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Monoclonal Antibodies to the Influenza A Virus Nucleoprotein Affecting RNA Transcription

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Monoclonal antibodies were used to map the antigenic domains of the A/WSN/33 (H0N1) influenza virus nucleoprotein. Three nonoverlapping antigenic regions of the nucleoprotein were identified by using competitive-binding enzyme-linked immunosorbent assays. Monoclonal antibodies to two nucleoprotein domains inhibited in vitro transcription of viral RNA, suggesting that these specific regions of the nucleoprotein are topographically or functionally involved in RNA transcription.

The influenza A virus nucleoprotein (NP) is a major internal component of the virion, comprising approximately 20% of the total viral protein. In both infected cells and mature virions, the NP is located in ribonucleoprotein (RNP) complexes, which are composed of viral RNA, NP, and three polymerase proteins. It has been demonstrated that RNP complexes have transcriptase activity and that the NP is an integral part of the functional RNP complex (2). Furthermore, studies with A/WSN/33 (H0N1) mutants with temperature-sensitive lesions in the NP gene (17, 18) suggest that the NP is an essential component of transcriptase complexes (7).

The NP was originally characterized as a type A-specific, antigenically invariant protein (13). However, recent serological studies of the NP, using either heterogeneous antiserum (14) or monoclonal antibodies (19), demonstrated that it does undergo antigenic variation. We recently produced monoclonal antibodies to five different antigenic determinants on the NP molecule of the A/WSN/33 (H0N1) influenza virus and demonstrated that four of these determinants are antigenically variable in other influenza A viruses (19).

To extend our analysis of the NP, we examined the distribution of determinants on the NP molecule and compared the involvement of each of the NP determinants in in vitro RNA transcription. Competitive-binding enzyme-linked immunosorbent assays (ELISAs) indicated that the NP possesses a minimum of three nonoverlapping antigenic domains, one of which is composed of at least three individual determinants. Monoclonal antibodies to two NP domains inhibited in vitro transcription of viral RNA, suggesting that these particular domains are either functionally or topologically involved in the synthesis of influenza virus messenger RNA.

Hybridoma cell lines were produced by the methods of Kohler and Milstein (6). Cell lines secreting antibodies directed to five distinct determinants on the influenza virus NP were identified by radioimmunoprecipitation and ELISA, as described previously (19). ELISA tests of these antibodies with NP purified by polyacrylamide gel electrophoresis, and also with disrupted X37 and X47 recombinant influenza viruses (1), eliminated the possibility that any of the antibodies were directed to influenza virus P1, P2, or P3 proteins (data not shown).

Hybridoma cell lines were grown as ascites tumors in pristane-primed BALB/c mice. Purified monoclonal antibodies were obtained by chromatography of 0.1 ml of ascites fluid on 1.0-ml columns of *Staphylococcus aureus* protein A-conjugated Sepharose 4B (Pharmacia) (4). Elution of bound immunoglobulins was in glycine buffer (pH 3.0). Immunoglobulin concentrations were determined by the method of Lowry et al. (8).

Competitive-binding ELISAs were performed to investigate the distribution of five distinct antigenic determinants on the influenza A virus nucleoprotein. These experiments were designed to test the ability of each of the five monoclonal antibody preparations to sterically inhibit the binding of antibodies to four other determinants on the NP molecule. Monoclonal antibodies to the influenza A virus NP were conjugated to horseradish peroxidase according to the method of Nakane and Kawaoi (9). Before competitive-binding assays, each conjugated antibody preparation was titrated by direct ELISA. This was done by incubating dilutions (50 μl) of conju-

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gates in individual wells of assay plates for 30 min. After washing to remove unbound conjugated antibodies, 100 μl of substrate solution (0.05 M citrate buffer [pH 4.0] containing 0.008% hydrogen peroxide and 40 mM azino-di-3-ethylbenzothiazoline-6-sulfuric acid) was added. Optical density of the resulting mixture at 405 nm was read on a multichannel photometer. In this manner, the antibody concentration required to saturate all available NP binding sites was determined for each conjugated antibody preparation.

Competitive-binding ELISA tests were performed using mixtures containing a fixed concentration (25 μl) of conjugated monoclonal antibodies (determined as above) and increasing concentrations (25 μl) of unconjugated, protein A-purified monoclonal antibodies. Mixtures (50 μl) were added to wells of ELISA plates and assayed as described above. The results of these experiments are illustrated by competitive binding curves (Fig. 1). The binding of two of the conjugated antibodies, 5/1 and 7/3 (Fig. 1D and E), was inhibited only by homologous antibody preparations, indicating that each recognizes an antigenic determinant which is physically distinct from the other four NP epitopes. In contrast, a group of three monoclonal antibodies, 3/1, 469/6, and 150/4 (Fig. 1A, B, and C), exhibited competitive binding with each other. The results of these assays indicate that the influenza A virus NP possesses at least three nonoverlapping antigenic domains. One of these domains is composed of three distinct epitopes, all of which have been shown to vary independently of each other in influenza field isolates (19). The other two domains are currently defined by single antigenic determinants.

The NP is an integral part of the transcriptase complex associated with influenza virions (2, 12), but its function during RNA synthesis and its physical relationship with other proteins in the transcribing complex are unclear. To approach these questions, we examined the effects of monoclonal antibodies to the NP on in vitro RNA synthesis by A/WSN/33 (H0N1) influenza virions. Increasing concentrations of each of the five protein A-purified monoclonal antibody preparations were added to standard RNA transcriptase reaction mixtures (10). Reactions were stopped after 15 min, and radioactivity incorporated into newly synthesized RNA was measured. The results (Fig. 2) indicated that antibody 7/3 had no effect on the RNA polymerase reaction. In contrast, antibody 5/1 caused a 80% reduction in the amount of RNA synthesized. Antibodies 3/1, 150/4, and 469/6 also inhibited RNA transcription, although they did so less effectively than antibody 5/1. This inhibition was not due to RNase contamination of the antibody preparations; inhibition of the B/Hong Kong/77 influenza virus transcriptase was not observed (data not shown). Kinetic studies (Fig. 3) demonstrated that addition of antibodies to virions before initiation of transcription or to transcribing virions at intervals after initiation produced comparable and immediate reduction in the rate of RNA synthesis. These results, analogous to those obtained with antibodies to N protein of vesicular stomatitis virus (3), suggest that anti-NP antibodies inhibit elongation of nascent RNA, although the possibility also exists that they prevent initiation of RNA synthesis. These data differ from those of Scholtissek et al. (16), who reported that heterogeneous antiserum to influenza NP did not inhibit RNA synthesis. The most likely explanation for this difference is that their antiserum did not contain an effective concentration of antibodies directed to the same NP epitopes recognized by our monoclonal antibodies.

The mechanism whereby anti-NP antibodies inhibit RNA synthesis is unknown. One possibility is that antibodies interfere directly with NP function. Although the precise function of NP is unclear, several lines of evidence suggest that NP is involved in influenza virus replication. For example, rescue experiments with temperature-sensitive mutants have shown that NP is involved in virion RNA synthesis (11). Others have shown that mutants possessing lesions in the NP gene are defective in RNA transcription at nonpermissive temperature (7). Furthermore, it is well documented that NP has a high binding affinity for influenza virus RNA (15) and is an integral component of functional transcriptase complexes (12). Since it appears likely that NP is an important cofactor in transcriptase activity, it is conceivable that the inhibition of RNA synthesis is due to direct inhibition of NP function. Alternatively, anti-NP antibodies could indirectly inhibit RNA synthesis by steric hindrance. For example, antibodies bound to NP might distort the helical NP-RNA structure or prevent the interaction of NP with polymerase enzymes, resulting in less efficient RNA synthesis. On the other hand, these antibodies might sterically block either the binding or enzymatic site(s) of polymerase enzymes in a manner analogous to the steric hindrance of influenza virus hemagglutination by anti-neuraminidase antibodies (5).

Antibodies to three NP domains clearly differ in their ability to inhibit RNA transcription. This difference is not due to the immunoglobulin class of individual monoclonal antibodies; all of

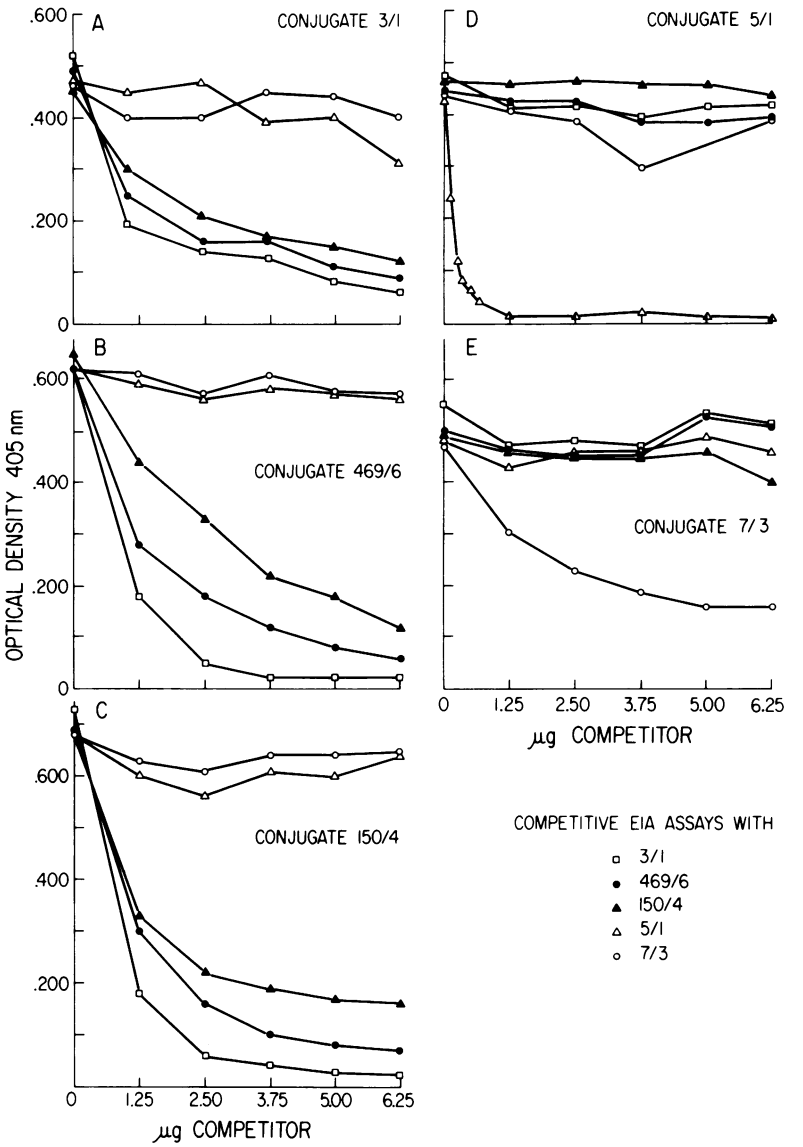

FIG. 1. *Determination of the number of nonoverlapping antigenic NP domains by competitive-binding ELISA. Fixed concentrations of conjugated antibodies were mixed with increasing concentrations of purified unconjugated antibody preparations. These mixtures were tested in direct ELISA with A/WSN/33 (H0N1) virus as described in the text.*

the antibodies are of the immunoglobulin G1 or $G2_a$ subclass (data not shown). Rather, our results suggest that only antibodies directed to specific NP domains inhibit RNA synthesis. The different levels of inhibition produced by antibodies to distinct NP domains could be an effect of individual antibody affinity, or could reflect the degree to which topographically different domains of NP affect transcription. Further experiments are necessary to discriminate between these possibilities. The data also suggest that the epitope corresponding to antibody 5/1 may represent an NP domain which is important for either structural or functional integrity of transcribing RNPs and may account for the apparent conservation of this epitope in influenza A viruses (19).

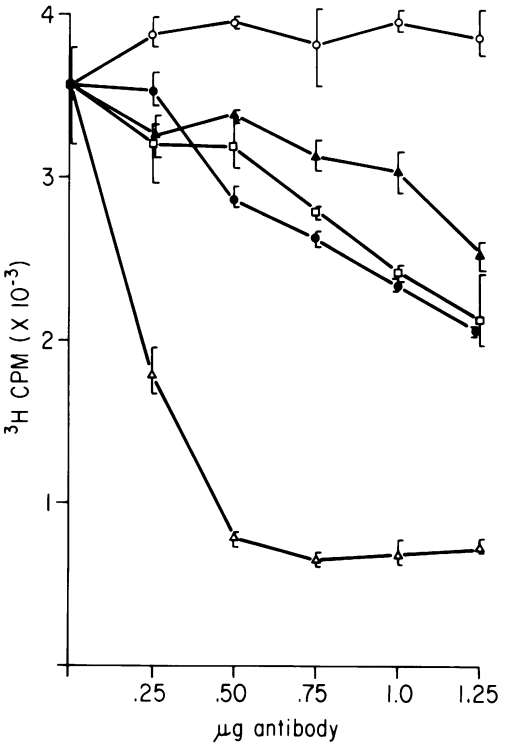

FIG. 2. *Inhibition of transcription by monoclonal antibodies to NP. Increasing concentrations of protein A-purified monoclonal antibodies (5 μl) were added to 40 μl of reaction mixture A (64 mM Tris-hydrochloride [pH 8.0], 80 mM NaCl, 0.15% Triton X-100, 0.8 mM dithiothreitol, and 8.4 μg of purified A/WSN/33 [H0N1] influenza virus) and incubated at 4°C for 15 min. The transcriptase reaction was started by the addition of 5 μl of reaction mixture B (0.8 mM $MgCl_2$; 0.64 mM ATP, GTP, and CTP; 0.04 mM UTP; 0.4 mM adenyl-3',5'-guanosine [Boehringer Mannheim]; and 2 μCi of 5'-[^{3}H]UTP [New England Nuclear Corp.]). Reactions were stopped on ice after 15 min, and RNA was precipitated by the addition of trichloroacetic acid. Precipitates were washed three times with trichloroacetic acid and radioactivity was quantitated. Each point represents the average of three experiments; bars show the range of individual measurements. Symbols: □, antibody 3/1; ●, 496/6; ▲, 150/4; △, 5/1; and ○, 7/3.*

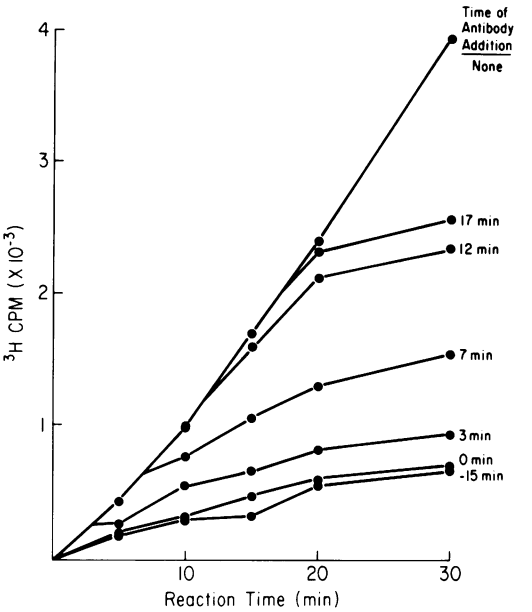

FIG. 3. *Kinetics of inhibition of in vitro transcription by anti-NP monoclonal antibodies. Antibody 5/1 was added to individual transcriptase reaction mixtures at the indicated time intervals before or after initiation of viral RNA synthesis. [^{3}H]UTP incorporation into viral RNA was determined at the time of initiation and at 5, 10, 15, 20, and 30 min thereafter.*

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