

## Varicella-Zoster Virus IE63, a Virion Component Expressed during Latency and Acute Infection, Elicits Humoral and Cellular Immunity

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Varicella-zoster virus (VZV) latency in human dorsal root ganglia is characterized by the transcription of large regions of its genome and by the expression of large amounts of some polypeptides, which are also expressed during lytic cycles. The immediate early 63 protein (IE63) is a virion component expressed very early in cutaneous lesions and the first viral protein detected during latency. Immune response against IE63 has been evaluated among naturally immune adults with a history of chickenpox: Specific antibodies were detected in serum, and most subjects who had a T cell proliferation with unfractionated VZV antigens had T cell recognition of purified IE63. The cytotoxic T cell (CTL) response to IE63 was equivalent to CTL recognition of IE62, the major tegument component of VZV, whose immunogenicity has been previously described. T cell recognition of IE63 and other VZV proteins is one of the likely mechanisms involved in controlling VZV reactivation from latency.

As a member of the Alphaherpesviridae, varicella-zoster virus (VZV) shares many characteristics (e.g., neurotropism or the ability to become latent after a primary infection and to reactivate many years later) with the other members of this virus subfamily (i.e., herpes simplex type 1 or 2 [HSV-1 or -2]). However, the mechanisms involved in latency of VZV and HSV-1 and -2 seem to be completely different: During HSV latency, the viral genome is found in neurons only, and transcription is limited to one region of the genome that produces latency-associated transcripts (i.e., antisense of the ICP-0 transcripts) [1]. These transcripts do not seem to be translated, and no viral protein has been detected during HSV latency.

On the contrary, the nature of cells in which VZV remains quiescent is still debated because in patient samples, the genome has been detected in neurons only [2, 3], in satellite cells surrounding neurons only [4], and in both cell types [5]. The most striking difference between HSV and VZV latency is that several immediate early (IE) and early genes of VZV, such as genes encoding for IE4, IE62, IE63, and MDBP (major DNA binding protein), are transcribed in latently infected rat dorsal root ganglia [6] and in human ganglia [7, 8]. Moreover, VZV latency is characterized by the expression of large amounts of IE63, as demonstrated in the rat experimental model [9] and

then confirmed in human dorsal root ganglia [10]. This protein is one of the virion components [11]; it is expressed very early in culture [9] and in skin lesions, in which it is detectable much earlier than are late proteins, and sometimes it is expressed in the absence of a lesion [12]. During acute infections, IE63 shows mostly a nuclear localization, while it seems to accumulate in cytoplasm of latently infected neurons [10]. However, it is still unknown whether the protein has the same characteristics during productive and latent infections or whether differences (e.g., phosphorylation) can be observed in either case.

More recently, other proteins corresponding to the transcripts previously detected during the dormant phase have been detected in human material [13].

These observations indicate that the mechanisms involved in VZV latency are completely different from those acting in other alphaherpesvirus-infected cells, and the role of viral proteins alone or in interaction with cellular proteins has to be hypothesized.

One of the parameters that could be critical to the control of VZV infection is host immunity, as suggested by Hope-Simpson in 1965 [14]: VZV primary infection is limited by the host immune response, which remains high for many years, with a slow decrease in time. According to his hypothesis, frequent virus reactivations or contact with infected persons could counterbalance this progressive decrease; however, host resistance may still reach a critical level that is too low to control virus reactivation. Many clinical observations show that the frequency and the severity of virus reactivations increase in patients whose cellular immunity is impaired due to age, pathologic disorders, or immunosuppressive treatments prior to transplantation. These observations confirm Hope-Simpson's hypothesis on the role of host immune response, even if it now appears obvious that it is mostly the cell-mediated immunity that limits virus reactivation; this is suggested by the fact that virus reactivates despite high anti-VZV antibody titers and

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since the herpes zoster episode is not correlated with hypogammaglobulinemia.

Previous studies have shown that protein IE62 (the major tegument protein) and gC, gE, gG, and gI (the major glycoproteins) are highly immunogenic and elicit a long-term cellular immune response after natural VZV infection or immunization with a live attenuated vaccine strain (see [15] for review). The immunogenicity of IE63 has not been evaluated, and its expression during latency and acute infection suggests that this protein probably plays a crucial role and could be an important target for the immune system.

As previously described [16], we have evaluated the immune response against IE63 among 15 healthy adults with a history of chickenpox as verified by detection of anti-VZV IgG antibodies. As controls, we used 2 healthy adults who had no history of varicella and no measurable anti-VZV antibodies. All experiments were done with the IE63 protein, which was expressed in bacteria and fused to glutathione-S-transferase (GST), as a stimulating antigen [9].

## Materials and Methods

**Study populations.** Blood was obtained from 15 healthy adults with a history of varicella as confirmed by detection of IgG antibodies to VZV by ELISA. Two healthy adults who had no history of varicella and no measurable VZV IgG antibodies served as controls.

**GST and GST-IE63 production and purification.** The GST and GST-IE63 proteins were produced from the plasmids pGex-3X and pGex-3X/ORF63, which have been described [9]. In brief, plasmid-transformed bacteria were induced by 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside for 3 h at 37°C, pelleted, and sonicated in PBS Triton X-100 (1% wt/vol). The GST and GST-IE63 proteins that were released into the supernatant were purified by affinity chromatography on glutathione-Sepharose-4B (Pharmacia-LKB, Uppsala, Sweden) and eluted with reduced glutathione (10 mM). The proteins were quantified by use of a Bradford assay (Bio-Rad, Hercules, CA) and loaded onto SDS-10% polyacrylamide gel to verify their purity.

**Whole VZV antigen preparation.** Melanoma cells cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum were infected with a laboratory strain (Chase) of VZV, dispersed with glass beads when 90% cytopathic effect was observed, washed with PBS, and sonicated for 60 s. After centrifugation at 4°C for 10 min, supernatants were frozen and thawed three times, and aliquots were stored at -70°C. An uninfected cell control was prepared in parallel.

**Western blot assay.** Western blot analysis was used to determine if anti-IE63 antibodies were in the sera. Purified GST and GST-IE63 were electrophoresed and transferred onto a nylon membrane. To avoid nonspecific labeling, the membrane was saturated with nonfat milk (5 g/L). The membrane was incubated for 1 h at room temperature with the donor's serum (1/250), washed, and incubated with a peroxidase-conjugated anti-human IgG (1/2000, Dakopatts, Glostrup, Denmark). Antibodies were detected using a Western blotting detection reagent (ECL; Amersham Life Science, Amersham, UK).

**Assays for cellular immunity to VZV.** Peripheral blood mononuclear cells (PBMC), which had been separated from 50 mL of heparinized blood by ficoll-hypaque gradient, were used for T lymphocyte proliferation, cytokine, or cytotoxicity assays.

Specific T lymphocyte proliferation was assayed as previously described [17]. In brief, PBMC were recovered from 50 mL of heparinized blood by ficoll-hypaque gradient. Cells were cultured in 96-well plates at  $3 \times 10^5$  cells/well and stimulated in triplicate with VZV or control antigens or with GST or GST-IE63 fusion proteins (3–25  $\mu$ g/mL) whose concentration had been optimized previously. Preliminary experiments done with a broad range of fusion protein concentration (3–100  $\mu$ g/mL) indicated that with  $>25$   $\mu$ g/mL, the response to GST alone was too high. Phytohemagglutinin (100  $\mu$ g/mL) and RPMI 1640 were used as positive and negative controls, respectively. After 5 days, T cell proliferation was measured by [ $^3$ H]thymidine uptake and expressed as a stimulation index (i.e., the ratio of mean counts per minute [cpm] in VZV or GST-IE63-stimulated wells to that of control or GST wells). A stimulation index  $\geq 2.0$  was conventionally defined as a positive response.

The IE63-specific cytotoxic response was detected by use of limiting-dilution assays and compared with the cytotoxic T lymphocyte response to IE62 as previously described [16]. Effector cells were either CD4<sup>+</sup> and CD8<sup>+</sup> cell populations or unfractionated T cells recovered from PBMC by separation with Lymphokwik-T (One Lambda, Los Angeles). CD4<sup>+</sup> and CD8<sup>+</sup> cells were purified by positive selection with magnetic cell sorting (MACS; Miltenyi Biotec, Auburn, CA) [16], and their purity was verified by fluorescence-activated cell sorter analysis: Positive selections yielded  $\geq 90\%$  CD4<sup>+</sup> or CD8<sup>+</sup> cells. The contaminating population was  $\leq 5\%$ , and CD16<sup>+</sup> cells constituted  $\leq 10\%$  of the effector cells in all experiments.

VZV-specific effector T cells were generated by incubation (2 weeks in 24-well plates) of  $2 \times 10^6$  cells/well with whole VZV antigen in the presence of  $10^6$  autologous irradiated PBMC (2700 rads) as feeders. Cells were refed every third day with 10% fetal calf serum, VZV antigens, and 10 U/mL recombinant human interleukin (IL)-2 (R&D Systems, Minneapolis). Target cells were autologous Epstein-Barr virus-transformed B cells that were infected with vaccinia recombinants expressing IE62 or IE63 [15]. Mock-infected targets or cells infected with vaccinia virus devoid of any VZV gene were used as controls. After 14 h of incubation at 37°C, targets were centrifuged, resuspended in culture medium, and incubated with 300  $\mu$ Ci of  $^{51}$ Cr (Amersham) for 4 h. Labeled cells were washed, resuspended at  $3 \times 10^4$  cells/mL, and added to 96-well V-bottom plates at  $3 \times 10^3$  cells/well in 0.1 mL.

To measure cytotoxicity, effector cells were added to lymphoid cell line targets at effector-to-target ratios of 30:1, 15:1, 3:1, 1:1, and 1:3 in 8-well replicates. After 4 h at 37°C,  $^{51}$ Cr release was measured by counting 100  $\mu$ L of supernatant in a gamma counter. The spontaneous release of  $^{51}$ Cr and the maximum release after lysis with 1% Nonidet P40 detergent were determined for each target cell preparation. The percentage of specific lysis was calculated for each target as the [(mean experimental release) - (mean spontaneous release)]/[(mean maximal release) - (mean spontaneous release)]  $\times 100$ .

Lysis of VZV-specific targets was considered specific when it was at least 10% higher than lysis of control targets. The responder cell frequency (RCF) for each assay was determined by applying

the maximum likelihood method to standard limiting-dilution plots, using a computerized analysis [18]. Replicates were scored as positive if the cpm was higher than the mean cpm for the corresponding spontaneous release plus 3 SD.

**Assays for cytokine production.** Cytokine production was evaluated by stimulating  $3 \times 10^5$  PBMC with GST-IE63 antigen or GST (1.25  $\mu\text{g}/\text{well}$ ) in 96-well plates. Supernatants were harvested from replicate wells on days 2, 4, and 6 and tested for IL-2,  $\gamma$ -interferon ( $\gamma$ -IFN), and IL-4. Cytokine release was quantitated by use of commercial ELISAs with sensitivities of detection defined by reference standards in each assay. The cytokine response was calculated as the amount of cytokine produced with GST-IE63 minus the amount elicited by GST. Commercial assays were used to measure IL-2 (Genzyme, Cambridge, MA) and  $\gamma$ -IFN (Endogen, Cambridge, MA). The presence of IL-4 was assessed using an ultrasensitive assay (Cytoscreen; BioSource International, Camarillo, CA).

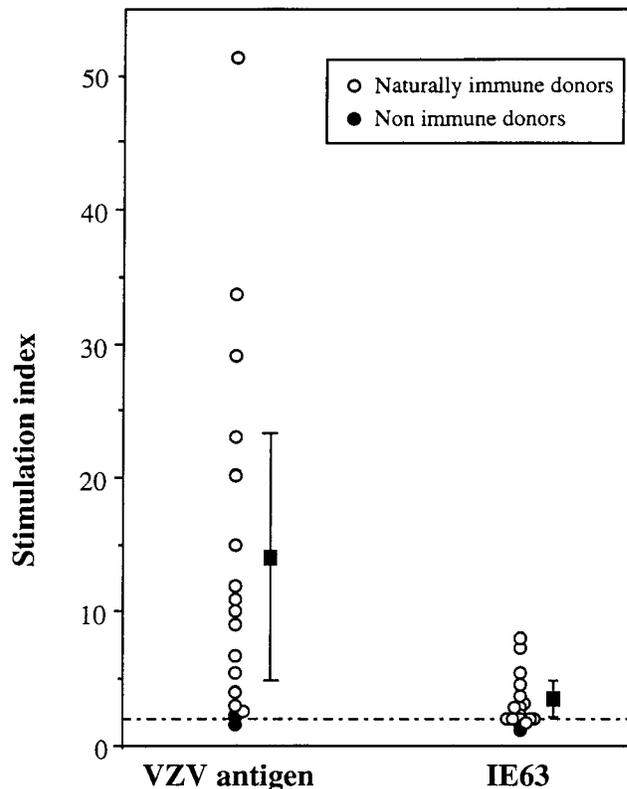
## Results

The presence of anti-IE63-specific antibodies was determined by use of Western blot analysis with GST-IE63: Serum from all VZV-immune subjects bound to a band corresponding to IE63, indicating the presence of specific antibodies. This anti-IE63 humoral immune response was not quantified, but the faint reactivity in Western blotting indicated that concentrations of IE63-specific antibodies were low in naturally immune subjects. No reactivity to IE63 was observed with control sera.

Since cell-mediated immunity seems to be the most important criteria in latency, as previously discussed, we searched for a memory immune response to IE63 by T cell lymphoproliferation and cytotoxicity assays.

After samples were stimulated with VZV antigens, the stimulation index (SI) in those from the VZV-immune subjects ranged from 2.1 to 52 (mean,  $14.3 \pm 10.2$  [SE]) (figure 1), while the SIs in samples from the nonimmune subjects were 2.1 and 1.6. The SI after stimulation with GST-IE63 was 1.2 and 1.4 for the nonimmune subjects and ranged from 1.8 to 8.0 (mean,  $3.4 \pm 1.5$ ) for the VZV-immune subjects. Among the 15 immune subjects, 9 (60%) had a clear T cell proliferation in response to GST-IE63; the remaining 6 (40%) had an SI of 2.0 despite SIs to VZV antigen ranging from 5.4 to 11.0. However, the latter showed a strong stimulation with GST alone, making difficult any interpretation of the specific stimulation by IE63. No correlation was found between the magnitude of the SI measured in response to GST-IE63 and total VZV antigens (data not shown).

For 6 immune and 2 nonimmune subjects, we measured the cytokine release by PBMC stimulated with IE63 for 2, 4, or 6 days. IL-2, IL-4, and  $\gamma$ -IFN released in the culture supernatant were quantified by ELISA. The results were expressed as the difference between the cytokine concentration detected after stimulation by GST-IE63 and by GST alone (table 1). IL-2 and IL-4 were not detected; however, significant amounts (50–1400 pg/mL), with a peak on day 4, of  $\gamma$ -IFN were measured



**Figure 1.** Proliferation induced by stimulation of T cells with unfractionated VZV antigens or with immediate early (IE) 63 protein. Stimulation index (SI) for each subject was calculated as ratio between mean counts per minute for GST-IE63-purified protein or VZV antigens and GST-purified protein or control antigen. Mean SI  $\pm$  SE is indicated by black square with error bars.

in all samples from VZV-immune subjects. This  $\gamma$ -IFN production was lower in the PBMC cultures of the 2 VZV-immune subjects, who had a low SI in response to IE63 stimulation. These results have to be compared with the absence of  $\gamma$ -IFN in the samples from nonimmune subjects and are in accordance with the pattern observed in T cells stimulated in standard PBMC culture with unfractionated VZV antigens [19].

However, the absence of measurable IL-2 and IL-4 could be explained by the fact that these cytokines could be produced very early and could be bound directly to their receptors, thus being undetectable by the techniques used in this study. In this case, it would be useful to search for the cytokines earlier after stimulation or to search for their transcripts by reverse transcript-polymerase chain reaction. It is also likely that the production of some cytokines is regulated by other ones. Even if the detection of other cytokines is needed to confirm these preliminary observations,  $\gamma$ -IFN production indicates that T cell stimulation by IE63 leads mostly to a Th1 proliferation and that the cytolytic component of cellular immunity is enhanced.

Using unfractionated T cells from 4 patients, we observed the lysis of targets expressing IE63 (27%–43%). This lysis

**Table 1.** Interferon- $\gamma$  (IFN- $\gamma$ ) production by IE63-stimulated peripheral blood mononuclear cells from 6 VZV-immune and 2 nonimmune subjects with no history of varicella infection.

Subject no.	SI	IFN- $\gamma$ * (pg/mL)
Immune subjects		
1	2.9	750
2	2.2	1400
3	7.4	500
4	8.0	625
5	2.0	175
6	2.0	50
Nonimmune subjects		
7	1.2	<50
8	1.2	<50

NOTE. IFN- $\gamma$  was measured on days 2, 4, and 6 of stimulation, and levels correspond to maximum amount detected. SI = stimulation index.

\* Expressed as difference between IFN- $\gamma$  concentration detected after stimulation by glutathione-S-transferase (GST)-IE63 and by GST alone.

was equivalent to that observed with targets expressing IE62 (25%–48%) and was significantly higher than that observed for mock-infected or vaccinia-infected targets used as controls. The mean effector frequency for T cells that recognized IE63 was  $1:31,000 \pm 16,000$  SE and was not significantly different ( $P = .8$ ) than the RCF for cells expressing IE62 ( $1:44,500 \pm 22,500$  SE).

Both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte populations lysed IE63 and IE62 targets, as demonstrated by assays using purified CD4<sup>+</sup> and CD8<sup>+</sup> populations as effectors. The RCF of CD8<sup>+</sup> cells recognizing IE63 ( $1:30,500 \pm 13,700$  SE) was not statistically different ( $P = .9$ ) from the RCF of CD8<sup>+</sup> cells recognizing IE62 ( $1:28,500 \pm 10,100$  SE), indicating that both viral proteins were recognized with the same efficiency by CD8<sup>+</sup> cells. In the case of IE63 recognition, the frequency of T cells lysing IE63 targets was equivalent ( $P = .97$ ) for CD4<sup>+</sup> ( $1:31,450 \pm 7500$  SE) and CD8<sup>+</sup> populations of effector cells.

## Discussion

Our experiments demonstrate that most VZV-immune individuals have memory immunity against IE63, as shown by specific antibody detection, and by proliferation of T cells when stimulated by a GST-IE63 fusion protein. The cytokines produced by these proliferating T cells suggest that the stimulation by IE63 leads mostly to a proliferation of Th1 cells and that the cytolytic component of the cellular immunity is enhanced, which is confirmed by the lysis of targets expressing IE63. As with IE62 and VZV glycoproteins, IE63 protein has an amino acid sequence that can be presented by the class I and II major histocompatibility complex (MHC) pathways, as indicated by the participation of CD4<sup>+</sup> and CD8<sup>+</sup> in the cytotoxic response.

IE63 is thus highly immunogenic and leads to a long-term immune response after natural infection that is comparable to

the immune response to other major VZV immunogenic proteins, such as IE62 and gE.

The implication of IE63 recognition by the immune system in the control of latency has to be documented using animal models or by studies involving more subjects, especially subjects with a high probability to reactivate the virus. In fact, one of the main characteristics of IE63 is the fact that it is expressed during productive infection and during latency. This raises the question of the relationship between the immune system and the nervous system. Indeed, the nervous system is protected from immune recognition by physical barriers first and then by the absence of classical MHC molecules at the surfaces of neurons. On the contrary, satellite cells surrounding neurons express MHC and could play an important role in antigen presentation. It is therefore critical to clearly define in which cells the virus remains quiescent: Viral genomes have been detected in neurons only, in satellite cells only, and in both cell types, whereas IE63 protein has been observed only in neuron cytoplasm during latency. If neurons are the only cells expressing viral antigens, the mechanisms leading to the recognition of viral peptides has to be clarified. It is possible that nonclassical MHC proteins are expressed at the cell surface in response to viral infection as has been suggested for HSV infection [20]. However, in HSV-infected cells, peptide presentation by MHC molecules seems to be impaired because of a viral protein that inhibits peptide processing [21–22]. Such a mechanism has not been demonstrated for VZV and must be explored.

It will be of interest to characterize the immune response to IE63 in elderly and immunocompromised subjects, who have a high incidence of herpes zoster, to examine possible correlations between the risk of virus reactivation and diminished responses to IE63. Enhancing the immune response to IE63 protein may be an important strategy to prevent VZV reactivation from latency. If it is, IE63 protein could be a suitable candidate for incorporation into a therapeutic VZV vaccine for administration to a population at high risk for herpes zoster.

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