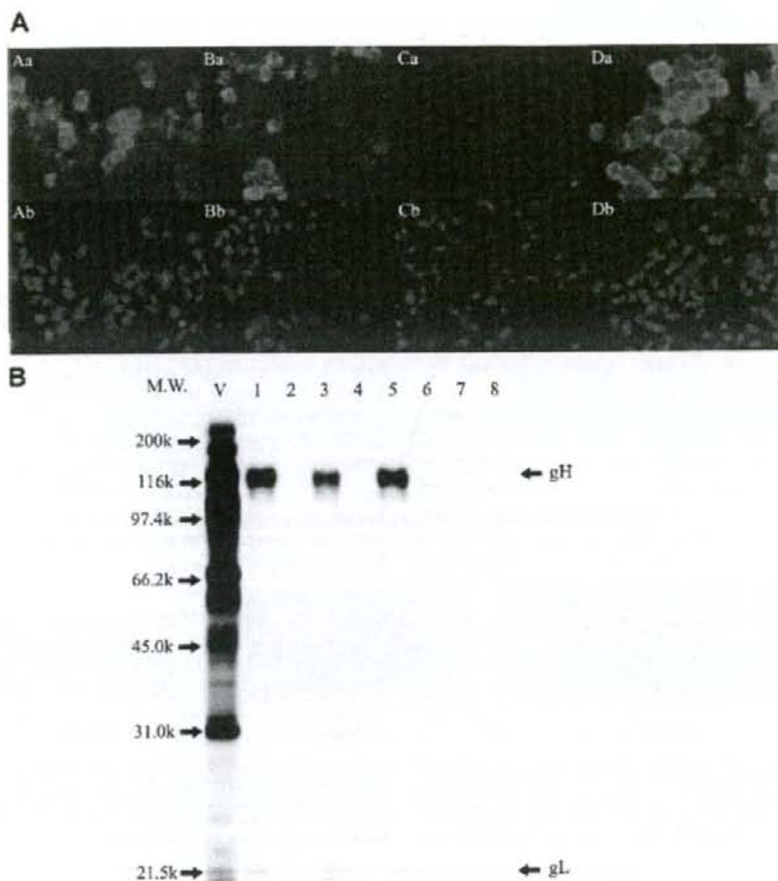


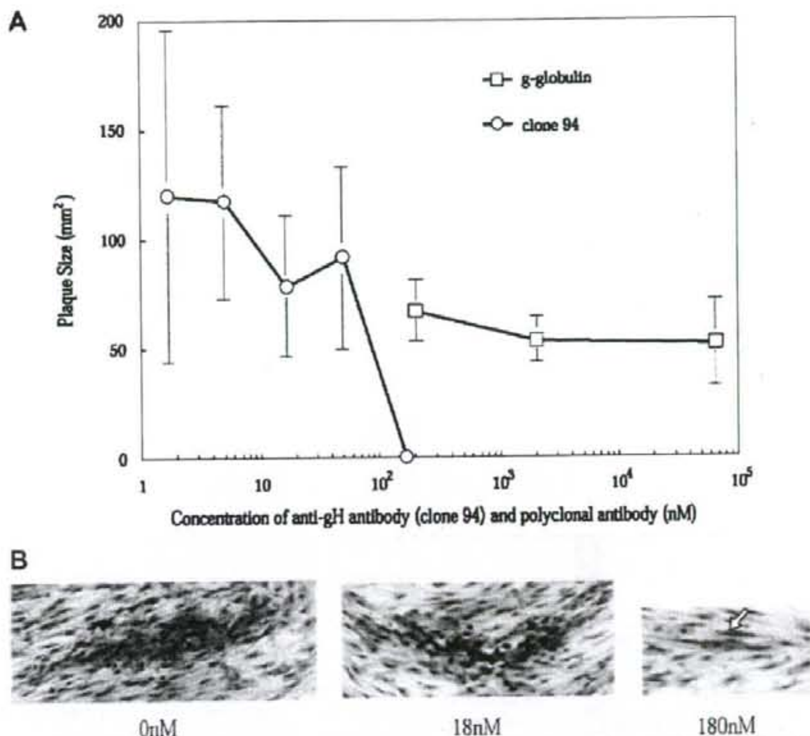
Fig. 4. **A**: Immunostaining of VZV-infected cells with various antibodies. VZV-infected cells were stained with fluorescent antibody [series (a)] and then with DAPI [series (b)]. Clone 24 (A), clone 94 (B), an anti-influenza HA antibody as a negative control (C), and a commercially available human γ-globulin (Baxter, Deerfield, IL) as a positive control (D). **B**: Identification of gH:gL complex in precipitates with neutralizing antibodies. Radiolabeled proteins were immunoprecipitated with human monoclonal antibody TI-57 [Sugano et al., 1991] (1 and 2), the IgG forms of clone 24 (3 and 4) and clone 94 (5 and 6), or anti-influenza HA monoclonal antibody (7 and 8). Lanes 1, 3, 5, and 7, or 2, 4, 6, and 8 indicate the results of VZV-infected HEL cells and those of mock-infected HEL cells, respectively. The molecular weights (MW) are indicated in kiloDaltons (kDa).

shown in Table I, the number of the amino acid substitutions of clones 24 and 60 was the same in CDR1, CDR2, and CDR3, and the total number of substitutions was larger in clone 60 than in clone 24. The number of amino acid substitutions in clone 24 was larger than those in clones 10 and 36, and the neutralizing activity of the clone 24 L chain was intermediate between those of clones 10 and 36. Thus, the neutralizing activity may be dependent on the configuration of the antigen-binding site created by L and H chains, and it is difficult to predict the neutralizing activity by the L chain class of kappa or lambda, a simple comparison of the number, or the nature of amino acid substitutions from the authentic L chain of clone 94.

DISCUSSION

In the present study, eight kinds of antibodies showed VZV-neutralizing activities in the library. While clones 94 and 24 showed the strongest activities, they gave only weak signals on ELISA. This apparent discrepancy could be due to the characteristics of the gH:gL complex. The AIMS library contains many VZV-specific antibodies acquired through natural exposure to the viruses. This made it possible to isolate the potent neutralizing antibody clones, such as clones 94 and 24, that had functioned naturally to recover from the illness in vivo.

The isolated antibodies showed three kinds of neutralizing activities against VZV and VZV-infected cells.



0nM 18nM 180nM

Fig. 5. Effects of IgG form of clone 94 and human γ-globulin on plaque size. **A**: VZV-infected cells were treated with various concentrations of the IgG form of clone 94 (○) and human γ-globulin (□) after cell-free virus infection and the size of plaques stained with immunoperoxidase was determined on the fourth day by the NIH Image program. The plaque size with each treatment was determined as the mean of more than 10 plaques; the bar indicates the SD. **B**: The plaques and a single infected cell formed in the presence of 0, 18, and 180 nM of anti-gH monoclonal antibody were stained with immunoperoxidase. White arrow indicate a single infected cell.

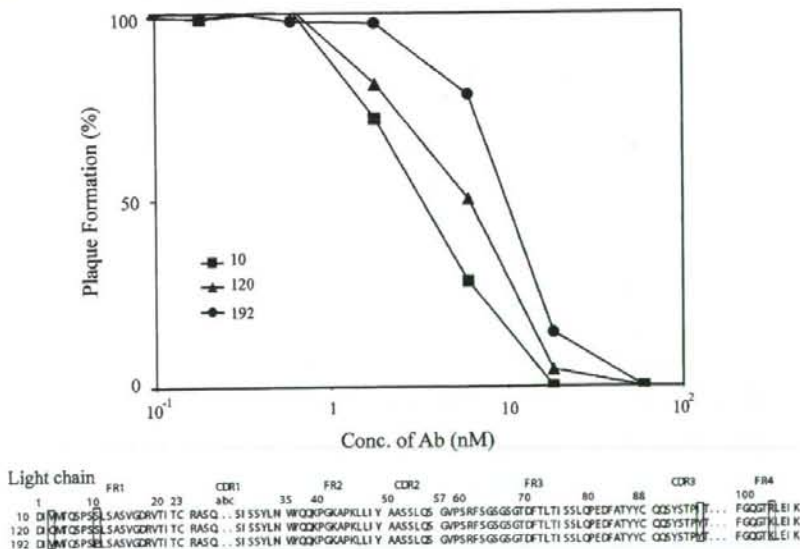


Fig. 6. Neutralizing activity and amino acid variation of purified Fab-PP forms created by the different kappa-L chain of clones 10, 120, and 192 with the common H chain. Dose-response neutralizing activity of clones 10, 120, and 192 and their L chain sequences are shown in the upper and lower figures, respectively.

TABLE I. The Number of Amino Acid Substitutions of L Chains of Clones 10, 24, 26, and 60 With That of Clone 94

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	Total
10	2	2	0	0	0	5	1	10
24	11	10	2	5	9	8	3	43
36	13	3	2	4	6	2	2	32
60	12	10	2	5	10	9	4	51

They prevented plaque-forming activities of the cell-free virus. While Drew et al. [2001] reported that monomeric antibodies do not neutralize VZV, the results shown in Figures 2 and 3 clearly indicated that divalency is not required for neutralizing activity. Monomeric (Fab) and dimeric (IgG) forms of antibodies possessed similar neutralizing activities. The ED_{50} in this neutralizing assay was estimated to be relatively low, 0.65 nM for clone 94 and 1 nM for clone 24 (Fig. 3). As a therapeutic agent, however, simple neutralization of cell-free viruses may not be adequate. After infection, VZV spreads not through production of cell-free virus but mainly by cell-to-cell transmission through syncytia formation [Duss et al., 1995]. Clones 94 and 24 have been shown to possess antibody dependent cellular cytotoxicity (ADCC) activity against VZV-infected cells and an antibody concentration higher than 20–30 nM is required for effective cell killing by ADCC activity (data not shown). Moreover, clone 94 prevented the virus from spreading. This suggests that virus particles or gH expressed on the infected cell surface are directly involved in cell-to-cell transmission. The concentration of antibodies necessary to block this process is around 100 nM (Fig. 5).

A number of groups have reported isolation of human monoclonal antibodies against VZV-gE [Foung et al., 1985; Sugano et al., 1987; Lloyd-Evans and Gilmour, 2000; Kausmally et al., 2004; Shankar et al., 2005] and VZV-gH [Sugano et al., 1987; Drew et al., 2001]. Human and mouse hybrid cells were established by cell fusion. Foung et al., [1985] fused B cells of a patient recovering from acute varicella infection with a human-mouse cell line. Among the wells containing hybridomas, one-third of them had VZV-specific antibodies in the supernatant. Two monoclonal antibodies were isolated whose VZV-neutralizing activities (ED_{50}) ranged from 1 to 5 µg/ml, which correspond to 6–30 nM. The characteristics of the antibody against gH reported by Sugano et al. [1991] appeared to be very similar to those of the antibodies (clone 94 and 24). The ED_{50} in a neutralization test

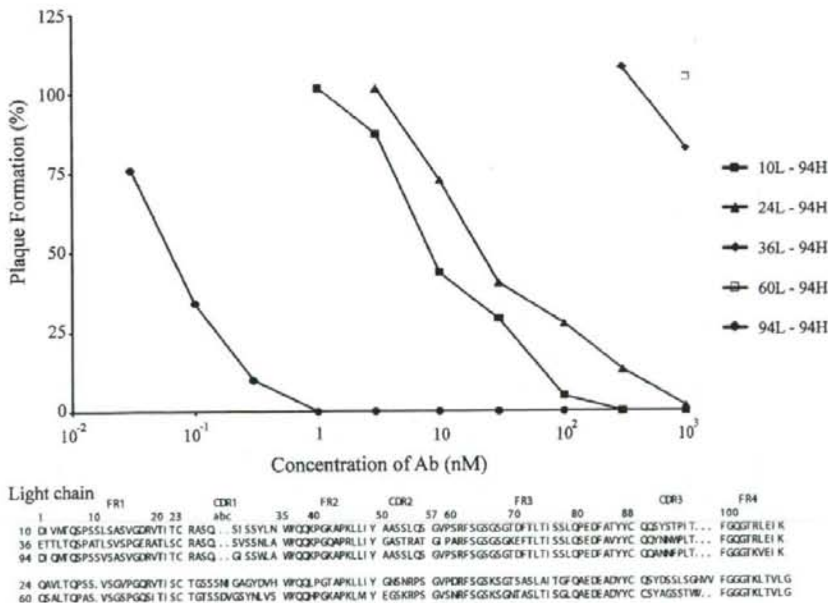


Fig. 7. Quantification of VZV-neutralizing activities shown by purified Fab-PP forms created by replacing the L chain of clone 94 by the L chain of clones 10, 24, 36, and 60. Dose–response neutralizing activity of replaced Fab-PP forms of the L chain of clone 94 by L chains from clones 10, 24, 36, and 60 and the sequences of the L chains are shown in the upper and lower figures, respectively.

against the Oka strain was reported to be 0.15 μg/ml, which corresponds to 1 nM. This strength is almost the same as that of clones 94 and 24 (Fig. 3). Both antibodies showed ADCC against VZV-infected cells, and moreover inhibited virus spread. It is suggested that the clones 94 and 24, like the clone of Sugano et al. [1991], represent antibodies that had matured in vivo. Kausmally et al. [2004] isolated human monoclonal antibodies with neutralizing activities. In this case, an extracellular part of the gE molecule was used as the Ag. The ED_{50} of their clones were 0.23–11 μM for the monomeric form and 1.3–30 nM for the IgG form. Complements were required for neutralization, as predicted from the results of experiments using murine monoclonal antibodies.

The role of L chains in the neutralizing activity of antibodies was characterized by comparing clones with the different L chain and the same H chain obtained from the AIMS4 library and artificial replacement of the L chain of clone 94 by L chains from the neutralizing clones 10, 24, 36, and 60. Although the number of clones or combinations examined was limited, amino acid substitution in not only the CDR3 but also in various parts of the L chain affected neutralizing activity. Artificial replacing of the L chain of clone 94 by the limited number of L chains from the other clones did not improve neutralizing activity, and this suggested that these L chains were selected for their authentic H chains and were not so suitable as the authentic L chain of clone 94. Interestingly, when the kappa-L chain of clone 94 was replaced by the lambda-L chain of clone 24, the replaced clone retained neutralizing activity. This important finding encourages the random replacement of the L chain for the neutralizing H chain by the L chain clones to select more active antibody clones.

This study describes successful isolation of human monoclonal antibodies with strong neutralizing activities against VZV. Judging from the wide versatility of this method, human monoclonal antibodies against various infectious diseases could be developed as therapeutic drugs. While this method has already been applied to HIV type1 [Barbas et al., 1992], type 2 [Björling et al., 1999], Puumala hantavirus [de Carvalho Nicacio et al., 2000], Measles virus [de Carvalho Nicacio et al., 2002], Ebora virus [Maruyama et al., 1999], RSV [Tsui et al., 1996] and rotavirus [Higo-Moriguchi et al., 2004], none of them has been made available as therapeutic drugs for patients. When the characteristics of clones 94 and 24 were compared with a humanized monoclonal antibody, MEDI-493, that has been developed as a prophylactic drug against RSV [Johnson et al., 1997], both showed the similar concentration of antibody required for virus neutralization and that for inhibition of virus spread. Furthermore the human monoclonal antibody clones are candidates as prophylactic or therapeutic drugs against VZV infection in those who are susceptible to severe VZV infection, especially, in patients with the bone marrow transplant recipients with cord blood.

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Clinicopathologic understanding and control of varicella-zoster virus infection☆

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ABSTRACT

As reflections by the recipient for the Japanese society for vaccinology Takahashi award, clinicopathologic understanding and control of varicella-zoster virus (VZV) infection was briefly reviewed. In 1974, a live varicella vaccine was developed by attenuating the Oka strain of VZV after the passages in non-human cells at a low temperature. The vaccine was immunogenic, well tolerated, and effective, and distributed worldwide so far. For post-exposure prophylaxis of varicella, the vaccine and acyclovir is effectively used in the incubation period of varicella. The delayed type hypersensitivity to VZV skin test antigen was applied for evaluation of cell-mediated immunity to VZV which is commercially available in Japan. The biphasic viremia during incubation period of varicella and airborne spread of VZV from varicella patients and from herpes zoster patients with localized lesions were shown by the virus isolation or by detection of the virus DNA.

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1. Introduction

Varicella-zoster virus (VZV) causes two distinct diseases with varicella occurring after primary viral infection and zoster resulting from a secondary infection due to reactivation of latent VZV in the sensory ganglia. Varicella is generally believed to be a benign disease with occasional complications in immunocompetent individuals, but infection can be severe or fatal in immunocompromised hosts. More than 30 years ago there were no effective tools, such as a vaccine or acyclovir, to control primary VZV infection. In the early 1970s, I started clinicopathologic and virological research on VZV infection in order to understand the pathogenesis of primary VZV infection, to diagnose and treat the disease, as well as to prevent the disease. Over the past several decades, our understanding of this virus has greatly improved with new techniques for virological study and global implementation of a VZV vaccine. Reflections and results from these 30 years of research will be briefly introduced in this article.

2. Development of a live attenuated varicella vaccine

The vaccine virus was isolated from a vesicle of a 3-year-old otherwise healthy boy called Oka (his family name) who had typical varicella. This virus was passaged through human embryonic lung cells, guinea pig embryonic cells at a low temperature, and human diploid cells (WI-38). It was then adapted to MRC-5 human diploid cells for vaccine preparation. The vaccine contains cell-free virus with a minimum of 1000 plaque forming units per dose and suitable stabilizers [1]. In 1974 the vaccine was administered to hospitalized children immediately after the occurrence of an index varicella case because preventive methods, such as administration of VZV immune globulin, were unavailable at the time. The vaccine prevented the spread of varicella throughout the children's ward of the hospital. Subsequently, the vaccine was shown to be immunogenic, well tolerated, and efficacious even in high-risk children [2]. The vaccine has been studied extensively with largely favorable results as shown in Table 1 [3–16].

The vaccine was initially licensed in Japan in 1987 for high-risk children but was extended just after licensure to include normal children based on the needs of parents and physicians. Because varicella vaccination is not compulsory in Japan, only approximately 36% of Japanese children received the vaccine in 2006. This low level of coverage was not sufficient to alter the circulation of wild-type VZV, and the epidemiology of natural varicella has not changed since the vaccine was introduced. The most dramatic changes were reported in the USA after the introduction of a universal immunization strategy in 1996 [17–21], causing vaccine coverage to increase to 89% in 2006. As a result, there have been substantial declines among both children and adults in the incidence of varicella, hospitalizations and ambulatory visits for varicella, mortality due to varicella, varicella-related complications, and overall expenditures for varicella-related illnesses. The vaccine is now commercially available worldwide and was administered to approximately 14 million individuals in more than 100 countries in 2005.

☆ Reflections by the recipient for the Japanese society for vaccinology Takahashi award.

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Table 1
Properties of the live attenuated varicella vaccine (Oka strain)

1.	Causes little or no clinical reactions[a]
2.	Induces antibody (measurable by CF, NT, FAMA, IAHA tests)
3.	Induces CMI (delayed-type skin reaction, lymphocyte transformation)
4.	Lack of contact infection in most cases
5.	Induces long-term protective immunity
6.	Prevents disease when administered up to 3 days after exposure
7.	Does not enhance the incidence of herpes zoster

[a] Mild clinical reactions in 15–75% of leukemic children.

3. Development of VZV skin test antigen

Cell-mediated immunity (CMI) to VZV is important in order to recover from VZV infections. In 1977 Kamiya et al. developed a skin test antigen from virus-infected cells that was used to evaluate CMI to VZV [22]. This is a very convenient method and VZV immune status can be determined by measuring the diameter of erythema 24–48 h after intradermal inoculation of the skin test antigen. Subsequently, an improved VZV skin test antigen was developed from the supernatant of virus-infected cells, which has far less protein content than the former test that was prepared by sonicating infected cells [23]. The varicella vaccine is now administered to the elderly to enhance VZV CMI in order to prevent herpes zoster and postherpetic neuralgia with favorable results. The skin test antigen has been successfully used to evaluate CMI to VZV [24,25].

4. Viremia during varicella

An understanding of the pathophysiology of VZV infection has been limited because there is no suitable experimental animal model to produce clinical varicella [26,27]. Asymptomatic viremia preceding the onset of a varicella skin rash is assumed to be a distinct phase of varicella [28]. Using sensitive culture techniques, the virus was isolated from peripheral blood mononuclear cells 1 day after disease onset [29]. This viremia could be detected between 5 days before varicella onset and 1 day after disease [30,31], with the most frequent detections occurring just before disease onset, which probably corresponds to secondary viremia in the logical schema by Grose [28]. The viremia disappeared 2 days after disease onset and was undetectable thereafter when specific immunity to VZV developed [31,32]. However, viral DNA was detected for a longer period after the introduction of polymerase chain reaction (PCR) amplification [33]. Although virus could not be isolated from the blood early in the incubation period, viral DNA was detected at the same stage by PCR amplification and was assumed to represent primary viremia [33]. After primary viremia, the virus replicates in the internal organs even in immunocompetent children with varicella [34]. The number of vesicular skin rashes on the body correlates with the maximum body temperature, duration of fever, and degree of viremia (the number of infected cells in the blood) [35]. The virus was not isolated from the blood of vaccine recipients at any time after immunization using the same technique [31]; however, viral DNA was occasionally detected in the blood of vaccine recipients from days 7 to 28 after immunization [36]. Our understanding of the pathophysiology of primary VZV infection is summarized in Fig. 1.

5. Virus spread from varicella and herpes zoster

Varicella is one of the most contagious infectious human diseases. Several observations suggest that infection occurs after exposure to aerosolized VZV from patients with varicella or herpes zoster, although the mechanism by which the virus is shed and the sites of viral shedding are unknown. It is generally accepted that typical varicella is infectious for 1–2 days before disease onset and for 4–5 days thereafter. However, it is difficult to isolate VZV from throat swabs just after the onset of disease [37], although viral DNA can be frequently detected [38]. Viral DNA has been detected on several environmental surfaces, including an air conditional filter, occasionally, intermittently or persistently just after disease onset and thereafter [38]. In the case of the viral DNA positive air conditional filter, this contaminated surface could not be directly touched by the family members, suggesting the airborne spread of VZV. Varicella patients, even when routinely treated with oral acyclovir for 5 days, excreted the virus from their respiratory tract or vesicles and disseminated the virus to the environment via an aerosol route [39].

After primary infection with VZV, the virus becomes latent in cells of the dorsal root ganglia and can be reactivated to produce herpes zoster. It is generally accepted that patients with localized herpes zoster are less contagious than those with varicella or

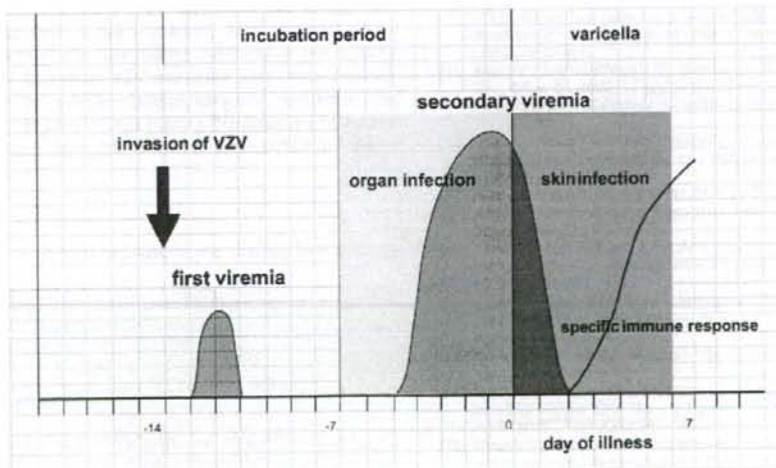

Fig. 1. Viral replication between invasion of VZV and clinical signs of varicella. *Note:* Biphasic viremia during the varicella incubation period and two stages of organ and skin infections.

Table 2
Detection of VZV DNA by PCR in patients with herpes zoster localized to the thoracic region whose skin lesions were covered with either hydrocolloid dressing agents or conventional bandage gauzes

Group and patient #	Day of illness			
	4	5	6	7
Hydrocolloid group				
1	−/+/−	−/−/−	−/+/−	ND/−/−
2	−/−/−	−/−/−	−/−/−	−/−/−
3	−/−/−	−/−/−	−/−/−	−/−/−
4	−/−/−	−/−/−	ND/ND/ND	ND/ND/ND
5	−/−/−	−/−/−	−/−/−	−/−/−
6	−/−/−	−/−/−	−/−/−	−/−/−
7	−/−/−	−/−/−	−/+/−	−/−/−
Gauze group				
8	+/−/+	+/+/-	+/−/+	+/−/+
9	+/+/+	+/−/+	+/+/+	+/+/+
10	+/−/+	+/−/−	+/−/+	+/−/−
11	ND/ND/ND	+/−/−	+/−/+	+/−/+
12	+/+/−	+/−/−	+/+/+	+/+/+
13	+/+/−	+/+/−	−/+/+	+/+/−

Note: PCR results are from samples of the surface of the lesion coverings/throat swabs/surface of air purifier filters. The day of appearance of vesicular skin lesions was defined as day 1. +, positive; −, negative; ND, not none (from Ref. [42]).

disseminated herpes zoster. It was, however, recently shown that the virus from herpes zoster patients spreads to the environment rapidly and widely, suggesting that even localized herpes zoster can contribute to airborne spread of the virus [40–42]. A recent report has suggested that skin lesions were a possible source of airborne spread of the virus, even in patients with localized herpes zoster, and that hydrocolloid dressing agents prevented dissemination of aerosolized VZV DNA from the skin lesions of patients with localized herpes zoster (Table 2) [42].

6. Post-exposure prophylaxis of varicella by oral acyclovir

In a family setting, when one family member develops varicella, the remaining members who are susceptible to VZV will likely develop disease 2 weeks later. Thus, the attack rate of varicella is high in family members susceptible to VZV, and these cases are generally more severe than the initial cases. If the live Oka varicella vaccine is inoculated in the remaining susceptible family members within 72 h after exposure, the disease can be prevented or modified [4,10]. However, until recently, there was no effective method for controlling varicella in susceptible individuals between 72 h after VZV exposure and disease onset. In order to prevent or modify clinical varicella, acyclovir was administered to susceptible siblings during the disease incubation period. The results were favorable and are summarized in Table 3 [43–48].

Table 3
Summary of post-exposure varicella prophylaxis with oral acyclovir in family settings

1. Can control clinical varicella when administered late in the incubation period
   Acyclovir, 40 or 80 mg/kg daily in 4 divided doses
   Starting 7–9 days after exposure to the index cases
   Administered for 7 days
2. Can control clinical varicella when administered for the last 7 days but not for the first 7 days of the incubation period
3. Modification of clinical varicella is related to the doses of acyclovir
   Geometric mean FAMA antibody titers are higher in the low dose group
   Incidence of subclinical infection is higher in the high dose group
4. VZV immunity persists for a long time after the observation period

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Characterization of Neutralizing Epitopes of Varicella-Zoster Virus Glycoprotein H$^{\nabla}$

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Varicella-zoster virus (VZV) glycoprotein H (gH) is the major neutralization target of VZV, and its neutralizing epitope is conformational. Ten neutralizing human monoclonal antibodies to gH were used to map the epitopes by immunohistochemical analysis and were categorized into seven epitope groups. The combinational neutralization efficacy of two epitope groups was not synergistic. Each epitope was partially or completely resistant to concanavalin A blocking of the glycomoiety of gH, and their antibodies inhibited the cell-to-cell spread of infection. The neutralization epitope comprised at least seven independent protein portions of gH that served as the target to inhibit cell-to-cell spread.

Varicella-zoster virus (VZV) glycoprotein H (gH) is the major target for neutralization (4, 5, 7, 9, 18), and it plays an important role in viral entry and cell-to-cell spread of infection (1, 3, 11, 12, 15). We isolated human monoclonal antibodies (MAb) to gH using an antibody library called AIMS4 constructed from B-lymphocyte-rich tissues of several dozen people (6, 19). Nine clones were selected for their neutralizing ability and their Fab sequences of heavy (H) and light (L) chains and used, in addition to TI-57, an anti-gH human MAb from a hybridoma, to characterize the neutralization epitopes of gH (18). Our system makes it possible to use the Fab form, which has about one-third the molecular weight of immunoglobulin G (IgG), in order to eliminate the spatial interaction between the Fc or other unreacted Fab of IgG molecules on one gH molecule. The neutralizing epitopes of gH are conformational, making gH hardly detectable by Western blot or enzyme-linked immunosorbent assay, and therefore, the conformational epitopes were mapped immunohistochemically. The combinational neutralizing activity between two species of Fab protein A (Fab-pp) forms and the inhibition of cell-to-cell infection were characterized, and the neutralization domain of gH was found to comprise a cluster of the seven neutralization epitopes and to prevent cell-to-cell infection.

Human embryonic lung cells were used to propagate Oka varicella vaccine, and cell-free virus was obtained by sonication of infected cells in SPGC medium (phosphate-buffered saline [PBS] containing 0.1% sodium glutamate, 5% sucrose, and 10% fetal bovine serum) followed by centrifugation (13, 14, 16).

Except for TI-57, each MAb was expressed in two forms: Fab-pp and Fab with an avidin tag (Fab-Avi-tag). Fab-pp corresponds to an Fab molecule fused with two domains of the Fc-binding protein A from *Staphylococcus aureus* (8) and purified on an IgG-conjugated column (19). Fab-Avi-tag is composed of an Fab bearing a 23-amino-acid-long peptide tag that can be biotinylated by the bacterial BirA biotin ligase (1). Fab-Avi-tag antibodies were purified by using SoftLink soft release avidin resin (Promega, Madison, WI).

To map the neutralizing epitope by Fab-pp, VZV-infected cells in 24-well plates were fixed by air-drying and then with 50% methanol and 50% acetone. The Fab-pp form (5 μg/ml in 0.5 ml of PBS with 3% skim milk) was used to block gH epitopes for 24 h at 4°C, and then 0.1 ml containing 1 to 10 μg Fab-Avi-tag was added and incubated at 4°C overnight. After incubation with streptavidin conjugated with peroxidase, competition for the gH epitope by the first Fab-pp and the challenging Fab-Avi-tag reaction was visualized by using a Dako liquid diaminobenzidine substrate chromogen detection system (17).

To assess the relationship between the glycomoiety and epitope, VZV-infected cells in eight-chamber culture slides were fixed by air drying and 50% methanol and 50% acetone. Then, the cells were treated with 0.5 ml/well of 200 μg/ml concanavalin A (ConA) (Wako Pure Chemical Industries Ltd., Osaka, Japan) in PBS for 1 h and with bovine serum for 1 h. After being washed with PBS, the cells were incubated with 1 μg/ml Fab-pp from each clone or 1:50-diluted zoster serum at 37°C for 1 h, washed with PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (H+L) rabbit serum (Wako) at 37°C for 1 h. The cells were observed under a fluorescence microscope.

The cells in six-well plates were infected with 50 PFU/0.05 ml of cell-free virus for 1 h and incubated for 1 h without antibody after washing the cells and then in the medium containing 500 μg/ml of the Fab-pp of clones 10, 11, 24, 36, 60, or 94 for 4 days without a change of medium (19). After fixation with 5% formalin, the cells were stained with methylene blue.

Blocking with PBS failed to inhibit the staining with each

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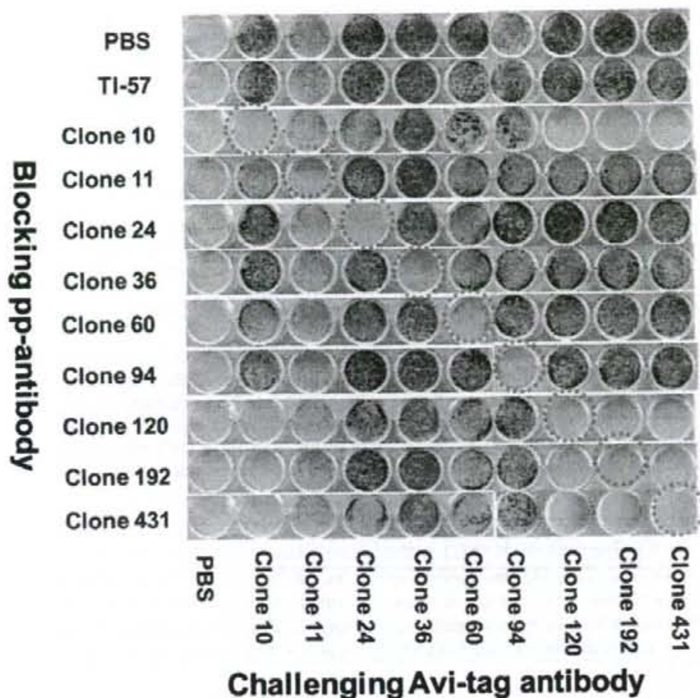

FIG. 1. Epitope mapping of gH by competitive immunostaining. Infected cells were first blocked by Fab-pp and then challenged by Fab-Avi-tag for visualization of the reaction of gH and Fab-Avi-tag. When PBS was used as the blocking agent, the challenging Fab-Avi-tag recognized the gH epitope, resulting in positive staining of infected cells, as shown in the top lane. When an Fab-pp successfully blocked the reaction with Fab-Avi-tag, the staining was blocked, as marked by dotted red circles around cultures with the homologous antibody combinations. When the Fab-pp and Fab-Avi-tag recognized different epitopes, infected cells were stained. Clones 10, 120, 192, and 431 showed identical reaction profiles, indicating that they belonged to the same epitope group. TI-57 blocking allowed staining by all kinds of Fab-Avi-tag, indicating that TI-57 recognized different epitopes than the other Fab-pp.

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Avi-tag antibody, and all the infected cells were positively stained (Fig. 1). Blocking with a homologous Fab-pp blocked the immunostaining with Avi-tag antibody, as shown by the red circles. The Fab-pp of clones 11, 24, 36, 60, and 94 failed to block binding by the clone 10 Avi-tag antibody, and the Fab-pp of clones 120, 192, and 431 blocked binding by the clone 10 Avi-tag antibody, indicating that the epitope of clone 10 was similar to those of clones 120, 192, and 431 but different from those of clones 11, 24, 36, 60, and 94. The Fab-pp of clones 24, 36, 60, and 94 blocked only homologous combinations with each Avi-tag antibody and failed to block binding with the other Avi-tag antibodies. Altogether, epitope mapping of clones indicated the presence of six epitope groups, 10, 120, 192, and 431; 11; 24; 36; 60; and 94, in the neutralization domain of gH.

TI-57 antibody did not block any reaction of Avi-tag antibodies with clones 10, 120, 192, 431, 11, 24, 36, 60, or 94, even when used at 20 μg/ml to block the epitope of gH. This suggested that the epitope recognized by TI-57 was different from those recognized by the nine clones. TI-57 is not produced any more, and the amount of TI-57 was not sufficient to perform further work with TI-57. The target epitopes of gH for neutralization were defined by the 7 groups of gH antibodies, 10, 120, 192, and 431; 11; 24; 36; 60; 94; and TI-57.

ConA presents as a tetramer with a molecular mass of approximately 108,000 Da, while the molecular masses of Fab-pp and gH are 50,000 and 120,000 Da, respectively. Tetrameric ConA efficiently inactivates viral infectivity (8) and may interfere with the interaction between Fab-pp and the epitope because of its spatial bulkiness when it reacts with the glycomoiety near the target epitope (20). Figure 2 shows the specificity of immunofluorescence and a comparison of the immunofluorescent staining by each antibody clone with and without ConA treatment. The intensity of staining by zoster serum was reduced by ConA treatment, possibly due to blocking of the interaction of the antibody with viral glycoproteins. Staining of infected cells by clone 36 with and without ConA and the contrast between infected and uninfected cells were not affected by ConA treatment, while those parameters in the other clones were reduced slightly or greatly by ConA treatment. Six epitopes were located near the glycomoiety of gH and not in the glycomoiety itself, and the epitope recognized by clone 36 was remote enough to evade spatial blocking by the tetrameric ConA bound to the glycomoiety. This indicated that the epitopes rec-

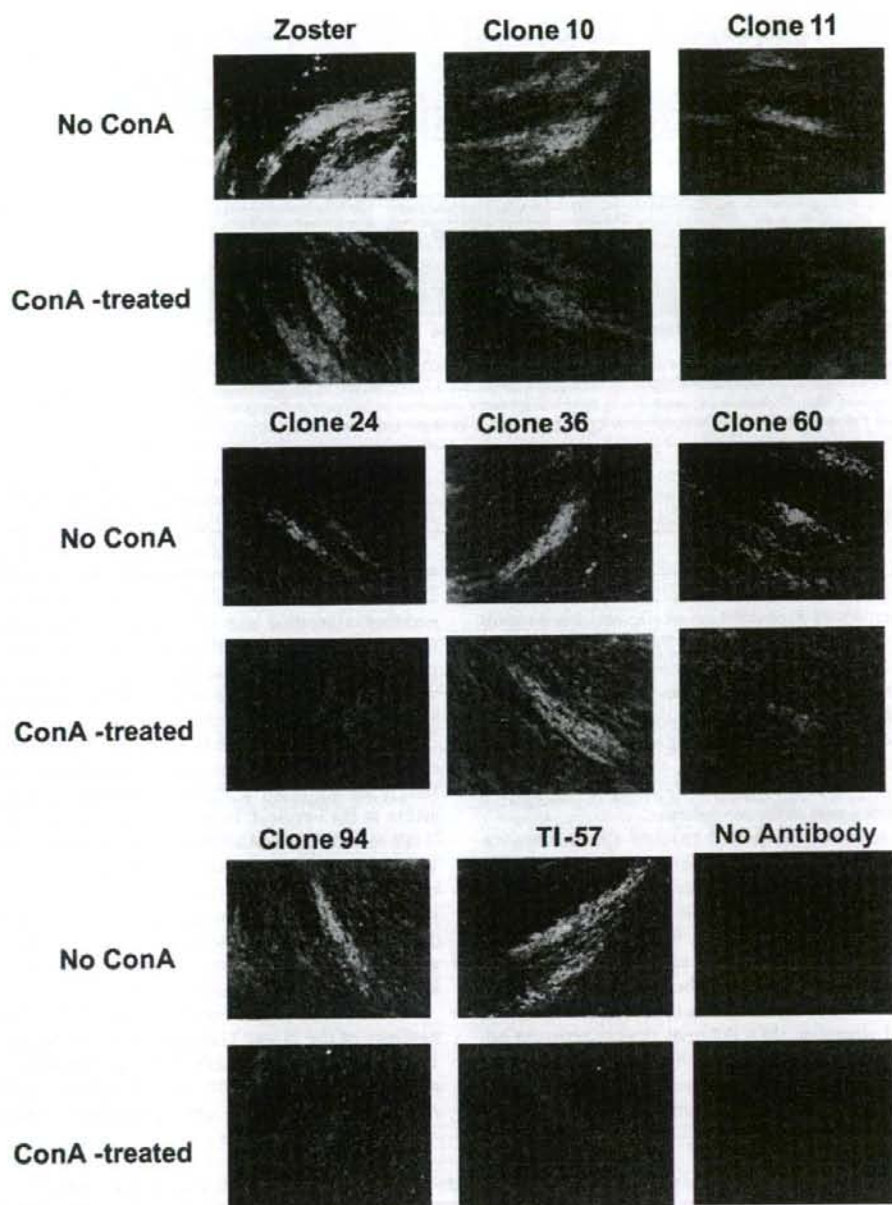

FIG. 2. Interference with epitope recognition by binding of ConA to gH glycomoieties. The cells on eight-chamber glass slides were treated with 0.5 ml/well of 200 μg/ml ConA or PBS and then with Fab-pp, followed by staining with FITC-labeled anti-human IgG (H+L) rabbit serum. "No Antibody" indicates that infected cells were directly stained with FITC-labeled anti-human IgG (H+L) rabbit serum to determine the specificity of anti-gH MAb. The FITC staining of infected cells without and with ConA treatment is shown. The specificity of FITC staining of infected cells among surrounding uninfected cells and the FITC staining contrast with and without ConA treatment illustrate the effects of ConA treatment on the interaction of anti-gH MAb with infected cells.

ognized by anti-gH neutralizing MAbs were protein portions or at least not glycomoieties of gH that interact with ConA.

Figure 3 shows the successful inhibition of plaque formation by the six clones representing six epitope groups. The infected culture without antibody showed extensive cytopathology, but the typical cytopathology did not develop in the infected cultures treated with each Fab-pp.

Table 1 shows the genetic characterization of the H chains of each clone. The variable H chain (VH) gene sequences from framework region 1 to framework region 3 of the seven anti-

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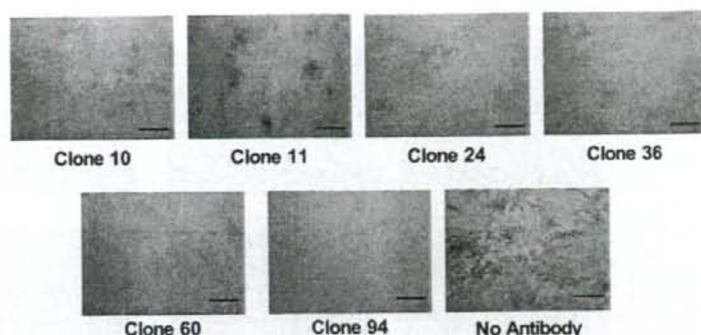

FIG. 3. Inhibition of cell-to-cell spread of infection and plaque formation by Fab-pp treatment. The cells were infected and incubated for 1 h without antibody and then treated with 500 μg/ml of Fab-pp of clone 10, 11, 24, 36, 60, or 94 for 4 days. The cells were fixed with formalin and stained with methylene blue. Plaques with extensive cytopathology were observed in infected cultures without antibody treatment, while treatment with 500 μg/ml of Fab-pp inhibited the spread of cytopathology. Bars indicate 1 mm.

body clones were compared with the germ line sequences listed in VBASE (VBASE Directory of Human V Gene Sequences; http://vbase.mrc-cpe.cam.ac.uk/). The original germ lines for clones 10 and 431, which recognize one epitope, were DP51 and DP50, respectively, and their complementarity-determining region 3 sequences were different in size and amino acids (19). Tyrosine-tyrosine in clone 10 and phenylalanine-tyrosine in clone 431 may form a hydrophobic core and recognize the same antigenic structure. Antibody clone 11, with no mutation in its VH gene, showed low neutralizing activity (50% inhibitory concentration, 8,000 nM), indicating a naïve antibody. The other clones had 18 to 29 nucleotide mutations in the VH genes, which may have contributed to efficient neutralizing activity by reacting with different epitopes.

Because these clones recognized multiple epitopes, we examined the combinational neutralizing activity of different Fab-pp forms by using a plaque reduction assay (14, 15, 19, 23). Eight kinds of Fab-pp clones were mixed in all possible combinations of two different clones, 36 in total, and the mean neutralizing efficacy was determined seven times. No combination of two clones reduced the number of plaques more than expected (data not shown), indicating that no combination of two clones had synergism. In a different virus system, the addition of MAbs to different functional domains, the V2, V3, or CD4 binding site of human immunodeficiency virus glycoprotein gp120, produced synergistic neutralization (21, 22). In contrast, the multiple neutralizing epitopes of gH might have recognized one functional domain, resulting in no synergism.

Murine MAb 206 to gH neutralizes VZV and inhibits cell-to-cell fusion in gH+gL-transfected cells (2, 3). Prior reports indicate that gH can endocytose on its own, without gE (10), and that interaction with gE may lead to *trans*-Golgi network targeting. gE can increase endocytosis of gH lacking a YNKI endocytosis motif (11). The inhibitory mechanism of gH in the virus-to-cell or cell-to-cell interaction by neutralizing anti-gH MAbs is not clear. Some combinations of our MAbs that recognized six epitopes might be antagonistic, and further analysis of the relationship between neutralization and cell-to-cell infection among these MAbs might elucidate the gE-gH interaction in the virus-cell interaction and cell-to-cell infection.

All the anti-gH MAbs that had neutralizing activity against VZV blocked entry and egress of the viruses (19), suggesting that both infection by viruses and syncytium formation after infection would be mediated by the same single functional domain on the gH molecule (3–5, 7, 9, 11, 12, 15, 18). In conclusion, the neutralizing domain comprises at least seven independent protein portions of gH.

Nucleotide sequence accession numbers. The accession numbers of the H and L chains for clones 10, 24, 36, 60, 94, 120, 192, and 431 are AB063700 and AB064076, AB063703 and AB064219, AB063705 and AB064116, AB063707 and AB063990, AB063708 and AB064045, AB063700 and

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TABLE 1. Genetic characterization of the variable H chains compared with germ line sequences in VBASE[a]

Clone	Germ line	No. of mutated nt/total no. of nt[b]	No. of mutated nt in:					Neutralization titer[c] (IC_{50} nM)
			FR1	CDR1	FR2	CDR2	FR3	
010	DP-51	24/291	9	6	2	1	6	40
011	DP-10	0/296	0	0	0	0	0	8,000
024	DP-35	29/296	3	5	4	9	8	3.0
036	DP-67	27/294	9	2	2	6	8	400
060	DP-49	28/294	3	3	4	11	7	25
094	DP-46	21/294	2	2	1	8	8	0.12
431	DP-50	18/296	1	2	1	10	4	200

[a] nt, nucleotides; FR; framework region, CDR; complementarity-determining region.
[b] The regions covered by the primers used for PCR were excluded.
[c] Neutralization titers are from Suzuki et al. (19). IC_{50}, 50% inhibitory concentration.

AB063929, AB063700 and AB063932, and AB355876 and AB355875, respectively.

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