Brief Definitive Report

Novel Packages of Viral and Self-antigens Are Generated during Apoptosis

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Summary

Immune context is an essential determinant of the host response to potential autoantigens. The clustering of the autoantigens targeted in systemic lupus erythematosus within surface blebs of apoptotic cells generates high concentrations of autoantigen within discrete subcellular packages. We demonstrate here that when apoptosis is induced by Sindbis virus infection, viral antigens and autoantigens cocluster exclusively in small surface blebs of apoptotic cells. The surface of these blebs is rich in viral glycoproteins, and virions can be seen blebbing from their surface. We propose that these blebs of mixed foreign and self-origin define a novel immune context that may challenge self-tolerance.

Apoptosis is a morphologically and biochemically distinct form of cell death that occurs in many different cell types in response to a diverse range of stimuli (1, 2). When apoptosis occurs in response to DNA damage, growth factor deficiency, or ligation of a family of cell surface receptors, the resulting apoptotic cellular fragments contain only self-components, and the response of the organism to these fragments is typically noninflammatory and nonimmune (3). Apoptosis may also be induced by viral infection, but the host-response to apoptotic fragments in this setting is unknown (4–6). The previous demonstration that complexes of self- and viral antigens are able to break tolerance to self has suggested that viral infection might act as an initiating stimulus in autoimmunity, but the site and context in which significant concentrations of these complexes may be encountered in vivo has not been identified (7).

We have recently demonstrated that when apoptosis is induced by ultraviolet irradiation, the autoantigens targeted in SLE are clustered in two discrete populations of surface structure on apoptotic cells: (*a*) apoptotic bodies that arise from the condensed, fragmented nucleus and contain nucleosomes, the ribonucleoproteins Ro and La, as well as the small nuclear ribonucleoproteins (snRNPs); and (*b*) numerous small surface blebs that arise from fragmented rough endoplasmic reticulum (ER) and contain ribosomal and ER autoantigens, as well as Ro (8). Since infection with Sindbis virus (SV) induces apoptosis of multiple cell types in vitro (4), we evaluated the distribution of viral components within the two populations of surface blebs in apoptotic cells. These studies demonstrate that virus-induced apoptosis results in the coclustering of autoantigens and viral antigens exclusively in small surface blebs of apoptotic cells. Of note, the small blebs also contain abundant viral glycoprotein in their surface membranes. We propose that these small blebs, rich in viral antigens and budding virions, define a novel immune context for the self-antigens clustered within these apoptotic structures. Specific complexes of viral and self-antigens encountered in this setting may provide a unique challenge to tolerance.

Materials and Methods

Cell Culture and Viral Infection. HeLa cells were cultured in medium containing 10% calf serum using standard procedures. Confluent monolayers were infected with wild-type SV strain AR339 (multiplicity of infection of 20), followed by incubation for a further 18–20 h at 37°C in a 5% CO_2 humidified incubator before performing biochemical or morphologic analyses.

DNA Extraction and Gels. DNA from whole cell populations was extracted, electrophoresed on 1.5% agarose gels, and visualized after staining with ethidium bromide as described (9).

Antibody Screening Procedures. Reactivity of human autoimmune and control sera with saline soluble extracts of human spleen and rabbit thymus was determined by Ouchterlony double immunodiffusion using standard reference sera to Ro and nRNP (10). Sera were also characterized by immunoblotting, as previously reported (8).

Immunofluorescence and Confocal Microscopy. Immunofluorescence microscopy was performed on cells grown on #1 glass coverslips as described (8). Cells were fixed in 4% paraformaldehyde (5 min, 4°C) before permeabilization with acetone (30 s, 4°C), and antibody staining. Ro and U1-70-kD RNP were stained with human sera of defined specificities (8) (diluted 1:160) and were visualized with fluorescein-conjugated goat anti-human $F(ab')_2$ (Organon-Teknika Corp./Cappel, Durham, NC). Sindbis virus proteins were visualized with affinity-purified rabbit antibodies to SV (4) or mAb 4F directed against Sindbis capsid protein (11). Visualization was with Texas red–conjugated goat anti-rabbit or anti-mouse antibodies (The Jackson Laboratory, Bar Harbor, ME). Coverslips were mounted on glass slides with Permount (Lipshaw, Pittsburgh, PA), before viewing and photography on a microscope (Axiophot, Carl Zeiss Inc., Thornwood, NY) equipped with phase contrast and epifluorescence optics. Confocal microscopy was performed on a

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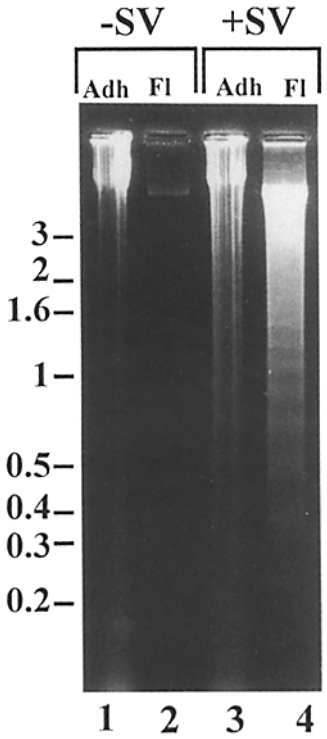

Figure 1. SV infection of HeLa cells induces a nucleosomal ladder pattern that is characteristic of apoptosis. HeLa cells were mock infected (lanes *1* and *2*) or infected with SV (lanes *3* and *4*) (5). After incubation for 18 h at 37°C, adherent (*Adh*) and detached, floating cells (*fl*) were collected. DNA was extracted, electrophoresed on 1.5% agarose gels, and visualized by staining with ethidium bromide (9). Note that the nucleosomal ladder pattern is seen exclusively in the SV-infected cells, and is especially prominent in the floating cell population (lane *4*). Migration positions of marker DNA are shown on the left.

scanning confocal microscopy system (model MRC600; Bio-Rad Laboratories, Richmond, CA).

Electron Microscopy. Cells were grown on 35-mm tissue culture dishes, infected with SV, and processed 18 h after infection. Monolayers were washed very gently with PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, and 8 mM Na_2HPO_4) and fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, accompanied by microwave irradiation as described (8) so that a final fixative temperature of 50°C was obtained. Postfixation was in osmium tetroxide. Monolayers were stained en-bloc with 1% uranyl acetate before dehydration in ethanol, and embedding in Epon. Thin sections were cut, viewed, and photographed in an electron microscope (model TEM type 1A; Zeiss).

Results and Discussion

SV, a positive strand RNA virus which causes a human syndrome characterized by fever, skin rash, and arthritis (12), was used to infect HeLa cells or human keratinocytes. Within 20 h of infection, islands of cells in the cultures showed characteristic apoptotic morphology with cytoplasmic contraction, marked surface blebbing, nuclear condensation, and fragmentation. Apoptosis of infected cells was confirmed by transmission electron microscopy (see Figure 4 *A*), and by demonstrating the characteristic nucleosomal ladder pattern

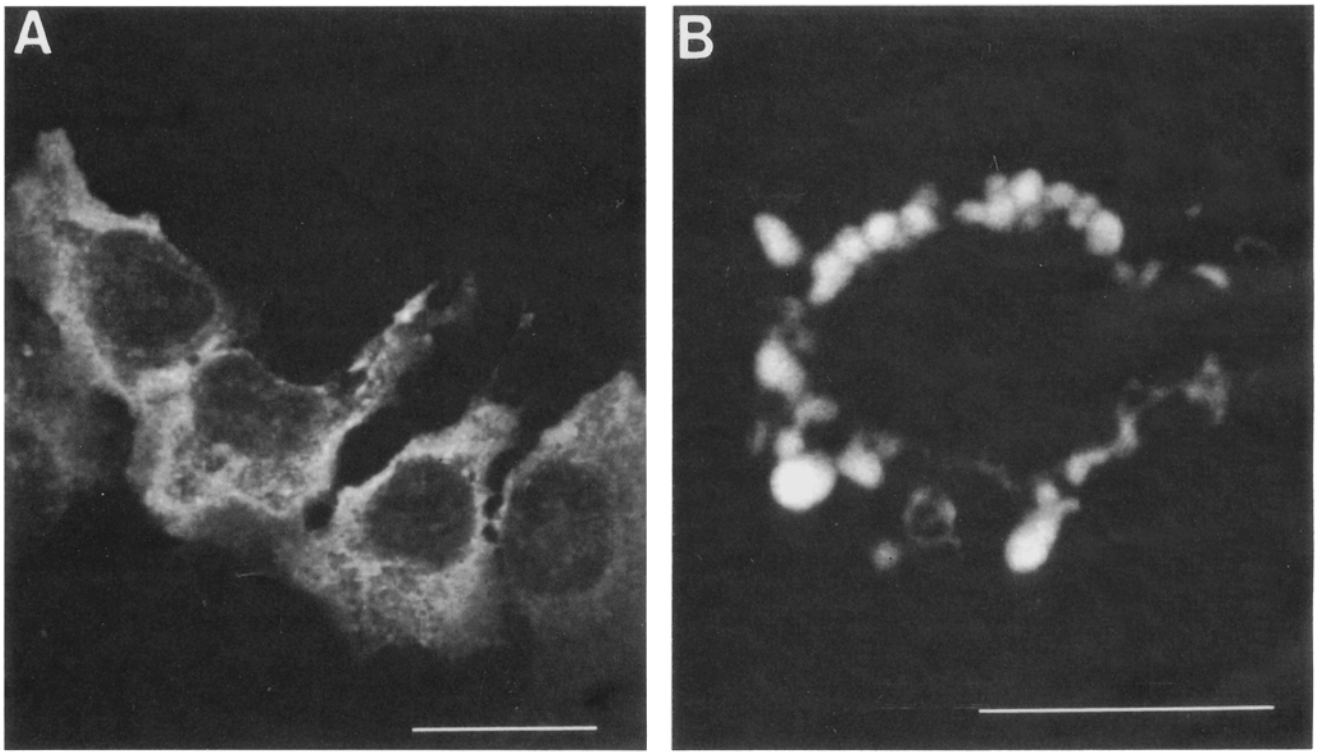

Figure 2. Immunofluorescent staining of SV proteins in Sindbis-infected HeLa cells shows redistribution of viral antigens into small surface blebs in apoptotic cells. HeLa cells were infected with SV, incubated for 20 h at 37°C, then fixed and and permeabilized as described in Materials and Methods. SV was stained using an affinity-purified polyclonal rabbit anti-SV antibody (4) followed by Texas red–conjugated goat anti–rabbit IgG, and was visualized using confocal immunofluorescence microscopy as described (8). (*A*) An island of SV-infected cells that do not yet exhibit apoptotic morphology show diffuse cytoplasmic and ER staining. (*B*) An SV-infected cell with classic apoptotic morphology (1) showing accumulation of viral protein in small surface blebs. In uninfected apoptotic HeLa cells, SV staining was never detected (data not shown). Similar results were obtained in four separate experiments. Scale bar, 10 μm.

of DNA upon gel electrophoresis (Fig. 1). Immunofluorescence of Sindbis-infected cells was performed with a panel of defined polyclonal and mAbs recognizing SV capsid protein or surface glycoproteins (4, 11). Infected islands of cells, 1–10 in number, could be identified at 20 h after infection. The staining pattern of viral components in individual cells was influenced markedly by whether the cell had become apoptotic, and how far in this process it had progressed. Thus, viral antigens in infected cells that had not yet become apoptotic stained with a typical ER and Golgi distribution, and were excluded from the nucleus (Fig. 2 *A*). In infected cells that demonstrated prominent surface blebbing, consistent with the morphology of early apoptosis, viral antigen was concentrated in the small surface blebs (Fig. 2 *B*). There was a concomitant decrease in staining of viral antigen within the cytosol (Fig. 2 *B*). In these cells, mAbs to surface glycoproteins E1 and E2 (11) strongly stained the surface of small blebs (data not shown). In infected cells with the morphology characteristic of late apoptosis (that is, nuclear fragmentation and formation of large surface apoptotic bodies), viral antigens remained localized within small surface blebs, but were excluded from the surface apoptotic bodies containing nuclear fragments (Fig. 3 *F*). Complete lack of staining on uninfected cells rendered apoptotic by irradiation with ultraviolet B confirmed the specificity of viral antibody staining (data not shown).

The autoantigen contents of the two populations of surface blebs reflects their subcellular origin in the apoptotic cell. Thus, small blebs contain antigens derived from fragmented, rough ER, and apoptotic bodies possess autoantigens originating in the apoptotic nucleus (8). To directly address whether viral proteins colocalize with autoantigens in the surface blebs of apoptotic cells, HeLa cells rendered apoptotic by infection with SV were double labeled with mAb to the virus capsid antigen (11) and monospecific human sera that highlight the two different populations of surface structures. Examination by fluorescence confocal microscopy showed that within small surface blebs, viral capsid antigen costained with the RNP autoantigen Ro (Fig. 3, *A–C*; overlapping pixels seen as yellow). These small blebs also stained strongly with propidium iodide, and were observed by electron microscopy to contain numerous ribosomes and fragmented ER (Fig. 4, and data not shown). No overlapping staining was observed in the nucleus or apoptotic bodies, which stained strongly for Ro but excluded virus (Fig. 3, *B* and *C*).

These findings were verified when apoptotic cells were double stained for Sindbis capsid antigen and the 70-kD protein component of the U1-snRNP (U1-70-kD RNP), that has an exclusively nuclear distribution, and has previously been demonstrated to segregate only into large surface blebs in apoptotic cells (8). A nonoverlapping pattern of staining with antibodies to U1-70-kD RNP and Sindbis capsid an-

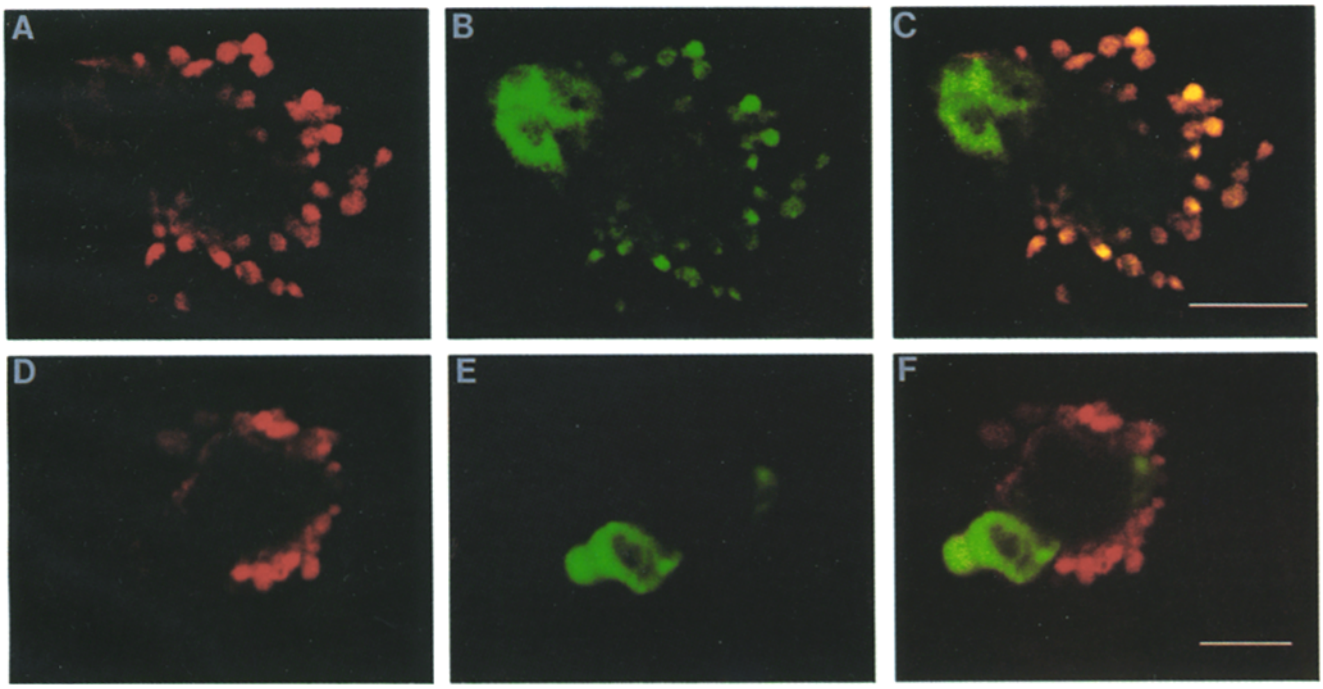

Figure 3. SV capsid protein is found in small surface blebs of infected apoptotic cells, where it colocalizes with Ro, but not with U1-70-kD RNP. HeLa cells were infected, fixed, and permeabilized as described in Materials and Methods. The cells were then double stained with mAb (11) to SV capsid protein (*A* and *D*) and monospecific human sera to the following autoantigens: 52-kD Ro (*B*) and U1-70-kD RNP (*E*). The stained cells were examined by confocal immunofluorescence microscopy. Ro (*B*) and U1-70-kD RNP (*E*) were visualized with fluorescein-conjugated goat anti-human $F(ab')_2$ and assigned the color green, whereas SV staining (*A* and *D*) was visualized with Texas red–conjugated goat anti-mouse IgG, and assigned the color red. When red and green images were merged (*C* and *F*), overlapping pixels appeared yellow/orange (*C*). In the small surface blebs of apoptotic cells, SV colocalizes with Ro but not with U1-70-kD RNP. Similar results were obtained in three separate experiments. Scale bars, 10 μm.

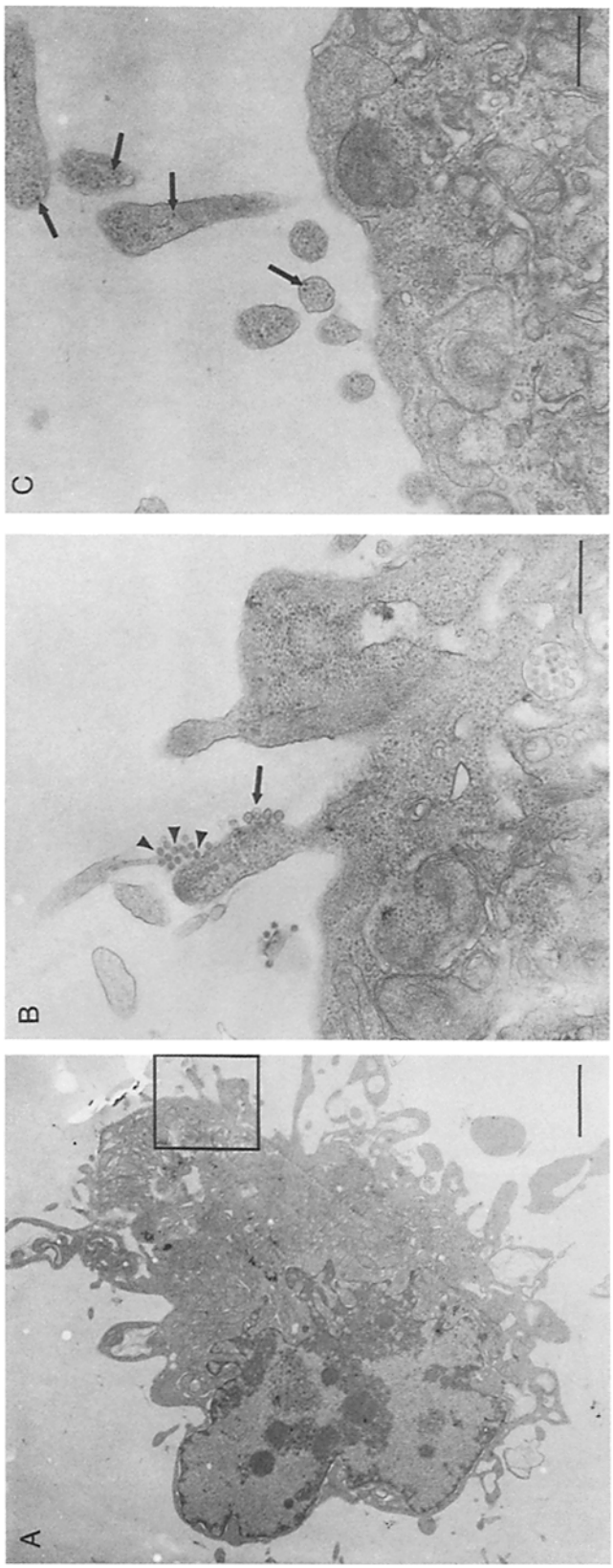

Figure 4. Electron microscopy of SV-infected HeLa cells shows characteristic apoptotic morphology, multiple nucleocapsids within small surface blebs, and occasional virions budding from these structures. HeLa cells were infected with SV, incubated at 37°C, and then fixed in glutaraldehyde using microwave irradiation (8), and processed for electron microscopy as described in Materials and Methods. (*A*) SV-infected HeLa cell showing condensed, fragmented nucleus and surface blebbing characteristic of apoptosis (1). (*B*) Enlargement of the boxed area in *A* to show detail of virions budding from the surface of a small bleb (*arrowheads*), as well as spherules, the site of assembly of the viral replication machinery (*arrow*); (*C*) multiple nucleocapsids are observed within small surface blebs (*arrows*). Bar *A*, 2.6 μm; *B* and *C*, 0.5 μm.

tigen was observed (Fig. 3, *D–F*). Thus, whereas the nucleus and apoptotic bodies stained prominently with antibodies to U1-70-kD RNP, they were never stained with antibodies to viral antigen (Fig. 3, *E* and *F*). Furthermore, small surface blebs stained intensely for viral products but not with U1-70-kD RNP (Fig. 3, *D–F*). Identical findings were obtained using affinity-purified rabbit polyclonal antibodies against SV (data not shown).

To determine whether the accumulation of viral components within small surface blebs of apoptotic cells reflected the production and budding of viral particles from these structures, or merely the presence of viral proteins within them, we performed transmission electron microscopy of SV-infected cells. Virus budding from apoptotic cells was clearly visualized, both from the cell surface (data not shown) as well as from small surface blebs (Fig. 4, *B* and *C*). Within small surface blebs, both nucleocapsids (Fig. 4 *C*, *arrows*) and budding virions (Fig. 4 *B*, *arrowheads*) were observed; virion size was 50 nm, which is characteristic of Sindbis. Although nucleocapsids were observed in most small surface blebs, actual virion budding from these structures was seen in a minority. Apoptotic bodies did not contain nucleocapsids or budding virions (data not shown). In some cases, light bulb-shaped "spherules" characteristic of alphavirus infection were noted at the surface of small blebs (Fig. 4 *B*, *arrow*). These structures, believed to represent the site of assembly of the viral replication machinery (13–15), were also noted within intracellular cytopathic vacuoles (data not shown).

Taken together, our data demonstrate that the relocation of Sindbis viral components in cells undergoing apoptosis is highly ordered, with capsids, nucleocapsids, and virions becoming concentrated exclusively in small blebs of cytoplasmic origin, where they colocalize with autoantigen clusters (8). Furthermore, the surface membrane of these apoptotic structures, rich in viral E1 and E2 glycoproteins (data not shown) and supporting virion budding (Fig. 4), clearly reveals their viral content, and potentially defines a novel immune context for these apoptotic cellular fragments. Formation of complexes of viral and self-antigens has recently been demonstrated to result in the breaking of tolerance to the self-molecule (7). For example, SV40 large T antigen forms a specific complex with the tumor suppressor molecule, p53. Upon inoculation of complexes of these two components into animals, specific high titer autoantibody production to p53 is initiated. Once tolerance is broken, the autoantibody response may be driven by p53 alone (7). It is therefore of great interest that calreticulin, an ER autoantigen in SLE, has recently been shown to form complexes with rubella and al-

phaviruses during viral replication (16, 17). The ability of complexes of these molecules to break tolerance to calreticulin remains to be addressed.

These observations suggest that each of a growing number of viruses known to trigger apoptosis (4–6, 18) may generate novel sources of concentrated foreign and self-antigens, the nature of which will be influenced by the tropism and life cycle of a given virus. Since coimmunization with complexes of viral and self-antigens can break tolerance (7), we propose that the specific clustering and concentration of such components within defined apoptotic cell fragments may result in the autoimmune targeting of specific self-molecules. The autoantibody pattern characteristic of a specific disease may, in part, reflect those host cell molecules that were clustered with viral antigens at the time of programed cell death, and hence initial immunization.

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