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Research Note—

Appearance of Serum Antibodies Against the Avian Influenza Nonstructural 1 Protein in Experimentally Infected Chickens and Turkeys

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SUMMARY. In order to support eradication efforts of avian influenza (AI) infections in poultry, the implementation of "differentiation of infected from vaccinated animals" (DIVA) vaccination strategies has been recommended by international organizations. These systems enable the detection of field exposure in vaccinated flocks, and through this detection, infected flocks may be properly managed, thus interrupting the perpetuation of the infectious cycle. A promising system, based on the detection of antibodies to the nonstructural 1 (NS1) protein of AI, has been deemed a good candidate. However, there are presently no data available, in support of this DIVA system, with regard to the kinetics of antibody production against the NS1 proteins in poultry following infection. The present investigation was undertaken to establish the dynamics of the appearance of anti-NS1 antibodies in a naïve population. Following experimental infection of turkeys, antibodies to a peptide spanning the c-terminal of the NS1 protein were detected by enzyme-linked immunosorbent assay (ELISA) starting between day 3 and day 5 postinfection. In contrast, no antibodies to the NS1 peptide could be detected in chickens over the test period. In addition, the turkeys and chickens reacted differently at a clinical level to the infection by the H9N2 challenge virus. Taken together, these findings indicate that there is a significant difference in the viral replication in turkeys and chickens, resulting in a variation in the production of antibodies to NS1, as detected by the peptide-based ELISA used. This fact must be taken into consideration when using a DIVA system based on the identification of antibodies to the NS1 protein.

RESUMEN. *Nota de Investigación*—Aparición de anticuerpos séricos contra la proteína no estructural 1 del virus de influenza aviar en pollos y pavos infectados experimentalmente.

Con la finalidad de apoyar los esfuerzos para la erradicación de las infecciones de influenza aviar en las aves domésticas, la implementación de estrategias de vacunación que permitan la diferenciación entre aves infectadas y aves vacunadas (por sus siglas en Inglés DIVA), ha sido recomendada por parte de las organizaciones internacionales. Estos sistemas permiten la identificación de exposiciones de campo en aves vacunadas. Mediante esta detección, las parvadas infectadas pueden ser manejadas de manera apropiada, interrumpiendo de esta forma la perpetuación del ciclo de infección. Un sistema prometedor, basado en la detección de anticuerpos contra la proteína no estructural 1 del virus de influenza aviar, ha sido considerado como un buen candidato. Sin embargo, actualmente no hay datos disponibles que apoyen este sistema DIVA en relación a la cinética de la producción de anticuerpos contra la proteína no estructural 1 en aves domésticas después de la infección. La presente investigación se llevó a cabo para establecer la dinámica de la aparición de anticuerpos contra la proteína no estructural 1 en una población sin exposición previa. Comenzando entre el día tres y el día cinco posteriores a la infección experimental de pavos, se detectaron anticuerpos contra un péptido que abarca la porción c-terminal de la proteína no estructural 1, mediante la prueba de inmunoensayo asociado a enzimas. En contraste, no fue posible encontrar anticuerpos contra la proteína no estructural 1 en pollos durante el periodo de evaluación. Adicionalmente, a nivel clínico, los pavos y pollos reaccionaron de manera diferente a la infección con el virus de desafío H9N2. Estos hallazgos indican que existe una diferencia significativa en la replicación viral en los pavos y pollos que resulta en una variación en la producción de anticuerpos contra la proteína no estructural 1, según se pudo detectar mediante la prueba de inmunoensayo asociado a enzimas basada en péptidos, utilizada en el presente experimento. Este hecho debe ser tomado en cuenta cuando se utiliza un sistema DIVA basado en identificación de anticuerpos contra la proteína no estructural 1.

Key words: avian influenza, NS1, kinetics, antibodies

Abbreviations: AGID = agar gel immunodiffusion test; AI = avian influenza; DIVA = differentiation of infected from vaccinated animals; ELISA = enzyme-linked immunosorbent assay; EID_{50} = embryo infectious dose; HI = hemagglutination inhibition; IgG = immunoglobulin G; LPAI = low-pathogenicity avian influenza; NS1 = nonstructural 1 protein; N = neuraminidase

Avian influenza (AI) virus poses significant threats to both animal and human health. Since 1997, AI viruses belonging to the H5 or H7 subtype have crossed the species barrier and caused fatal disease in humans in several Asian countries, the Netherlands, and, more recently, Turkey, Iraq, and Azerbaijan (4,20). In addition to the H5 and H7 subtypes, several outbreaks of AI caused by viruses of the H9N2 subtype have occurred in poultry in various parts of the world. Low-pathogenicity avian influenza (LPAI) H9N2 viruses have been reported in the Middle East since 1998, and they have been responsible for serious disease in commercial chickens in Iran and Pakistan (1,2). There have also been a number of reported human infections with H9N2 subtype viruses in Hong Kong and China, resulting in increasing concern about the pandemic potential of H9N2 subtype viruses (3).

International organizations have issued a series of recommendations aimed at bringing AI under control (10,14). In addition to direct control measures based on biosecurity, restriction policies, and stamping out, the appropriate use of vaccines is encouraged to

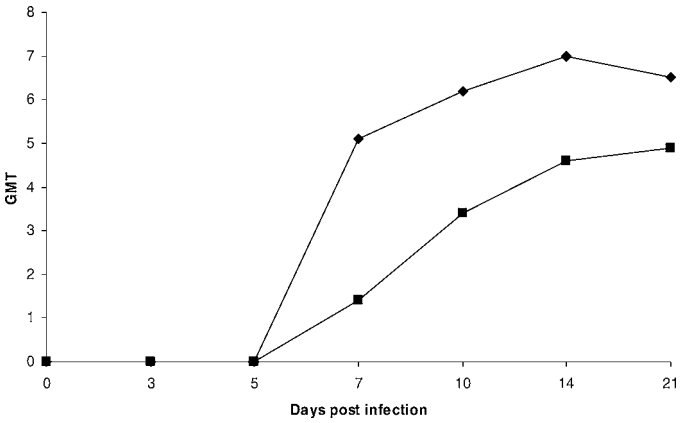

Fig. 1. HI geometric mean titers (log2) of turkeys (♦) and chickens (■) after infection with A/ty/Wisconsin/66 (H9N2).

maximize eradication efforts. It is known that vaccination prevents clinical disease, increases resistance to infection, and reduces virus shedding levels, but it does not prevent infection if birds are challenged with a sufficiently high dose of virus (6,17). For this reason, vaccinated birds may still become infected and shed virus into the environment without displaying any clinical signs, and, therefore, vaccinated birds represent a means of spreading infection. In order to achieve the goal of eradication, so-called ''differentiation of infected from vaccinated animals'' (DIVA) vaccination strategies have been recommended and must be implemented. These systems, coupled with an appropriate monitoring system, enable the detection of field exposure in vaccinated flocks, and through this process, infected flocks may be properly managed.

Several DIVA systems have been developed to date, although they have some limitations in the field (5,7,12,15). A promising system, based on the detection of antibodies against a specific antigen, the nonstructural 1 (NS1) protein of AI, has been deemed a good candidate (18,21). The NS1 protein is synthesized in large amounts in infected cells but is not incorporated into the mature virions, and for this reason, it represents the ideal candidate to elicit a specific immune response only in the presence of active viral replication.

Data generated in our laboratory, based on the work of Tumpey *et al.* (18), indicated that following experimental infection with LPAI A/ty/Italy/8000/H7N3, antibodies to the NS1 protein were detectable with an enzyme-linked immunosorbent assay (ELISA) test based on a synthetic peptide $NS1^{219–230}$ spanning the c-terminal of the NS1 protein (9). This test was able to detect antibodies in blood samples collected at day 21 postinfection. Since little is known about the kinetics of the antibody response to the NS1 protein in poultry, the present work was undertaken to investigate the production of antibodies against the immunogenic c-terminal of this protein in both turkeys and chickens.

MATERIALS AND METHODS

Challenge virus. An LPAI isolate of H9N2 subtype (A/ty/Wisconsin/66) was selected for this experiment. This strain has a full-length NS1 protein (GenBank accession number AAY52686) and was chosen because it was expected to induce a measurable antibody response to the entire NS1 protein. The virus was grown and titrated in specific-pathogen-free eggs, and the median embryo infectious dose (EID_{50}) was calculated according to the Reed and Muench formula (16).

Infection of turkeys and chickens. Nine commercial turkeys (BUT-6) and 14 chickens (White Leghorn specific pathogen free) that were 6 to 7 wk old and had been hatched and reared in isolation were oronasally and intraocularly infected with 10^6 EID_{50} of the challenge virus. Birds were examined daily for the appearance of clinical signs and were bled prior to infection and at days 3, 5, 7, 10, 14, and 21 postinfection.

Hemagglutination inhibition (HI) test and agar gel immunodiffusion test (AGID). To confirm the AI-free status of the test birds, the AGID test was performed on prechallenge sera. The HI test was performed using four hemagglutinin units of the isolate A/ty/Wisconsin/66 (H9N2) on all sera collected. Both tests were carried out according to EU Directive 92/40/EEC (8).

Peptide. Peptide $NS1^{219–230}$ (H-KRYMARRVESEV-OH) spanning the c-terminal of the NS1 protein was synthesized by Global Peptide Services (Fort Collins, CO). This peptide has previously been shown to be immunogenic in turkeys (9).

ELISA. The ELISA test used was described earlier (9). Briefly, Maxisorp ELISA plates (NUNC, Roskilde, Denmark) were coated with the $NS1^{219–230}$ peptide (0.5 μg/well) in bicarbonate buffer (pH 9.6) and incubated overnight at 4 C. The plates were blocked using 5% (w/v) nonfat skim milk in phosphate buffer solution plus 0.05% Tween 20 (PBST), and bound antibody was detected using anti-chicken immunoglobulin G (IgG) (ICN Biomedicals, Irvine, CA) conjugated to horseradish peroxidase (9).

Absorbance at 405 nm was measured following the addition of a peroxidase substrate solution (KPL, Gaithersburg, MD). Serum samples were considered positive for the peptide if they yielded an OD_{405nm} value of greater than the mean plus four standard deviations for the corresponding preimmune serum.

RESULTS

Clinical observations. No clinical signs were observed in the chickens infected with A/ty/Wisconsin/66. In contrast, the infected turkeys showed depression and respiratory signs starting on day 4 postinfection. They also exhibited sinusitis characterized by swelling of the infraorbital sinuses, and a drop in feed consumption was also recorded. All clinical signs, except for sinusitis, were self-limiting and disappeared by day 21 postinfection.

HI and AGID tests. All birds were seronegative to type A and H9 antibodies prior to challenge. For all the turkeys, the HI test confirmed infection by A/ty/Wisconsin/A66 starting from day 7 postinfection to the end of the experiment at day 21 (titer ranges 6–7 at day 21) (Fig. 1). The HI test confirmed infection in all except one of the 14 chickens (titer ranges 4–6 at day 21). The geometric mean titer values obtained for the turkeys were significantly higher at each sampling point than were those of the chickens, indicating a greater immunologic response to the virus, possibly because of the greater extent of viral replication in this species.

ELISA. Optimization experiments were carried out to determine the most suitable dilution of serum and secondary antibody for the two poultry species under study (data not shown). Without influencing the sensitivity of the test, the optimal dilutions determined were as follows: serum samples diluted 1:10 for turkeys and 1:20 for chickens in PBST; secondary antibodies diluted 1:1000 for turkeys and 1:2000 for chickens. Using these optimized conditions no antibodies to the $NS1^{219–230}$ peptide were detected in the preinfection sera of any of the turkeys or chickens (Fig. 2A,B). IgG antibodies against the $NS1^{219–230}$ peptide were detected in one of the nine turkeys beginning at day 3 postinfection. At day 7 all of the turkeys were positive in ELISA, but by day 14, the anti-$NS1^{219–230}$ antibodies in the sera of eight of the nine birds had returned to preimmune levels (Fig. 2A). No antibodies to the $NS1^{219–230}$ peptide were detected in the serum of any of the chickens except at day 3,

when three of the animals were positive (Fig. 2B). These positive samples were not confirmed on any of the other sampling days and were therefore considered to be false positives.

DISCUSSION

Since there are no data available on the kinetics of the anti-NS1 immune response in poultry, this study was designed to investigate the production of antibodies to the c-terminal of this protein in a naïve population of turkeys and chickens. The rationale behind the experiment was that following infection with an influenza virus containing a full-length NS1 gene, antibodies to the immunogenic c-terminus of the protein could be detected using an ELISA test containing a synthetic peptide corresponding to the c-terminus as an antigen. Such an investigation was supported by previous results that indicated that the peptide-based ELISA was suitable for this purpose (9).

The fact that the antibody response to the NS1 peptide in turkeys peaked at day 7 and did not continue beyond day 10 is puzzling and completely unexpected. The IgG response would be expected to start later and to certainly last longer than the 10 days of serologic positivity observed in the present study (11). The results obtained are, therefore, difficult to interpret and do not allow for conclusions to be reached regarding the onset and kinetics of antibody production against the $NS1^{219–230}$ peptide.

Neither are the data in complete agreement with two previous studies on the detection of anti-NS1 antibodies as markers of infection (18,21) or with the results generated in a previous experiment by our group (9). In all cases the authors were able to detect antibodies to the NS1 protein using either a synthetic peptide(s) or the whole protein produced as a recombinant in *Escherichia coli*. In the study performed by Tumpey *et al.* (18), chickens and turkeys were infected intranasally or intravenously with 10^6 EID_{50} of LPAI H5N9, H7N1, H1N1, and H7N2 subtype viruses. In all cases anti-NS1 antibodies could be detected using two detection methods (one peptide-based ELISA and one whole protein–based ELISA).

One limitation to using peptides to detect antibodies to the NS1 proteins is that variation in the sequence of the *ns1* gene does exist between AI viruses, and this most likely affects the sensitivity of the peptide-based ELISA (9). Indeed, the sequence of peptide $NS1^{219–230}$ (i.e., KRYMARRVESEV) has three amino differences when compared to the equivalent region of the NS1 of the challenge virus (A/ty/Wisconsin/66) used in this work (i.e., KRKMARTIESEV), and this may explain the apparent lack of sensitivity of the ELISA used in this work. Future work should involve the identification of a ''universal'' NS1 peptide that is conserved and immunogenic among different AI viruses.

In this present work, despite being infected with the same amount (10^6 EID_{50}) of virus, the chickens and turkeys reacted differently at both a clinical and immune level to infection. Turkeys have been shown to be highly susceptible to infection with both highly pathogenic AI and LPAI viruses, and they also develop a more severe clinical condition than do chickens (6,13,19). It therefore follows that the extent of viral replication in turkeys is probably greater than in chickens. The findings of the serologic investigations indicate that notwithstanding seroconversion, antibodies to the NS1 peptide were detected in the sera of the infected turkeys, taken as a group, only for a relatively short period of time (day 5 to day 10). The ELISA test was unable to detect antibodies to the NS1 peptide in chickens. This result could be due to the limited extent of viral replication occurring with this particular virus in this species. It is possible that because the presence of the NS1 protein is only associated with actively replicating virus, viruses that are not well adapted to particular species may have difficulty replicating and, therefore, will not be identified using the NS1 antibody detection system, despite the bird being infected.

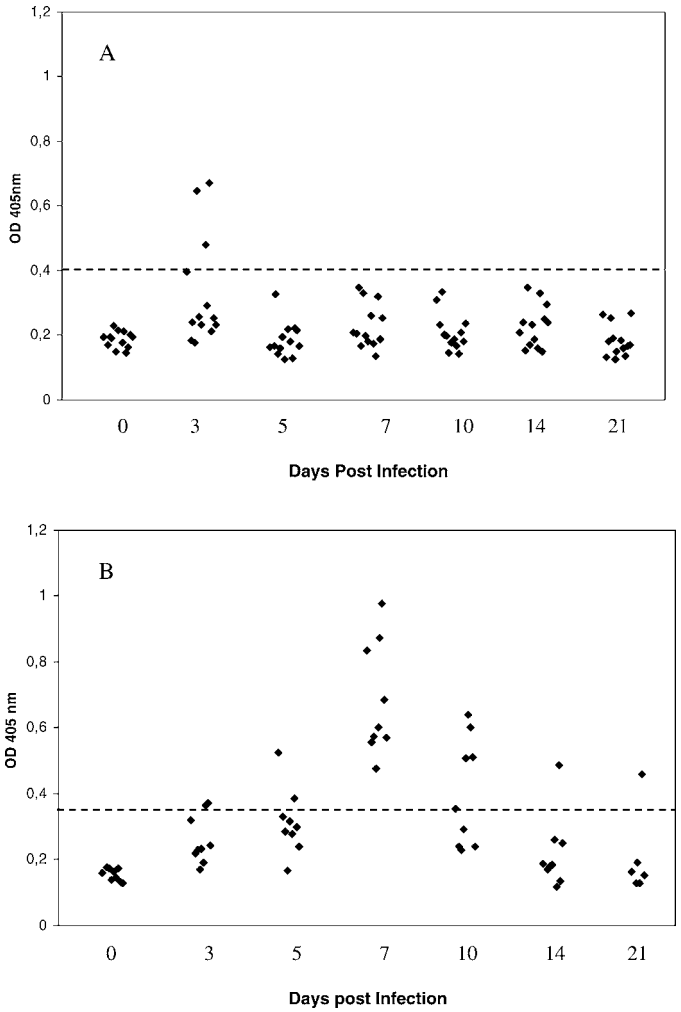

Fig. 2. Detection of anti-$NS1^{219–230}$ IgG antibodies by ELISA in chickens (A) and turkeys (B). The dashed horizontal line represents the minimum positive value calculated as an OD_{405nm} value greater than the mean plus four standard deviations (SD) for the corresponding preimmune serum. Values above this line are positive; those below this line are negative.

Nevertheless, if our experimental data reflects the situation in the field, the absence of detectable antibodies in chickens and the return of detectable NS1 antibodies in turkeys to preimmune levels by day 14 would allow a window of just a few days for the detection of infection in this species using this system, making it unsuitable, in its present form, as a surveillance tool to monitor infection.

In conclusion, the data from this work indicate that an ELISA system based on the detection of antibodies to the c-terminal of the NS1 protein appears to be unsuitable as a universal marker system for AI infections. However, the results of this experiment raise a series of questions that should be addressed. In addition to improving our knowledge on the appearance, duration, and extent of the immune response to the NS1 protein in poultry, further work should be carried out in order to increase the sensitivity of this peptide-based ELISA. These studies should also include comparison between experimental trials using different AI strains in diverse avian species.

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