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# Hemagglutinin Mutations Related to Attenuation and Altered Cell Tropism of a Virulent Avian Influenza A Virus

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**The H5 hemagglutinin (HA) of a highly virulent avian influenza virus, A/Turkey/Ontario/7732/66 (H5N9), was previously shown to have five neutralizing epitopes, and escape mutants within one epitope (group 1) were markedly attenuated (M. Philpott, B. C. Easterday, and V. S. Hinshaw, J. Virol. 63:3453–3458, 1989). To define the genetic changes related to these antigenic and biologic properties, the HA genes of mutants within each of the epitope groups were sequenced by using the polymerase chain reaction. The mutations in the attenuated group 1 mutants were located near the distal tip of the HA molecule in close proximity to the receptor-binding site, on the basis of alignment with the three-dimensional structure of the H3 HA. All group 1 mutations involved charged amino acids. The group 1 mutants, similar to the wild-type virus, spread systemically and were recovered from the spleens of infected chickens but, unlike the wild-type virus, failed to produce severe necrosis in the spleens. Viral replication in the spleens was investigated by in situ hybridization of spleen sections from chickens infected with the wild-type or attenuated mutants. Wild-type virus replication was demonstrated in large, mononuclear, macrophagelike cells; however, group 1 mutant virus was detected attached only to erythrocytes within the red pulp. These results suggest that the attenuated mutants differ in their cell tropism within the spleen.**

Highly virulent avian influenza viruses are an excellent system for the elucidation of mechanisms of virulence and are economically important to the poultry industry (1). Virulence is known to involve the interaction of many viral genes, but studies have demonstrated a pivotal role for viral hemagglutinin (HA) (for a review, see reference 32). One way HA contributes to severe disease is to promote the systemic spread of virus, which is primarily determined by the presence of multiple basic amino acids at the carboxyl terminus of the $HA_1$ polypeptide (4, 5, 8, 15). HAs with basic amino acids at this location are efficiently cleaved by host cell proteases (2, 4, 5, 15, 16), which is thought to allow the production of infectious virus in many tissues. Other contributions to influenza virus pathogenicity provided by HA are not well understood.

During an investigation into the number and distribution of neutralizing epitopes on the H5 HA, we observed the following: (i) the H5 HA has five neutralizing epitopes, (ii) all of the escape mutants selected with monoclonal antibodies in one epitope group (designated group 1 mutants) were attenuated relative to the wild-type virus, and (iii) attenuation of these mutants was unrelated to the cleavage properties of their HAs (23). Unlike other highly virulent avian influenza viruses, the wild-type A/Turkey/Ontario/7732/66 (H5N9) (Ty/Ont) produces severe necrosis of lymphoid organs, especially the spleen (30). The group 1 epitope of the H5 HA from Ty/Ont is a strain-specific epitope (23). Furthermore, group 1 mutants failed to produce necrosis of the spleen (23). Therefore, it was of interest to locate the genetic changes responsible for the loss of antibody binding associated with these phenotypes and to investigate the cell types infected in the spleens by examining the distribution of viral RNA.

In this paper, the nature and location of the attenuating mutations for group 1 mutants, as well as the changes in mutants representing the other four epitopes on the H5 HA, are identified. These data allowed construction of the first epitope map for the H5 HA of a virulent avian influenza virus. The distribution of viral RNA within the spleens of infected chickens was investigated by in situ hybridization. The results suggest that group 1 mutants have an altered cell tropism within the spleen and that this may be due to mutations in close proximity to the receptor-binding site of HA.

## MATERIALS AND METHODS

**Virus growth and purification.** The virus Ty/Ont was obtained from the original outbreak of disease (19) and was passaged twice in embryonated chicken eggs. The isolation of escape mutants of the wild-type Ty/Ont virus has been described (23). Viruses were propagated in the allantoic cavities of 11-day-old embryonated chicken eggs for 30 h at 35°C. The allantoic fluid was collected and clarified by centrifugation at 16,000 × *g* for 30 min. Polyethylene glycol (MW 8000; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 8% (wt/vol), and the fluid was stirred for 1 h on ice. The precipitate was collected by centrifugation at 16,000 × *g* for 30 min. The pellet was suspended in STE buffer (0.1 M NaCl, 50 mM Tris hydrochloride [pH 7.2], 1 mM EDTA) and pelleted through a pad of 30% (wt/wt) sucrose in STE at 35,000 × *g* for 2 h in an SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The final pellet was suspended in STE with 100 U of RNasin (Promega Biotec, Madison, Wis.) per ml. Viral RNA was extracted from virions as described previously (13).

**Molecular cloning of H5 HA.** All primers used in these experiments were prepared by the University of Wisconsin Biotechnology Center. First-strand cDNA synthesis was carried out at 42°C for 45 min in a 40-μl reaction volume containing 2 μg of freshly boiled viral RNA; 2 μg of primer; 50 mM Tris hydrochloride (pH 8.3); 75 mM KCl; 10 mM $MgCl_2$; 0.5 mM spermidine; 10 mM DL-dithiothreitol; 60 U of

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RNasin; 1 mM (each) dCTP, dGTP, dATP, and dTTP; and 10 U of avian myeloblastosis virus reverse transcriptase (Pharmacia LKB, Piscataway, N.J.) diluted as described elsewhere (17). An additional 5 U of reverse transcriptase was then added, and the reaction was incubated for 15 min at 50°C. Second-strand synthesis was carried out in a 100-μl reaction volume containing 1 μg of reverse primer; 20 mM Tris hydrochloride (pH 7.5); 5 mM $MgCl_2$; 100 mM KCl; 200 μM (each) dCTP, dGTP, dATP, and dTTP; and 20 U of polymerase I Klenow fragment (New England BioLabs, Inc., Beverly, Mass.) for 1 h at 37°C.

The cDNA synthesis products were blunt-end ligated into the *Sma*I site of pUC18 (36) in a 15-μl reaction volume containing 150 ng of *Sma*I-digested pUC18 treated with alkaline phosphatase (20), 66 mM Tris hydrochloride (pH 7.5), 6.6 mM $MgCl_2$, 10 mM DL-dithiothreitol, 0.4 mM ATP, 200 μg of bovine serum albumin per ml, and 2,000 U of T4 ligase (New England BioLabs) overnight at 16°C. The products were transformed into *Escherichia coli* JM107 (36), essentially as described previously (12), and selected on B medium (22) supplemented with 50 μg of ampicillin per ml, 20 μg of isopropyl-β-D-thiogalactopyranoside per ml, and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. Clones with HA inserts were identified by hybridization to $^{32}P$-labeled cDNA prepared as described above with an H5-specific primer in the presence of 10 μM unlabeled dCTP and 100 μCi of [α-$^{32}P$]dCTP (Amersham Corp., Arlington Heights, Ill.).

**Molecular analysis of escape mutants.** Single-stranded cDNA of each mutant was prepared as described above for the wild-type virus, and the RNA was removed by hydrolysis at 65°C in 0.2 M NaOH for 45 min. One-tenth of the cDNA was used as a template for the polymerase chain reaction (PCR) (26), which was done in a 100-μl volume containing 10 mM Tris hydrochloride (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 200 μM (each) dCTP, dATP, dGTP, and dTTP; 1 μM (each) forward and reverse primers; and 2.5 U of *Thermus aquaticus* DNA polymerase (*Taq* polymerase; United States Biochemical Co., Cleveland, Ohio). Cycle 1 was as follows: 5 min at 94°C, 1 min at 45°C, and 30 min at 72°C. This was followed by 39 cycles of 2 min at 94°C, 1 min at 45°C, and 4 min at 72°C and a final incubation at 72°C for 10 min. The PCR products were visualized on 1.5% SeaPlaque (FMC Corp., Rockland, Maine) agarose gels and recovered from melted gel slices by phenol extraction and ethanol precipitation. The recovered products were treated for 2 h at 37°C with 15 U of polynucleotide kinase (New England BioLabs) in a 50-μl reaction volume containing 50 mM Tris hydrochloride, (pH 7.6), 10 mM $MgCl_2$, 5 mM DL-dithiothreitol, 0.1 mM spermidine, and 0.4 mM ATP. After phenol extraction to remove residual kinase activity, the products were blunt-end ligated into pUC18 and screened as described above, except the hybridization probe was a transcript prepared from a 1,084-base-pair cDNA spanning the entire $HA_1$-coding sequence of the wild-type Ty/Ont cloned into pSP64 or pSP65 (21).

**Sequence analysis.** Plasmid DNA was prepared from clones by a minilysis method (20). The double-stranded DNA was denatured by treatment with 0.2 M NaOH at 37°C for 20 min, neutralized, and ethanol precipitated. A sample of this material was sequenced on both strands with a Sequenase kit (United States Biochemical Co.). Sequence data were stored and manipulated by using the programs developed by the University of Wisconsin Genetics Computer Group (9). Alignment with H3 HA was done by first generating a best fit of the translation products of H5 and H3, followed by three-dimensional alignment with an Evans and Sutherland model PS390 interfaced with a MicroVax 2000 computer. Output was generated on a Macintosh computer using the Mac Frodo program generously provided by Tom Smith, Purdue University, West Lafayette, Ind.

**In situ hybridization.** Plasmid pSP65/501m, which contains a cDNA clone of the nucleoprotein gene from influenza virus A/Puerto Rico/8/34, was kindly provided by M. Krystal and P. Palese, Mount Sinai School of Medicine, New York, N.Y., and was digested with *Bam*HI to generate a transcript of the appropriate size for in situ hybridization. In vitro transcription with SP6 RNA polymerase was carried out essentially as described elsewhere (21), except it was in the presence of 400 μCi of [α-thio-$^{35}S$]UTP (Amersham).

In situ hybridization was performed on paraffin-embedded spleen sections by the method of Haase (11), with minor modifications. Permeabilization of the sections with digitonin was not required, and the hybridization solution contained 50% formamide, 0.6 M NaCl, 10 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, and 1× Denhardt solution, to which was added 50 μg each of single-stranded carrier DNA and *Saccharomyces cerevisiae* RNA per ml. Prehybridization was carried out for 4 h at 37°C; then $2 \times 10^5$ cpm of probe in the same solution supplemented with 0.1% Triton X-100 was added, and the sections were incubated at 37°C for 72 h. Slides were washed four times for 30 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 1 mM EDTA and 0.1% Triton X-100 and then washed for 30 min in 50% formamide–0.3 M NaCl–5 mM Tris hydrochloride (pH 7.4)–0.5 mM EDTA. Slides were then washed for 45 min in 2× SSC, followed by four 30-min washes in 0.6 M NaCl–10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA. Sections were dehydrated in ethanol series with 0.3 M ammonium acetate, air dried, coated with emulsion (Ilford, Cheshire, United Kingdom), and exposed at 4°C for 1 week. The slides were developed in the dark for 8 min, rinsed, fixed for 5 min, rinsed, and stained with hematoxylin.

## RESULTS

**Sequence of H5 HA of Ty/Ont.** To determine the sequence of the H5 HA from Ty/Ont, cDNA was prepared and cloned in pUC18 by using primers complementary to the universal 12 (3′) and 13 (5′) nucleotides of all influenza A viruses (27). Clones containing HA inserts were initially identified by hybridization to probes prepared by reverse transcription of viral RNA by using an H5 primer. No full-length clones were obtained. The sequence of the HA (GenBank M30122), obtained from several overlapping clones, is presented in Fig. 1. Following established convention, the DNA sequence of the plus strand is shown, along with the predicted amino acid sequence of the translation product from mRNA.

The H5 HA gene segment of Ty/Ont was 1,773 nucleotides in length and coded for a predicted protein of 566 amino acids, with 343 amino acids in the $HA_1$ polypeptide and 223 amino acids in the $HA_2$ polypeptide. Like other virulent avian influenza viruses (4, 15), Ty/Ont has multiple basic amino acids at the carboxyl terminus of $HA_1$ (Fig. 1). The percentage of overall sequence identity with other virulent H5 influenza viruses is 90.8% with A/Chicken/Pennsylvania/83 (14), 80.4% with A/Turkey/Ireland/84 (15), and 83.1% with A/Chicken/Scotland/59 (7).

**Molecular analysis of escape mutants.** Sequence data from the wild-type Ty/Ont were used to design primers with which to amplify the $HA_1$ region of each of the escape mutants by PCR (26). Amplification of the entire $HA_1$ in a single

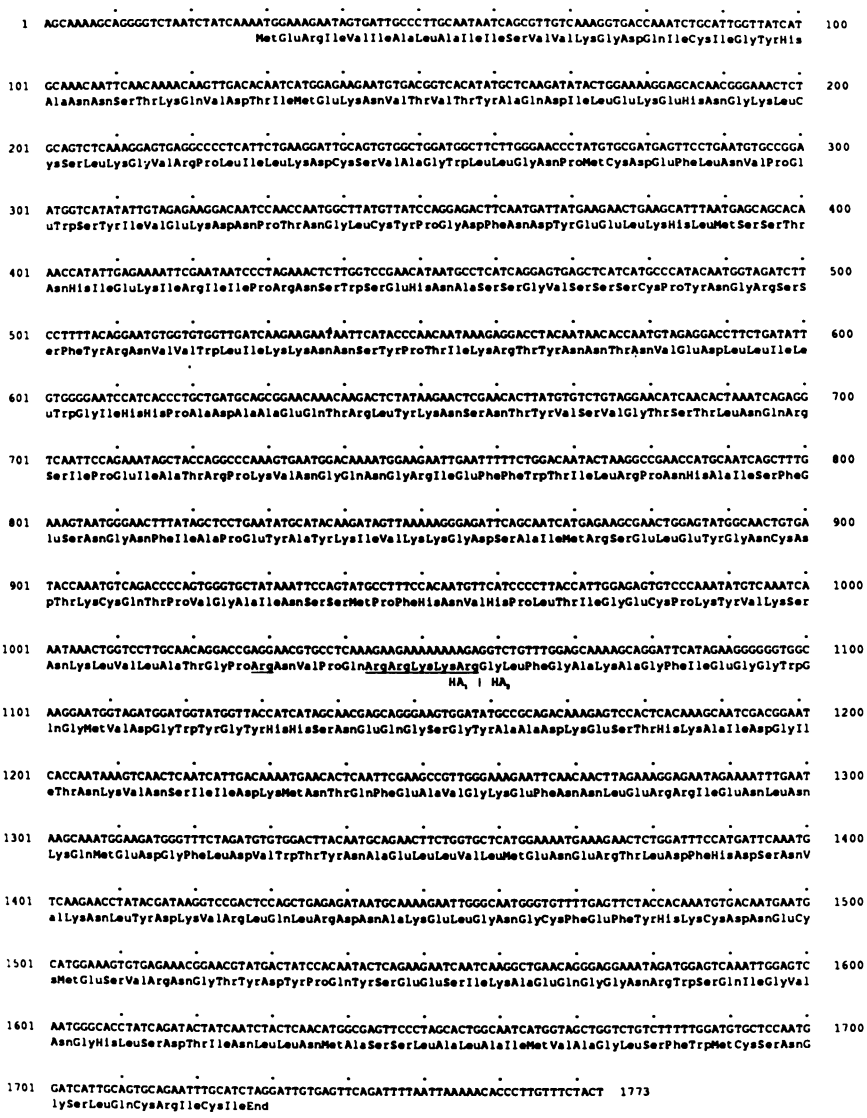

FIG. 1. Nucleotide sequence of the H5 HA gene segment from Ty/Ont. The figure displays the DNA sequence of the coding sense plus strand and the deduced amino acid sequence of H5 HA. The location of the cleavage by trypsinlike enzymes into the $HA_1$ and $HA_2$ polypeptides is marked, and the basic amino acids which participate in the recognition of the cleavage site (4, 16) by those enzymes are underlined.

fragment from cDNA was not achieved, possibly because of the extensive secondary structure in the $HA_1$ region of the gene, as indicated by computer analysis of the most favorable RNA structure (for nucleotides 1 to 1200; free energy, $\Delta G = -289$ kcal/mol [1 cal = 4.184J]). To circumvent this difficulty, the $HA_1$ of each mutant was amplified in three separate overlapping fragments: nucleotides 11 to 436, 361 to 709, and 600 to 1046. Of these three fragments, only the first could be amplified in the absence of RNA hydrolysis. Direct sequencing of linear PCR products recovered from gel slices was attempted, but the resolution of all bases was not possible from these templates. To obtain the complete and unambiguous sequence, PCR products were cloned into pUC18 plasmid DNA, and 5 to 10 clones from each fragment were sequenced in both directions. The products from several of the PCRs contained mutations presumably introduced by *Taq* polymerase during amplification (18). Such mutations were readily distinguishable from the mutations of interest, as they were not conserved among clones from the same reaction. The overall average frequency of mutations introduced by *Taq* polymerase in these experiments was 0.34%, or 1 of 294 bases. However, the frequency varied greatly among reactions from a high of 1 of 175 bases to a low of less than 1 of 700 bases (data not shown).

The mutations located for each of the eight escape mutants, representing five epitope groups (23), are shown in Table 1 and graphically represented by alignment with the three-dimensional structure of H3 HA in Fig. 2. Three of the four attenuated group 1 mutants had the same mutation at amino acid 168, a lysine-to-glutamic acid change. The fourth mutant in this group, 562G2v, had an arginine-to-isoleucine shift at amino acid 205. These two regions of the primary structure folded up to close proximity in the three-dimensional structure (Fig. 2).

Three of the mutants (42C3v and 67G1v from group 1 and 4F10v from group 3) had an additional mutation at amino

TABLE 1. Mutations identified in escape mutants of Ty/Ont representing five epitope groups[a]

| Group | Mutant | Nucleotide[b] | Nucleotide change[c] | Amino acid | Amino acid change |
|---|---|---|---|---|---|
| 1 | 77B1v | 530 | A to G | 168 | Lys to Glu |
| 1 | 56G2v | 642 | G to T | 205 | Arg to Ile |
| 1 | 42C3v | 530 | A to G | 168 | Lys to Glu |
| | | 620 | G to A | 198 | Ala to Thr |
| 1 | 67G1v | 530 | A to G | 168 | Lys to Glu |
| | | 620 | G to A | 198 | Ala to Thr |
| 2 | 4C9v | 233 | A to G | 69 | Lys to Glu |
| 3 | 4F10v | 182 | G to A | 52 | Glu to Lys |
| | | 620 | G to A | 198 | Ala to Thr |
| 4 | 76E1v | 420 | G to A | 131 | Arg to Gln |
| 5 | 24B9v | 497 | T to C | 157 | Ser to Pro |

[a] Details of the serological definition of the five epitopes have been described previously (23).
[b] As shown in Fig. 1.
[c] Changes are presented as the plus strand of DNA by established convention.

acid 198. This change (labeled ungrouped in Fig. 2) does not correlate with the serological grouping of the mutants (23) and therefore probably reflects heterogeneity in the original, uncloned stock of Ty/Ont from which the escape mutants were selected. Further evidence for such heterogeneity is provided by the finding of a silent C-to-T transition at nucleotide 277 which was present in five out of eight of the mutants.

**In situ hybridization.** To determine the location of viral RNA in vivo, spleens were examined by in situ hybridization by using an $^{35}S$-labeled RNA probe generated from an influenza virus nucleoprotein gene. Figure 3 shows photomicrographs of two spleens which had identical titers of virus ($10^{4.8}$ 50% egg infective doses per g) but were infected with the wild-type virus or mutant. The wild-type Ty/Ont virus appeared to replicate extensively in focal areas of the spleen. Of particular interest was the presence of large mononuclear cells which retained the probe (Fig. 3a). In contrast, cells such as these were not observed in any of four spleens examined by in situ hybridization and collected from chickens infected with the group 1 mutant 77B1v. In these spleens, viral RNA appeared to be primarily associated with erythrocytes found in the red pulp areas of the spleen (Fig. 3b).

## DISCUSSION

The HA genes of Ty/Ont and mutants from each of the five neutralizing epitopes were characterized genetically. Analysis of H5 HA from Ty/Ont indicates greater identity with the A/Chicken/Pennsylvania/83 virus than with other virulent H5 viruses from the British Isles, consistent with the proposal of two lineages for such viruses (15).

The amino acid changes found in the mutants were approximated on the three-dimensional structure of HA by alignment with H3 HA (35). The results indicate that three of the neutralizing epitopes of Ty/Ont correspond closely with those of human H3 HA (34): group 1 mutations are either in antigenic site B (56G2v) or between sites A and B (77B1v, 42C3v, and 67G1v), the group 5 mutation of 24B9v is located within site A, and the group 2 mutant 4C9v has a change within site E (3). However, the other two epitopes of H5 do not clearly correlate with those of H3, although the group 3 mutant 4F10v was found to have a change only 6 amino acids from Cys-52, which participates in the formation of antigenic

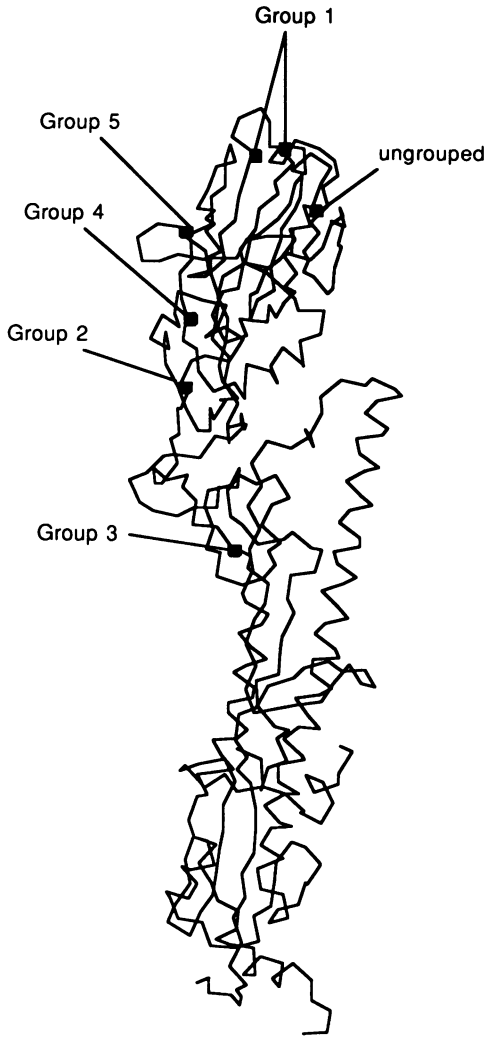


FIG. 2. Alignment of mutations identified in the escape mutants of Ty/Ont with the three-dimensional structure of H3 HA. The alignment procedure is described in the text. The amino acid changes are marked and labeled as groups 1 to 5, with each group defining a separate neutralizing epitope on the H5 molecule (23). The mutation labeled ungrouped did not correlate with B-cell recognition as described in the text. The amino acid positions of the mutations after alignment with the H3 structure are as follows: group 1, 156 and 193; group 2, 62; group 3, 46; group 4, within a 3-amino-acid gap in the alignment between 119 and 120 (120 is marked on the figure); group 5, 145; ungrouped, 186.

site C of H3 (34). Overall, the results of alignment of these two HAs suggest that although they share many common features, they are not recognized by murine B lymphocytes in an identical manner.

Because of the marked attenuation of group 1 mutants relative to that of the wild-type Ty/Ont, it was of particular interest to locate the amino acid changes in these mutants. There are several possible explanations for the attenuation of group 1 mutants. We have ruled out two obvious possibilities: inefficient cleavage of HA (23) and temperature sensitivity (M. Philpott and V. S. Hinshaw, unpublished data). Interestingly, the changes found in group 1 mutants clustered in close proximity to the receptor-binding region after alignment with the three-dimensional structure of H3

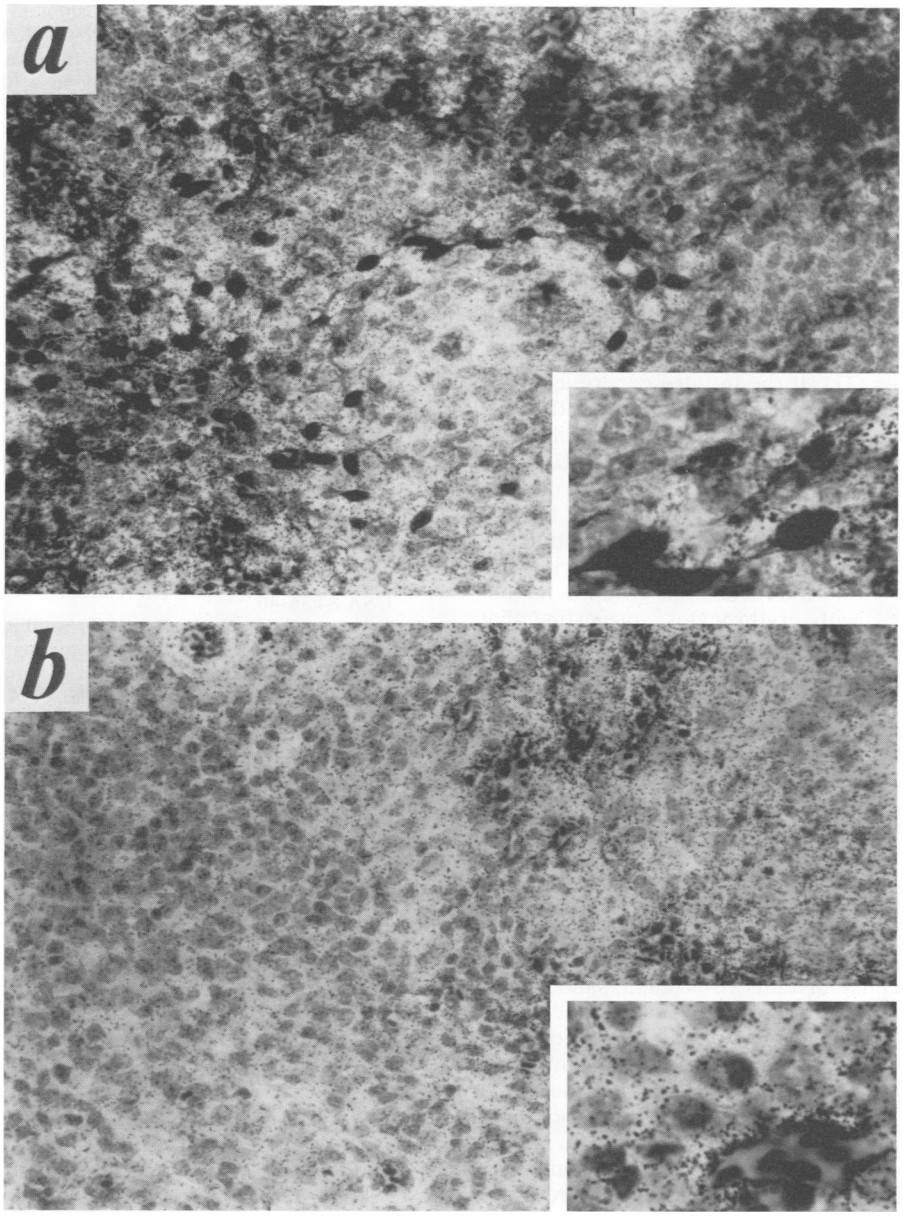


FIG. 3. In situ hybridization of spleen sections from chickens infected with wild-type Ty/Ont showing grains within large mononuclear cells in the vicinity of expanding regions of necrosis (a) or the group 1 mutant 77B1v showing grains on the surface of erythrocytes (b). Magnification, ×40; inserts of selected cells in the spleens, ×100.

HA. The cellular receptor for influenza A viruses has not been identified, but sialic acid residues complex with the receptor-binding pocket of HA (33) and are probably important to the initial virus-cell interactions. One might speculate that such interactions are altered by changes in the three-dimensional structure or local charge on the HA molecule. In this regard, it is striking how many of the changes involved charged amino acids. Changes from a basic residue to an acidic one were most common (in 77B1v, 42C3v, 67G1v, and 4C9v), with one change of an acidic residue to a basic one (in 4F10v) and two changes of basic residues to uncharged ones (in 76E1v and 56G2v). Since the changes in all four of the group 1 mutants involved charged residues and because of their proximity to the receptor-binding region, it is tempting to speculate that such shifts in charge influence the receptor-binding properties of the molecule and the cell type infected by the virus. In our previous study (23), each of the four group 1 mutants, 77B1v, 56G2v, 42C3v, and 67G1v, was reduced in virulence compared with the wild-type virus, producing mortalities of 30, 20, 80, and 70%, respectively. These variations in mortality among the mutants are not clearly explained by the results of the present study. Possible explanations are variations of reversion rates in vivo (i.e., revertant viruses as virulent as the parental virus arise during replication of group 1 mutants at different rates) or the influence of the second mutations (i.e., the change at amino acid 198 [which is not associated with antibody binding] may alter attenuation of mutants 42C3v and 67G1v, which had higher mortality rates than the other group 1 mutants).

The wild-type Ty/Ont exhibits a marked lymphotropism in

its avian host (30), with particularly damaging effects in the spleen (23, 30), where severe necrosis leads to virtually complete depletion of lymphocytes within that organ. Since the group 1 mutants failed to produce splenic necrosis (23), it was of interest to examine viral replication early in infection within that organ by in situ hybridization. While viral RNAs from both Ty/Ont and the group 1 mutant 77B1v were clearly present, the distribution of RNA was markedly different. The presence of mononuclear cells which retained the probe in spleens from birds infected with the wild-type Ty/Ont suggests that these cells actively replicate the virus, and other in vivo studies (31) in this laboratory support that conclusion. In vitro experiments indicate that adherent macrophages from the spleen can be infected with the wild-type Ty/Ont virus (31). If the in vitro experiments are an accurate reflection of the infected cell type within the spleen, then the cells observed by in situ hybridization may be splenic macrophages which replicate the virus. Unfortunately, in vitro infection of macrophages with the wild-type virus is nonproductive and therefore unsuitable for the comparison of wild-type and mutant viruses at this time. In contrast to the distribution of viral RNA for the wild-type Ty/Ont, most of the viral RNA from the group 1 mutant 77B1v was associated with erythrocytes, suggesting that replication may occur in other tissues and that the virus is passively carried to the spleen attached to erythrocytes.

The results of the in situ hybridization, considered with the location and nature of the changes in group 1 mutants, suggest that the attenuated group 1 mutants have an altered cell tropism. Whether an altered cell tropism is responsible for attenuation is not certain but is a likely possibility based on analogous observations in other virus systems. Escape mutants of reovirus have been shown to have an altered cell tropism in the central nervous system and to be attenuated (28, 29). Recently, attenuated escape mutants of rabies virus have been reported with changes in antigenic site II of the rabies virus glycoprotein (24). In addition, escape mutants of Theiler's murine encephalomyelitis virus (25, 37) and murine coronaviruses (6, 10) have recently been reported to be attenuated. Thus, it appears that certain areas of viral antigens which elicit neutralizing antibodies can influence pathogenesis in several virus-host systems. Furthermore, the viral molecules involved in each case are also those which are known or believed to function in host cell attachment. A reasonable hypothesis is that specific changes in these viral attachment molecules influence the receptor molecule(s) recognized by the virus.

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