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Influenza viruses induce autoantibodies to a brain-specific 37-kDa protein in rabbit

(autoimmunity/neurological disorders/encephalitis/molecular mimicry)

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ABSTRACT **Immunization of rabbits with certain H1N1 influenza viruses, including the neurotropic strains NWS/33 and WSN/33 and the New Jersey/76 strain, resulted in the production of autoantibodies to a brain-specific protein of 37 kDa that is present in various species, including humans. Autoantibodies were produced to brain only; various other tissues tested were negative. These antibodies were not elicited by other influenza A or B viruses, including closely related recombinant strains, but were elicited by the isolated hemagglutinin of A/Bellamy/42 strain and by formaldehyde-fixed WSN virus—demonstrating that infection was not essential for the induction of autoantibodies. In histological studies, reaction with anti-viral antisera was specific to gray matter and was confined to sera that recognized the 37-kDa protein. Antibody binding was prominent in regions comprised of neuronal cell bodies in cellular layers of the dentate gyrus, hippocampus, cerebral cortex, and cerebellum and was undetectable in myelin-rich regions, such as the corpus callosum. The 37-kDa protein, therefore, appears to be a neuronal antigen. Antibodies directed against this protein may be involved in the pathogenesis of one or more of the neuropsychiatric disorders that occur after infection with influenza.**

Recent work from several laboratories has demonstrated that various common microbial pathogens share epitopes with specific host antigens and may, under appropriate conditions, elicit autoantibodies. This is well illustrated by the M proteins of the group A β-hemolytic streptococci. Defined linear epitopes on these proteins elicit antibodies that crossreact with cardiac and renal autoantigens (1, 2). Viruses, too, have been shown to share structural elements with identified host proteins. In most cases this structure is restricted to short regions of analogous amino acid sequence of about 5–10 residues (for review, see ref. 3). However, more recently, striking functional analogies have also emerged. For example, cytomegalovirus encodes a molecule that is similar in its structural organization to the α chain of HLA class I antigens, and (like HLA class I α chain) can also bind to β_2-microglobulin (4). Other viruses may be structural analogs of naturally occurring ligands. Vaccinia, which contains an epidermal growth factor-like sequence, appears to infect its host cells by binding to epidermal growth factor receptors (5). Also, the rabies virus can bind to nicotinic acetylcholine receptors—apparently by virtue of the resemblance of the viral coat protein to snake neurotoxins (6, 7). Similar mechanisms may operate in the case of human immunodeficiency virus, where putative endogenous ligands of the CD4 virus receptor molecule (i.e., vasoactive intestinal peptide and HLA class II antigens) have corresponding sequences in the viral coat protein (8, 9).

In a relatively few cases, host-analogous viral sequences have been shown capable of inducing autoimmune reactions—e.g., hepatitis B DNA polymerase and myelin basic protein (10); type C retrovirus $p30^{gag}$ protein and small ribonucleoprotein autoantigen (11); human cytomegalovirus and the β chain of HLA DR (12); and human immunodeficiency virus type 1 gp41 and the β chain of HLA class II antigens (9). However, crossreactions of anti-viral monoclonal antibodies with host proteins are very frequent (>3%) (13), indicating that such antigenic mimicry is more common than our limited knowledge of protein sequence data would suggest. Influenza viruses exhibit a high degree of antigenic variation (14) and therefore have considerable potential for antigenic mimicry of host proteins. Furthermore, similarities in amino acid sequence have been described recently between influenza virus proteins and myelin proteins of the central and peripheral nervous systems (i.e., myelin basic protein and P_2 protein, respectively) (15) and also myelin proteolipid (16). However, predictions that influenza viruses can induce immune reactions that crossreact with host proteins have not yet been tested experimentally. In this report we studied the capacity of influenza viruses to induce autoantibodies and demonstrate that immunization of rabbits with certain strains of influenza A induces autoantibodies to a highly conserved 37-kDa brain-specific protein. These results are discussed with reference to the various neurological and psychiatric disorders that have been implicated with influenza infection.

MATERIALS AND METHODS

Viruses, Hemagglutinins and Antisera. Methods for the preparation of these reagents have been described in detail elsewhere (17, 18). Briefly, influenza A and B viruses were grown in the allantoic cavity of embryonated chicken eggs and were purified from the allantoic fluid by adsorption and elution with chicken erythrocytes. Eluted virus was pelleted in an ultracentrifuge, and rabbits were injected i.v. with 30,000 hemagglutinin units of active virus. One to two months later, the rabbits were given an identical dose of virus i.v. and were bled 5 and 7 days later. Adjuvant was not used for immunization with whole viruses, except where specifically stated. Hemagglutinins were purified from $NaDodSO_4$-disrupted virus by electrophoresis on cellulose acetate (17). The hemagglutinin band was cut out, eluted in saline, and precipitated with cold ethanol. The precipitated hemagglutinin was mixed with Freund's complete adjuvant and injected into the leg muscle and footpad of rabbits. The rabbits

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Abbreviation: PBS, phosphate-buffered saline.

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received two additional cycles of immunization at 1-month intervals. The last dose of antigen was given i.v. without Freund's adjuvant. The purity of isolated hemagglutinins was verified by gel electrophoresis. Detailed antigenic analysis of the resulting antisera by immunodiffusion for reaction with other viral products proved negative.

For some experiments, influenza A/WSN/33 was grown in Madin–Darby bovine kidney (MDBK) cells and purified as described (19). For immunization, formaldehyde-inactivated virus (30,000 hemagglutinin units) was mixed with Ribi adjuvant (Ribi Immunochem) and injected intradermally at five separate sites into New Zealand White rabbits. Rabbits were bled immediately before immunization to obtain control preimmune serum. A booster immunization was given 1 month later under the same protocol.

Preparation of Tissues and Cell Membranes. Crude synaptosomal/mitochondrial membranes were prepared from normal human cerebral cortex and corpus striatum by differential centrifugation. Human brain tissue was obtained at autopsy (16-hr postmortem) from a woman aged 42 years who died as a result of myocardial infarct. Tissues were dissected and homogenized in 5 vol of Tris·HCl, pH 7.4, in a Polytron (Brinkmann), and the homogenate was centrifuged at 200 × *g* for 5 min to sediment high-density material including nuclei. The resulting supernatant was centrifuged at 35,000 × *g* for 30 min to yield the crude membrane preparation. Crude membranes were prepared in the same way from various freshly dissected mouse tissues. All procedures were performed as quickly as possible at 0–4°C. Pelleted membranes were resuspended without dilution and were stored frozen in aliquots at −70°C.

Gel Electrophoresis. Electrophoresis in $NaDodSO_4$ was performed in polyacrylamide gels in the discontinuous buffer system of Laemmli (20), except that gradients of 10–20% acrylamide were used without stacking gels. Membrane samples were diluted 1:10 in sample buffer, and 30-μl samples were applied to gels. Virus samples were applied at a final protein concentration of 2 mg per ml. Molecular weight standard proteins (low range) were from Bio-Rad.

Immunoblotting. Transfer of electrophoretically separated proteins to nitrocellulose was performed as described (21), except that a current of 600 mA was used for 2.5 hr with cooling. Nitrocellulose blots were blocked overnight in 5% milk powder in phosphate-buffered saline (PBS) at 4°C, washed, probed with antiserum at a dilution of 1:500 in 5% goat serum in PBS at 37°C, washed again, and finally incubated with goat anti-rabbit IgG alkaline-phosphatase conjugate (Sigma) at 1:1000 in PBS containing 5% bovine serum albumin at 37°C. For some experiments a protein A-alkaline phosphatase conjugate was used at a dilution of 1:500, in which case sera were diluted in PBS containing bovine serum albumin. All incubations were performed for 1 hr with agitation, and all washes were performed at room temperature using PBS (six times at 5 min, with agitation). Bound antibodies were visualized with bromochloroindolyl phosphate/nitro blue tetrazolium (22).

Purification of Antibrain Autoantibody. Antibrain antibody was purified from anti-NWS antiserum using an antigen-bearing strip of nitrocellulose cut horizontally from a preparative blot of human frontal cortex membranes. The antigen strip was incubated overnight in 0.5 ml of antiserum at 1:200 in 5% goat serum with agitation. After extensive washing, bound antibody was eluted in 0.1 M glycine·HCl, pH 2.4, containing 5% bovine serum albumin and phenol red as pH indicator. The pH was adjusted to 7.4 using 1.0 M Tris. This antibody solution was used undiluted to probe blots in the same way as for diluted antiserum.

Tissue Autoradiography. Frozen unfixed mouse brain was cut into 30-μm sections that were dehydrated and processed for immunocytochemistry as described (23). Adjacent sections were incubated overnight at 4°C in antiserum at a dilution of 1:500 in PBS containing 0.5% carrageenin and 0.3% Triton X-100. After washing in PBS, bound antibody was crosslinked covalently to the sections with 0.2 mM bis-(sulfosuccinimidyl) suberate in PBS. Sections were washed again and incubated for 90 min at room temperature in ^{125}I-labeled protein A (ICN) at a radioactive concentration of 70,000 dpm per ml. Visualization was by autoradiography with Hyperfilm-^{3}H (Amersham). Films were exposed without intensifying screens for 4 weeks at −70°C.

RESULTS

Identification of a Brain-Specific Protein Recognized by Anti-influenza Antisera. During experiments designed to demonstrate the binding of neurotropic influenza viruses to putative receptors on nitrocellulose blots of brain membranes, we noted that an antiserum to the NWS/33 virus reacted directly with a single 37-kDa protein in the absence of virus. Further analysis of this observation revealed that this protein was specific to brain, because the antiserum failed to react with membranes from various other tissues (Fig. 1). The antiserum was of high titer—reaction with the 37-kDa protein being detectable at a dilution of 1:50,000 (data not shown). Furthermore, the 37-kDa protein was found to be highly conserved; a band of this molecular mass was detected in the cerebral cortex of all four mammalian species tested (Fig. 2). Comparison of this protein by gel electrophoresis in $NaDodSO_4$ with other known brain proteins of comparable molecular mass—i.e., synaptophysin (38 kDa) (24) and neuron-specific enolase (43 kDa) (25) demonstrated that these proteins were not identical with the 37-kDa antigen (data not shown), which therefore appears to be another protein. Analysis of two-dimensional electrophoresis with silver staining demonstrated that this protein was a single, minor component of brain tissue (P.L., unpublished work).

Identification of Viral Strains That Induce Antibrain Autoantibodies. The observation that the rabbit antiserum to NWS/33 reacted with rabbit cerebral cortex (Fig. 2) demonstrates that this is an autoimmune reaction. To test whether the capacity to induce antibrain autoantibodies was a general property of influenza A viruses, 23 different rabbit antisera made against diverse influenza A strains (including avian and equine isolates) and one antiserum against influenza B were tested for reaction against human brain proteins (Fig. 3). Of these, only five reacted with the 37-kDa band—i.e., anti-A/NWS/33 (two antisera), anti-A/WSN/33, anti-A/New

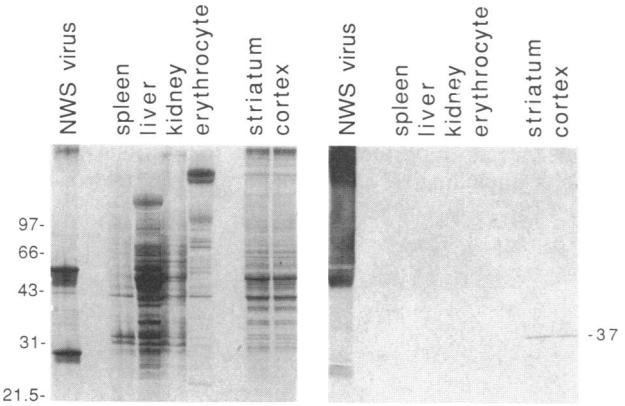

FIG. 1. Tissue distribution of 37-kDa antigen recognized by anti-A/NWS/33 antiserum. Crude cell membranes from various mouse tissues were separated by $NaDodSO_4$ electrophoresis. Replicate gels were stained with Coomassie blue (*Left*) or transferred electrophoretically to nitrocellulose (*Right*) and developed by the immunoblot technique using anti-immunoglobulin alkaline phosphatase. The positions of molecular mass calibration standards are indicated.

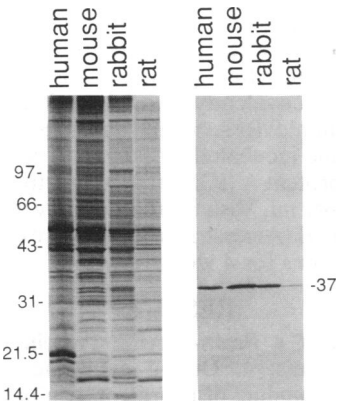

FIG. 2. Species distribution of 37-kDa antigen. Membranes from the cerebral cortex of various species were separated by $NaDodSO_4$ electrophoresis and stained with Coomassie blue (*Left*) or transferred to nitrocellulose and developed by the immunoblot technique using anti-A/NWS/33 antiserum (*Right*). Conditions were as for Fig. 1.

Jersey/11/76, and an antiserum against the purified hemagglutinin of A/Bellamy/42. Thus, of the 19 influenza A strains, the 4 that induced autoantibodies to the 37-kDa protein were the two neurotropic strains NWS (26) and WSN (27) and two other H1N1 viruses, all of which are human in origin. Although not an "early" H1 virus, A/New Jersey/76 "swine influenza" is antigenically related to the classical swine viruses responsible for the epidemics of this era (28). Notably, other H1N1 viruses, demonstrated by seroepidemiological studies to be less closely related to the 1918 strain (e.g., PR/8/34 and FM1/47) (29), did not induce autoantibody formation (Fig. 3).

Effect of Host Used for Virus Propagation on Autoantibody Induction. To determine whether autoantibodies were induced in response to virus *per se* or to a protein contaminant derived from the chicken embryo host, in which most viruses were grown, MDBK cells were used as an alternative host. (The 37-kDa antigen was not present in MDBK cells or in virus grown in this host system.) Thus, MDBK-grown WSN virus was also found to induce antibodies to the 37-kDa protein, even after formaldehyde fixation (data not shown), confirming our observation with the isolated Bellamy hemagglutinin that infection was not essential for the induction of autoantibodies.

Localization of Autoantigen in Brain. Autoradiographic analysis of the antigen in frozen sections of mouse brain (Fig. 4) showed that the antigen is located in regions containing a high density of neuronal cell bodies. Specific binding of antibody was very high in the dentate gyrus (granular cell layer) and in the hippocampus (pyramidal cell layer) and was also prominent in the superficial layers of the cerebral cortex and the cellular layer of the cerebellum. This pattern is very similar to that reported previously for various neuronal products, including receptors for insulin-like growth factor

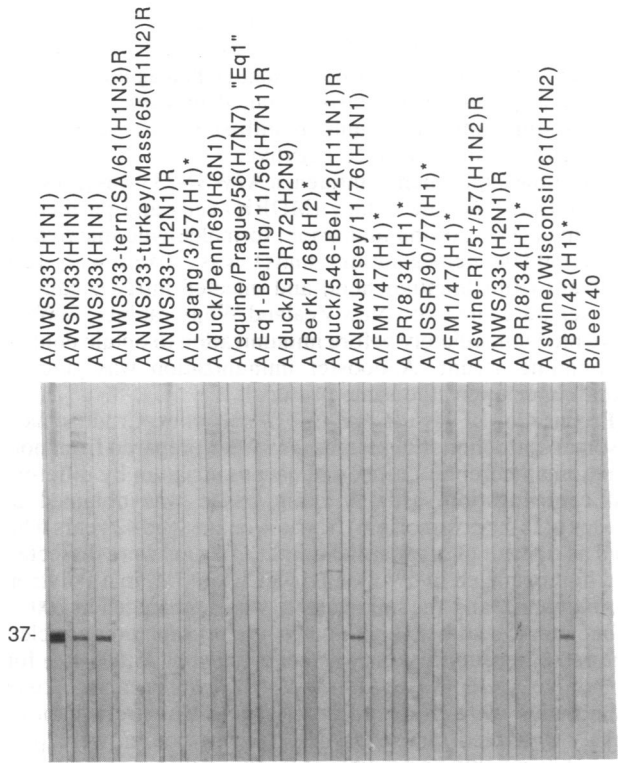

FIG. 3. Reaction of various anti-influenza sera with normal human brain. An immunoblot of human frontal cortex membranes was prepared and developed as for Fig. 1, except that protein A-alkaline phosphatase conjugate was used in place of the anti-immunoglobulin conjugate. Strips cut from the blot were probed with antisera against various influenza viruses including reassortant strains (R) and isolated hemagglutinins (*).

II, interleukin 1, and the surface antigen Thy-1 (30), and is also similar to the distribution of nerve growth factor β mRNA (31). The antigen distribution was strikingly different, essentially opposite, to that of myelin basic protein. This was particularly evident in the hippocampus and cerebellum, where different layers were stained (Fig. 4). Thus, the antigen appears to be neuronal in origin. When a larger number of antisera were tested, it became evident that the presence of antibodies to hippocampus assessed by autoradiography (Fig. 5) confirmed the results of immunoblotting (Fig. 3)—i.e., sera that were positive in one test were also positive in the other, suggesting that the same antigen is being recognized in both techniques.

Antigen Specificity of Virus-Induced Antibrain Autoantibody. Considering the high frequency of crossreaction of anti-viral antibodies with host tissues (>3%) (13), we investigated the possibility that the antibrain autoantibody was a

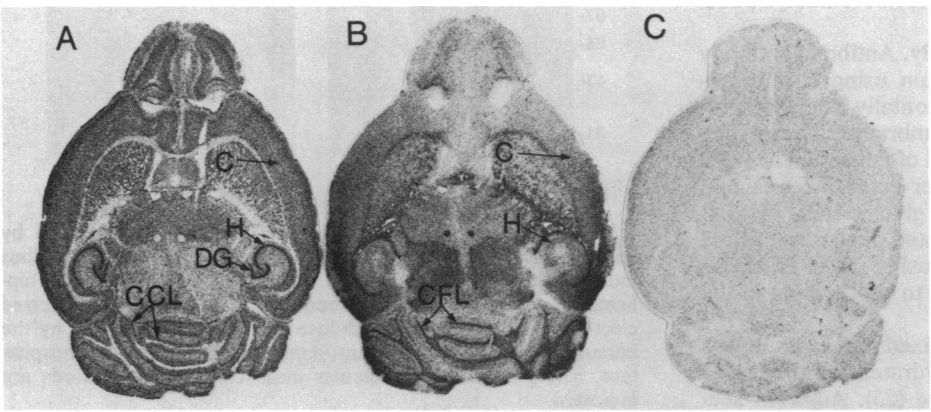

FIG. 4. Autoradiographic localization of antigen in mouse brain. Antisera were as follows: anti-A/NWS/33 (*A*), anti-myelin basic protein (*B*), and anti-A/PR/8/34 (*C*). Prominently labeled structures are indicated: C, cerebral cortex; H, hippocampus; DG, dentate gyrus; CCL, cellular layer of cerebellum; and CFL, fibrous layer of cerebellum.

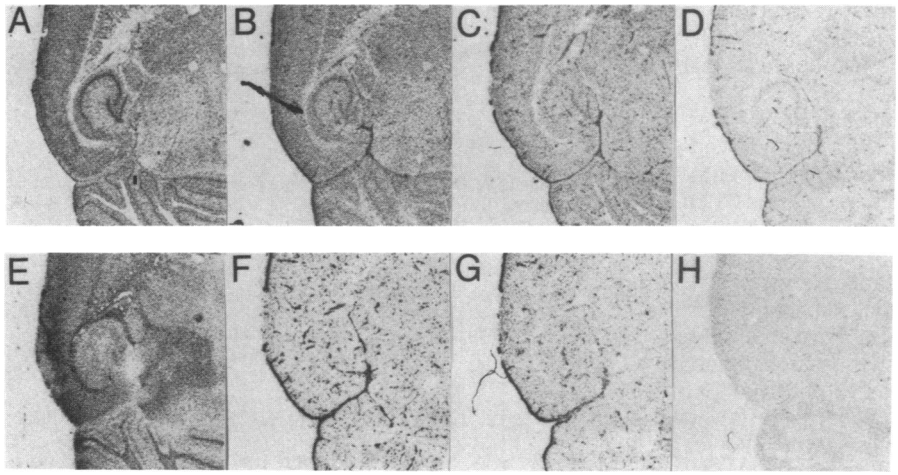

FIG. 5. Reaction of various anti-influenza antisera with hippocampus. Sera found to be positive by immunoblot analysis (*A–D*) were compared to controls (*E–H*). A, anti-NWS; B, anti-WSN; C, anti-New Jersey; D, anti-Bellamy hemagglutinin; E, anti-myelin basic protein; F, anti-B/Lee; G, anti-FM1 hemagglutinin; and H, anti-PR8.

crossreactive anti-viral antibody. When anti-NWS antiserum was tested for reaction with NWS virus, WS virus, and human frontal cortex membranes, it reacted strongly with all three as expected (Fig. 6). However, when antibrain autoantibody was affinity-purified from this antiserum, the purified antibody no longer reacted with either of the viruses, although it still reacted strongly with the 37-kDa brain antigen (Fig. 6). Also, antibrain autoantibody was not removed from the antiserum by adsorption with concentrated preparations of intact NWS virus (or ovalbumin). Attempts to immunoprecipitate ^{35}S-labeled virus proteins from detergent extracts of WSN-infected bovine kidney cells with antibrain antibody were similarly negative (data not shown). Thus, the virus-induced autoantibody against the 37-kDa brain protein does not demonstrably crossreact with virus under the conditions tested so far.

DISCUSSION

We have demonstrated that certain influenza A viruses, all of which are H1N1, can induce highly specific autoantibodies to a previously unknown brain protein. This protein is an autoantigen that is confined to gray matter in the brain, is found in human brain, and may therefore be relevant to a number of human diseases. However, because most viruses used in these studies were grown in embryonated chicken eggs, the antibrain antibodies we observed might result from contamination of viral preparations with chicken-derived proteins. For example, the 37-kDa antigen was also detected in chicken embryo brain but not in the chorioallantoic membrane or allantoic fluid (data not shown). Moreover, two of the four strains implicated in autoantibody induction are neurotropic (i.e., NWS and WSN), and these viruses may possibly have acquired neuronal antigens during growth in chicken embryo. Nevertheless, this hypothesis cannot explain why other strains of influenza virus (i.e., New Jersey/76 and Bellamy/42) also induced autoantibodies because there is no suggestion that these viruses are neurotropic. Nor does such a hypothesis readily explain why similar viruses prepared in the same way from embryonated eggs did not induce autoantibodies. Furthermore, the isolated hemagglutinin of A/Bellamy/42 induced antibrain autoantibody (Fig. 3), despite being purified under dissociating conditions in $NaDodSO_4$. Also, the 37-kDa antigen could not be detected in preparations of egg-grown virus representative of those that induced autoantibodies (Fig. 6). Finally, the induction of autoantibodies by MDBK-grown virus and the absence of 37-kDa antigen from MDBK cells strongly suggest that the autoantibodies are not due to a contaminant of the virus preparations. The induction of autoantibodies by isolated hemagglutinin and by fixed virus also excludes the possibility of crossreactivity with nonstructural proteins because these are produced only during infection with active virus.

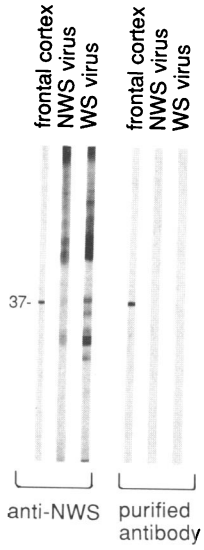

FIG. 6. Absence of reaction of isolated antibrain autoantibody with immunizing virus. Antibrain antibody was purified from anti-NWS antiserum using an antigen-bearing strip of nitrocellulose and was used to probe immunoblots of NWS virus, WS virus, and human frontal cortex membranes.

Considering that the isolated Bellamy hemagglutinin could induce autoantibodies, why reassortant strains carrying the hemagglutinin of the autoantibody-inducing NWS parental strain did not themselves evoke autoantibodies is therefore puzzling (Fig. 3). However, exhaustive trials might be necessary to establish whether any strain was definitively negative for autoantibody induction, and our results could be interpreted to mean that some strains are less able (rather than unable) to evoke autoantibodies. Also the hemagglutinins of the reassortant strains (though nominally the same) may differ antigenically from that of the NWS parental strain (32).

Considering the established ability of certain viruses to induce antibodies that crossreact with host proteins, we considered it particularly important to fully explore whether this mechanism was responsible for the production of autoantibodies to the 37-kDa antigen identified in this study; however, we were unable to obtain evidence in support of this concept. Thus, antibodies to the 37-kDa antigen did not crossreact with influenza virus proteins in a variety of test systems (i.e., immunoblot, immunoprecipitation, and preabsorption). Furthermore, antibodies to myelin were not detected (Figs. 4 and 5; P.L., unpublished immunoassay data), contrary to earlier predictions of antigenic mimicry based on sequence analogy between influenza virus proteins and myelin proteins (15, 16). Finally, we have tested 13 anti-WSN monoclonal antibodies (6 antihemagglutinin and 7 antinucleoprotein) for reaction with the 37-kDa antigen by immunoblotting; all were negative (unpublished observation). Nevertheless, antigenic mimicry cannot yet be excluded as a mechanism in the generation of this antibody because the

virus could become modified *in vivo* (e.g., proteolytically) in ways that were not reproduced by *in vitro* tests. Similarly, the property of crossreactivity could also be lost during clonal maturation of the antibody response (33).

Other mechanisms have been postulated to explain autoantibody formation by viruses. For example, the antireceptor autoantibodies (e.g., the anti-acetylcholine receptor autoantibodies of myasthenia gravis) have been suggested to be anti-idiotypes to virus antibodies (34). This concept has received experimental support for several viruses (35–38) but has not been shown to occur for influenza. However, active and inactivated influenza virus (PR/8) can induce erythrocyte autoantibodies in mice when administered with autologous erythrocytes (39), apparently by acting as an immunogenic carrier for an erythrocyte autoantigen. This latter finding illustrates the value of using polyclonal antisera to detect virus-induced autoantibodies, because autoantibodies of this type would not be represented among antiviral monoclonal antibodies.

To determine which of these mechanisms explains the induction of the autoantibodies described in our report requires further investigation. Formation of an immunogenic complex in the immunized rabbits between virus and the 37-kDa antigen is, superficially, an appealing idea. However, because the antigen was confined to brain, killed virus would (presumably) have little opportunity to form the putative complex, making the latter hypothesis unlikely. Further, the induction of anti-idiotypic antibodies that recognize virus receptors is less plausible for myxoviruses than for some other viruses because the receptor-binding site of the hemagglutinin is a pocket in the interior of hemagglutinin (40). Because this site is not accessible to antibodies, anti-idiotypes are therefore unlikely to resemble the hemagglutinin sufficiently to bind to influenza receptors. However, the autoantibodies we describe still could be anti-idiotypes of nonreceptor specificity. Despite our failure to obtain supportive evidence, the operation of antigenic mimicry seems the most probable mechanism to account for our findings.

Given epidemiological data linking neurological and psychiatric conditions with different epidemic strains of influenza [e.g., Reye syndrome (41); encephalitis (42, 43); psychosis (43, 44); parkinsonism (45, 46); and schizophrenia (47, 48)] and recent evidence suggesting that fetal exposure to influenza A may increase the risk of developing both schizophrenia (49–52) and Parkinson disease (53) in later life, virus-initiated autoimmune reactions (similar to that described here) may contribute to the pathogenesis of these disorders (54–56).

Whether autoantibodies to the 37-kDa protein occur after *infection* with influenza remains to be established. Nevertheless, the brain-specific localization of this protein and its autoantigenic nature suggest that this protein could be involved in the pathogenesis of central nervous system disorders that may have an autoimmune basis, including postinfluenzal syndromes.

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