

Infection of human embryonic myoblasts by West Nile virus (WNV), a flavivirus, caused significant upregulation of class I and II MHC expression as determined by flow cytometry. After 48 hours at a multiplicity of infection of 5 pfu/cell, a sixfold increase in MHC class I expression was induced from initially low levels of expression. In contrast, MHC class II was induced de novo to five times the control fluorescence level. At least 70% of the cells were infected as determined using fluorescence microscopy and anti-WNV antibody labeling. Myoblasts were >90% pure as shown by anti-Leu-19 labeling. MHC class I (but not class II) was increased threefold after exposure to virus-inactivated supernatant from 48-hour-infected cells, indicating the presence of factor(s) contributing to the MHC class I increase. These findings may be important in establishing a link between viral infection of human cells and induction of inflammatory autoimmune disease. We discuss the possibility of using WNV as an in vivo model. © 1992 John Wiley & Sons, Inc.

Key words: major histocompatibility complex • flavivirus • myoblasts • myositis • autoimmunity

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## FLAVIVIRUS INDUCES MHC ANTIGEN ON HUMAN MYOBLASTS: A MODEL OF AUTOIMMUNE MYOSITIS?

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**M**ajor histocompatibility complex antigens (MHC class I and II) are transmembrane glycoproteins. They bind viral antigen for recognition by cytotoxic (CD8<sup>+</sup>) T cells through MHC class I, and for presentation to T-helper (CD4<sup>+</sup>) cells through MHC class II. The ensuing cellular immune response consists of the controlled, specific eradication of virus-infected cells,<sup>41</sup> followed by suppression of this response. However, in certain individuals, autoimmune disease is thought to supervene.<sup>13</sup>

Several different organs may be involved simultaneously in autoimmune attack, but in some

diseases only a relatively narrow range of tissues may be affected. In the latter category, diseases such as polymyositis have been extensively investigated. Much work has uncovered evidence of humoral involvement in polymyositis,<sup>6,31,32</sup> but during the last decade it has become increasingly clear that cellular immunity may play a significant part in disease pathogenesis. Thus, increases in MHC class I<sup>22</sup> and II<sup>31</sup> on muscle tissue have been found, as well as local infiltrations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.<sup>1,2,11</sup> Significantly, antecedent virus infection is also thought to be an important factor in the precipitation of polymyositis.<sup>8,9,15,17,38</sup> Most viruses reduce cell surface MHC expression, either specifically or nonspecifically,<sup>10,28,34</sup> although some, for example, neurotropic coronaviruses, have been found to increase MHC expression on oligodendrocytes, astrocytes<sup>30,39,40</sup> and brain endothelium.<sup>21</sup> Viruses which induce MHC expression on normally MHC-negative cells would presumably be candidate etiologic agents in autoimmune polymyositis as well as other autoimmune diseases.

Recently, it has been found that flaviviruses increase MHC class I on fibroblasts<sup>26</sup> and class II on astrocytes<sup>29</sup> with little interference with cell metabolism for the first 24–48 hours. West Nile virus (WNV) is an arbovirus belonging to the family *Flaviviridae*. Transmitted by mosquitoes, this hu-

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man pathogen causes significant morbidity and mortality throughout the world.

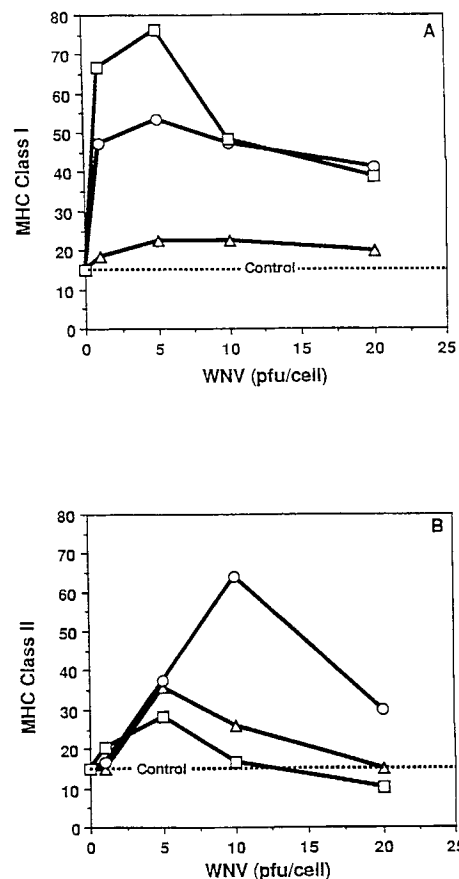
We report here that WNV infection of cultured human embryonic myoblasts increases MHC class I expression and induces de novo expression of MHC class II on these cells. We suggest that inappropriate expression and presentation of class I and II MHC by normally negative myoblasts/myotubes, particularly in the context of virus infection, could augment the pathogenesis of autoimmune disease. This virus may thus be useful as a tool to create a laboratory model for autoimmune muscle disease.

## MATERIALS AND METHODS

**Myoblast Infection by WNV and WNV Supernatant Treatment.** Human embryonic myoblasts, isolated from 14–17 week normal human embryos, were prepared as previously described,<sup>3</sup> and cultured for 1 week in Ham's F10 medium (CSL #733 2049) containing 10% fetal calf serum (HF10/FCS) under standard culture conditions (SCC). NH and MRC guidelines were strictly adhered to throughout. Myoblasts were then seeded into 50-mm plastic Petri dishes at  $5 \times 10^5$  cells/dish. The cells were infected 24 hours later with a multiplicity of infection (moi) of 5 plaque-forming units (pfu) of WNV/cell for 48 hours. The virus concentration and the time of infection was titrated to give maximum response for both class I and II MHC induction with minimum cytotoxicity (Fig. 1). Infected cells were incubated under SCC for 48 hours. Control cells were mock-infected using HF10/FCS only. A control for MHC induction consisted of myoblasts incubated with IFN- $\gamma$  (100 U/mL) for 48 hours. This was titrated for maximal MHC induction (Fig. 2).

Supernatants from WNV-infected cultures were irradiated with UV light at  $1600 \mu\text{W}/\text{cm}^2$  for 12 minutes. This destroys WNV without interfering with the activity of IFNs.<sup>26,27</sup> Supernatants were added to fresh myoblast cultures for 48 hours under SCC. Supernatants from mock-infected cultures were divided into two. Fresh WNV at 5 pfu/cell was added to half, the other was left untreated. Both were irradiated as above, and added to fresh cultures as controls.

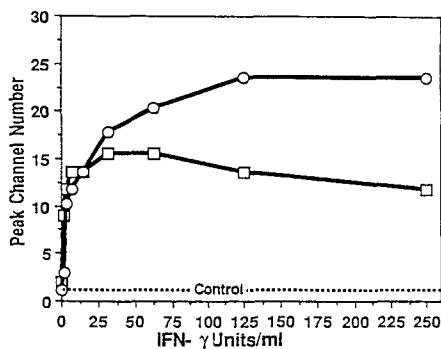
**Identification of Myoblasts Infected by WNV.** Coverslips placed in the same cultures were removed, the adjacent cells were fixed in acetone at  $-20^\circ\text{C}$  for 30 minutes, washed twice in calcium/magnesium-free Hank's buffer (CMF-HBSS) containing 1% bovine serum albumin, and once in HF10/



**FIGURE 1.** Titration of WNV multiplicity of infection on myoblasts. **(A)** MHC class I is maximally induced by 5 pfu/cell over a period of 24 hours ( $\Delta$ — $\Delta$ ), 48 hours ( $\circ$ — $\circ$ ), and 72 hours ( $\square$ — $\square$ ). **(B)** MHC class II is maximally induced by 5 pfu/cell over a period of 24 hours ( $\Delta$ — $\Delta$ ) and 72 hours ( $\square$ — $\square$ ), but maximally induced by 10 pfu/cell over 48 hours ( $\circ$ — $\circ$ ). The y-axis represents the peak channel fluorescence from FCM analysis of the myoblast population labeled for MHC class I (A) and MHC class II (B). The x-axis represents the WNV moi measured in pfu/cell.

FCS. The cells were then incubated at  $4^\circ\text{C}$  for 45 minutes with  $25 \mu\text{L}$  of a 1:20 dilution of polyclonal anti-WNV antibody and washed again. WNV antibodies were labeled using  $25 \mu\text{L}$  of 1:20 dilution of fluorescein isothiocyanate-conjugated sheep antimouse immunoglobulin (SAMIg-FITC) (Silenus). Hoechst 33342 (Sigma) was used at  $1 \mu\text{g}/\text{mL}$ ,  $25 \mu\text{L}/\text{coverslip}$ , as a counterstain. The percentage of WNV-infected cells was calculated as a ratio of FITC-labeled (infected) cells to Hoechst-labeled nuclei  $\times 100$ . Cells with double nuclei were counted as single cells.

**Preparation of Myoblasts for Flow Cytometry (FCM) Labeling.** Myoblasts in Petri dishes were washed twice in CMF-HBSS. One milliliter of trypsin



**FIGURE 2.** Titration of MHC class I and II induction by IFN- $\gamma$  on myoblasts. Class I (□—□) is maximally induced by 25–50 U/mL IFN- $\gamma$ , while class II (○—○) is maximally induced by 70–125 U/mL IFN- $\gamma$ . The y-axis represents the peak channel fluorescence from FCM analysis of the myoblast population labeled for MHC class I and II. The x-axis represents the concentration of IFN- $\gamma$  measured in units per milliliter.

(0.05%)–EDTA (1 mM) was added per dish and incubated at 24°C for 2 minutes, then 1 mL of HF10/FCS was added to stop the digestion. The myoblasts were centrifuged at 4°C at 400g, resuspended in HF10/FCS, and distributed into sample aliquots for labeling.

**Labeling of MHC Class I and Class II.** Cells were incubated with 100  $\mu$ L of a 1:20 dilution of monoclonal antibody (MAb) recognizing MHC class I (clone W6/32, Dakopatts) or class II (clone CR 3/43, Dakopatts) at 4°C for 45 minutes. Concentrations were titrated for maximal specific labeling. After incubation, myoblasts were centrifuged through 100  $\mu$ L FCS, the supernatant was removed, and the cells resuspended in 100  $\mu$ L of 1:20 SAMIg-FITC for 45 minutes. Cells were then centrifuged through FCS as above and resuspended in 300  $\mu$ L HF10/FCS for FCM. An isotype antibody recognizing glial fibrillary acidic protein (GFAP-Dako, Dakopatts) was used to control for nonspecific antibody labeling.

**Identification of Myoblasts.** Myoblasts were incubated with 50  $\mu$ L of a 1:2.5 dilution of antihuman Leu-19 conjugated to phycoerythrin (PE) (Becton Dickinson). This MAb recognizes myogenic cells.<sup>36</sup> In the absence of a PE-labeled primary antibody we used anti-GFAP as an isotype control and PE-conjugated SAMIg (Silenus).

**Flow Cytometry.** Fluorescence between 515 and 545 nm (FITC) or 575 and 595 nm (PE) was measured using a FACScan (Becton Dickinson). For-

ward and side scatter measurements were within the same range for all populations.

## RESULTS

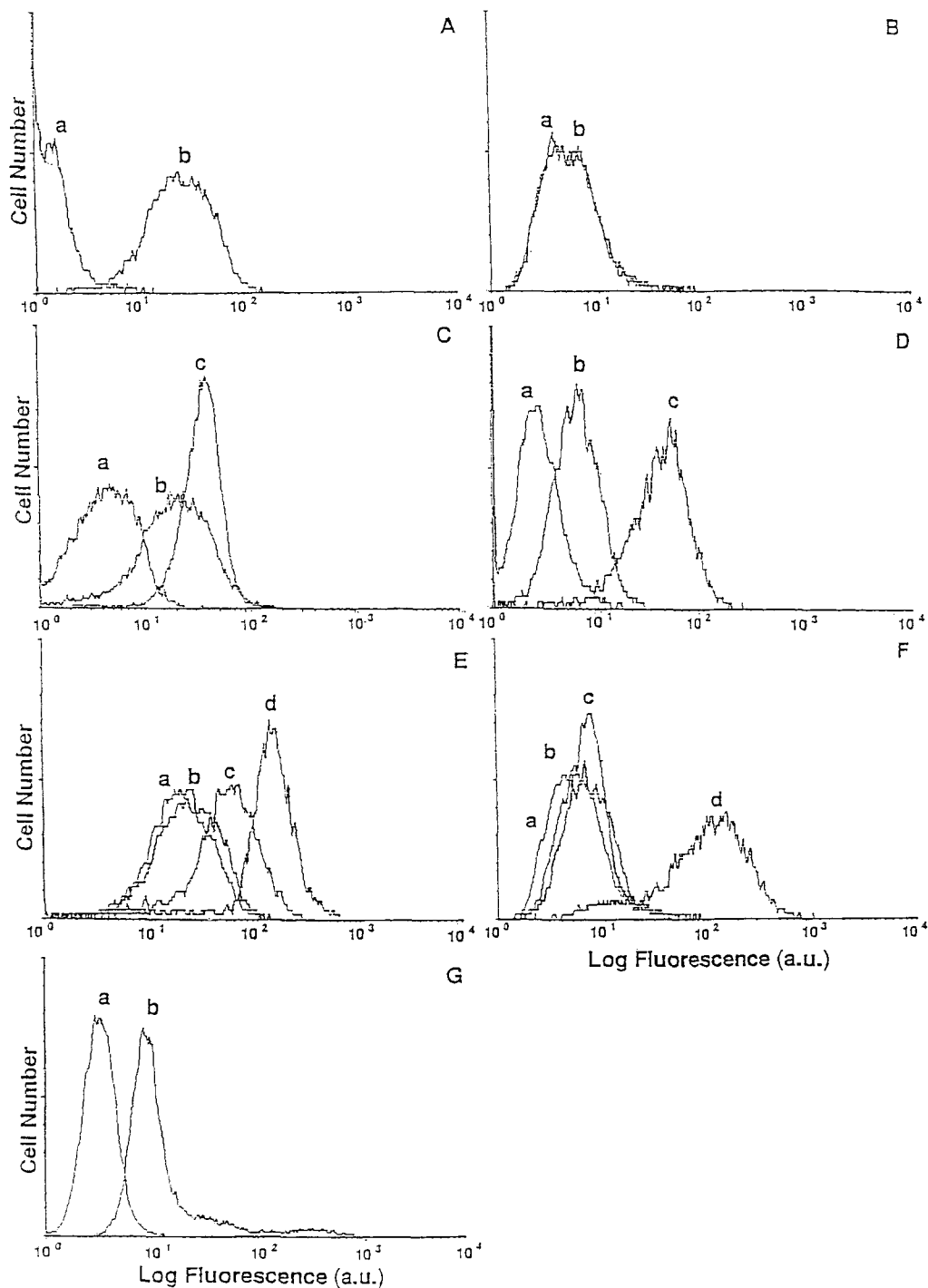
**MHC Expression on Normal Embryonic Myoblasts.** Previously, using quantitation by fluorescence microscopy, we reported low MHC class I expression and no class II expression on cultured normal human embryonic myoblasts.<sup>3</sup> Here we provide a more accurate quantitation of MHC expression using FCM.

Figure 3A illustrates the distribution of MHC class I expression on normal human myoblasts (b), compared with that of the isotype control antibody (anti-GFAP) (a). The difference in fluorescence is about tenfold. Thus, this level is not as low as was previously thought.<sup>7,22,31</sup>

Figure 3B shows the distribution of MHC class II expression on normal human myoblasts (b), compared with that of the isotype control antibody (a). In agreement with previous findings,<sup>3</sup> this demonstrates that there is no detectable MHC class II expression on these cells.

**Effect of WNV on MHC Expression by Myoblasts.** MHC class I and II expression was measured by FCM on 48-hour WNV-infected (5 pfu/cell), mock-infected, and IFN- $\gamma$ -treated myoblasts. Figure 3C shows a sixfold increase in MHC class I expression by infected myoblasts (b) compared with mock-infected myoblasts (a). MHC class I expression on IFN- $\gamma$ -treated myoblasts (c) was almost 10-fold higher than on mock-infected cells. Figure 3D illustrates the de novo induction of MHC class II expression after WNV infection (b). The fluorescence intensity is about fivefold greater than mock-infected myoblasts (a), while the IFN- $\gamma$ -treated group (c) showed a 16-fold increase in fluorescence. This demonstrates that the capacity for MHC class II induction on myoblasts is significantly greater than previously reported. This may in part reflect differences in technique<sup>3</sup> and/or tissue origin.<sup>19</sup>

**Effect of Supernatant from Infected Myoblasts on MHC Expression by Myoblasts.** To investigate whether soluble factor(s) secreted by infected myoblasts could upregulate MHC expression, UV-irradiated supernatants from WNV- or mock-infected cultures were added to fresh myoblasts for 48 hours. Figure 3E shows that MHC class I expression was increased threefold (c), while the mock-infected control groups, either with (b)



**FIGURE 3.** Flow cytometric analysis of MHC expression by human embryonic myoblasts. **(A)** MHC class I antigen expression (b) is tenfold higher than isotype control antibody (a). **(B)** No difference between anti-HLA-DR antibody (b) and isotype control antibody (a). Thus, normal myoblasts express little or no MHC class II antigen. **(C)** Expression of MHC class I in myoblasts infected with WNV (b) is approximately sixfold higher than in mock-infected myoblasts (a), while expression in the IFN- $\gamma$ -treated myoblasts (c) is about tenfold higher. **(D)** Expression of MHC class II in WNV-infected myoblasts (b) is about fivefold higher than control levels of mock-infected myoblasts (a), while expression in the IFN- $\gamma$ -treated myoblasts (c) is nearly 16-fold higher. **(E)** Expression of MHC class I on myoblasts cultured in UV-inactivated supernatant from WNV-infected myoblast cultures (c) is more than threefold higher than myoblasts cultured in the supernatant from mock-infected with (b) and without (a) UV-inactivated fresh WNV. Myoblasts treated with IFN- $\gamma$  (d) show about a tenfold higher expression than (a) and (b). **(F)** Expression of MHC class II on myoblasts cultured in UV-inactivated supernatant from WNV-infected myoblast cultures (c) is similar to the expression on myoblasts cultured in the supernatant from mock-infected with (b) and without (a) UV-inactivated fresh WNV. Myoblasts treated with IFN- $\gamma$  (d) show a greater than 20-fold higher expression than (a), (b), and (c). **(G)** More than 90% of cells are positively stained with antihuman Leu-19 directly conjugated to phycoerythrin (PE) (b) compared to an irrelevant antibody control (a). The abscissas represent log fluorescence intensity (arbitrary units). The ordinates represent the number of cells in each channel. Each result was obtained by counting  $10^4$  cells.

or without (a) UV-irradiated WNV, were unaffected. Thus, there was no apparent influence of irradiation breakdown products on MHC class I expression. The IFN- $\gamma$ -treated group (d) shows an 11-fold higher expression than mock-infected myoblasts.

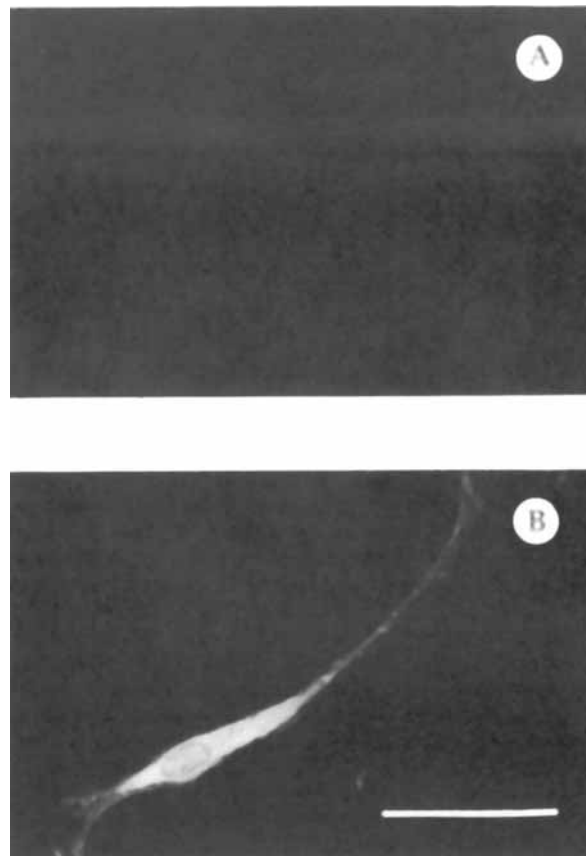
Figure 3F demonstrates that there is no increase in MHC class II expression in the presence of irradiated supernatant (c), mock-infected with (b) and without (a) UV-irradiated WNV. The IFN- $\gamma$ -treated group (d) shows a fluorescence intensity greater than 20-fold that of non-UV-irradiated WNV (a).

**Identification of Myoblasts.** The purity of myoblast cultures is shown in Figure 3G. More than 90% of cells were positively labeled by the myogenic marker, antihuman Leu-19 MAb (b), compared with an isotype antibody control (a).

**Detection of WNV Infection in Myoblasts.** Infection of myoblasts on coverslips was detected using anti-WNV antibody, labeled with SAMIg-FITC and fluorescence microscopy. Infection was detectable in at least 70% of myoblasts in all experiments as a bright specific perinuclear fluorescence (Fig. 4A) compared with uninfected controls (Fig. 4B).

## DISCUSSION

We report here that infection of human myoblasts by WNV significantly upregulates MHC class I and induces de novo class II expression. This was shown to be due, at least in part, to virus-induced secreted but unidentified soluble factors,<sup>26,40</sup> since the virus-inactivated supernatants from WNV-infected myoblast cultures were able to induce increased MHC class I expression in fresh myoblast cultures. This may also be due to a direct virus effect.<sup>26</sup> In contrast, MHC class II on myoblasts may be induced by the virus directly, as there was no induction of MHC class II in response to the virus-inactivated supernatant. The mechanism of this apparent direct induction by the active WNV is presently unknown. We think it may relate to the way some protein product(s) of viral transcription interact with the host cell. Investigation into possible mechanisms is presently being undertaken. Interestingly, and by contrast, UV-inactivated nonreplicating neurotropic coronavirus particles appear to be able to induce class II MHC in a small subpopulation of astrocytes *in vitro*.<sup>30</sup> This suggests different mechanisms of induction of class II by these two viruses and, if



**FIGURE 4.** Fluorescence micrographs of WNV-infected and mock-infected myoblasts. **(A)** Absence of viral antigen fluorescence in mock-infected myoblasts stained with anti-WNV antibody. **(B)** Distribution of WNV antigens recognized by anti-WNV antibody in WNV-infected myoblasts. White bar = 50  $\mu$ m.

true, would clearly have different implications for the respective antiviral immune responses.

As the concentration of MHC is directly related to the efficiency of both induction and execution of the cellular immune response,<sup>25,33</sup> increases in MHC expression due to IFN- $\gamma$  or virus (including WNV) are accompanied by increased lysis of target cells by virus-immune and alloimmune Tc cells.<sup>5,12,24,29</sup> In autoimmune muscle disease, long speculated to have a viral etiology, increased MHC class I<sup>22</sup> and class II<sup>32</sup> expression on muscle fiber membranes is frequently found in biopsies, and is associated with an influx of CD8<sup>+</sup> and CD4<sup>+</sup> T cells.<sup>1,2,11</sup> Evidence suggests that CD8<sup>+</sup> cells may mediate lysis of muscle cells.<sup>11</sup> However, although there is an association in inflammatory muscle disease with several different viruses (e.g., retroviruses,<sup>8,38</sup> Coxsackie virus,<sup>17</sup> picornavirus,<sup>14</sup> and influenza<sup>15</sup>), direct viral involve-

ment in the pathogenesis has been difficult to prove.

Hypothetically, viral infection resulting in a virus-specific cellular immune response would induce the release of IFN- $\gamma$  by T cells<sup>35</sup> and thus induce an increase in MHC expression on local uninfected muscle cells.<sup>20</sup> In susceptible individuals, this could facilitate crossreactive killing of uninfected muscle cells by antiviral T cells. Moreover, the induction of aberrant MHC expression might break self-tolerance and elicit an MHC-restricted antimuscle T-cell response.<sup>4</sup> Subsequent IFN- $\gamma$  induction of MHC by such autoimmune T cells would act as a positive feedback in this scenario.

Most viruses, however, in reducing cell surface MHC expression, would preferentially encourage the formation of high-affinity antiviral Tc cells.<sup>16,37</sup> These undoubtedly kill more efficiently if there is increased MHC expression on target cells that are infected.<sup>5,24,33</sup> Nevertheless, the high affinity of these Tc cells for virus-infected target cells would not usually result in crossreactive killing of uninfected cells despite the increased MHC expression of the latter. In theory, viruses which increase MHC expression in infected cells in vivo would induce an antiviral cellular immune response consisting mostly of low-affinity Tc cells with a high degree of crossreactivity for MHC alone. This occurs with WNV in vitro.<sup>23</sup> In addition, a tissue-specific, MHC-restricted response might simultaneously be induced in susceptible individuals in vivo. Such viruses would thus be candidate etiologic agents in autoimmune polymyositis and other autoimmune disease.

For reasons of host/virus survival, virus-induced autoimmunity cannot be a common event, although the reasons for this are not well understood. Nevertheless, under certain conditions, particularly in genetically susceptible individuals (e.g., HLA-B8 carriers<sup>18</sup>), it seems likely that autoimmune muscle disease could develop subsequent to such a viral infection. Ideally, we would like to be able to demonstrate a virus-specific and allospecific Tc-cell response in the human myoblast system reported here. However, although practicable in the murine system, there are severe ethical problems associated with doing this in the human embryonic system.

In conclusion, these findings suggest the use of WNV infection as a possible model for autoimmune muscle disease development in mice. Our preliminary findings suggest that murine embryonic myoblasts infected with WNV behave in the

same way as human myoblasts. It seems reasonable to suggest that investigation of such an animal model would shed useful light on events related to virus-induced autoimmune muscle disease pathogenesis in humans.

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