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Evolution of gp85 gene of subgroup J avian leukosis virus under the selective pressure of antibodies

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Abstract Subgroup J Avian leucosis virus (ALV-J) strain NX0101 was inoculated into chicken embryo fibroblasts (CEF) monolayers in 6-well plates. The six wells of CEF inoculated with NX0101 were divided into groups A (without anti-ALV-J serum in the medium) and B (with anti-ALV-J serum in the medium), then viruses from each well of both groups were separately passed in CEF every 6 d and formed their independent passage lineages. For each lineage of both groups, gp85 genes of the viruses in the 10th, 20th and 30th passages were amplified, cloned and sequenced. The sequence data indicated that the homologies of gp85 at aa level between the primary virus and the passed viruses of different passages of 3 lineages in group A were 97.7%－99.7%; and the homologies of gp85 between the primary virus and the passed viruses of different passages of 3 lineages in group B were 93.8%－96.1%. Analysis of the ratios of nonsynonium (NS) vs synonium (S) mutations of nucleic acids demonstrated that NS/S in 3 highly variable (hr-) regions at aa#110－120, aa#141－151 and aa#189－194 of gp85 in 3 lineages of group A were 2 (8/4), 1(3/3) and 1.3 (4/3), however, NS/S in the same 3 hr-regions of group B were 4.1 (13/3), 4.7 (14/3) and 3.3 (11/3). This study is the first demonstration of influence of immune selective pressure on evolution of ALV-J gp85 by specific antibodies under the controlled *in vitro* experiments.

Keyword: subgroup J avian leucosis virus, gp85 gene, evolution, immune selection.

In the interaction with the host, the virus continues to be in the evolution and mutations. Such mutations happen more easily and frequently in retroviruses than other viruses, because the retroviruses have no correction mechanism for their RNA polymerases activity, which makes the viruses genetically less conserved and more variable[1]. Various selective pressures in nature, especially immune selective pressures not only accelerate mutations of viruses but also help some virus variants become the dominant population through escaping host immune reactions by changing of viral antigenecity[2]. Recently, much attention was given to the influence of immune selective pressures in evolutions of viruses. For example, human immunodeficiency virus (HIV) envelope glycoprotein genes have high frequency of mutations, and their 3rd hr-region (V3 loop) could have mutations as high as 50%[3]. The V3 loop of HIV1 is closely related to its cell tropism, replication kinetics and cellular pathogenicity, and the mutations in V3 loop could help viruses escape from attacks by cytotoxic T cells or neutralizing antibodies[4,5]. Haemagglutinin glycoproteins of human respi-

ratory measles virus could also be changed in its antigenecity by the influence of immune selective pressure to help the virus escape from host immune reactions[6].

Avian leucosis virus (ALV) of subgroup J (ALV-J) was first isolated in England in 1988 and then identified as a new subgroup of ALV[7]. According to the recent epidemiologic studies, gp85 of ALV-J mutated much faster than ALV classical subgroups A, B, C, D. Venugopal *et al.*[8] compared 12 field strains of ALV-J isolated in United Kingdom with the prototype strain HPRS-103 for their gp85 amino acid sequences and demonstrated homology of 92.0%—98.8%. Silva *et al.*[9] also compared American strains of ALV-J with HPRS-103 and demonstrated some hr-regions in their *env* genes. During the past several years, we also analyzed mutations in gp85 of 8 ALV-J field strains isolated in China during 1999—2001 and compared them with UK and US strains. The results indicated that immune selective pressure had some influence on gp85 gene evolutions[10—12].

ALV is a group of retroviruses, which could induce tumors or leukemia in domestic and wild birds. Among several proteins encoded by ALV, the envelope glycoprotein could interact with receptor on the host cell and determine the subgroups of the viruses. Envelope gene (*env*) of ALV encodes two proteins gp85 and gp37. However, gp85 is located on the surface of virions and it is the major protein for sub-grouping of ALV. To demonstrate the influence of immune selective pressures on evolution of viral antigenic protein genes, the ratios of nonsynonium (NS) and synonium (S) mutations of a given glycoprotein gene were analyzed. It is recognized generally that there is some influence of immune selective pressure on evolution of the glycoproteins if the ratios of NS/S are more than 2.5, otherwise changes in nucleic acids belong to random mutations[8]. To further understand the role of host immune pressures in viral evolutions, some systematic studies in animal models are necessary. In this study, mutations in gp85 of ALV-J strain NX0101 under the selective pressures of specific antibodies were studied *in vitro* by a series of passages in cell cultures with or without anti-ALV-J serum.

1 Materials and methods

1.1 Preparation of cell cultures

Chicken embryo fibroblast cells (CEF) were prepared from 10—12 d-old embryos of SPF chickens and used to pass ALV-J.

1.2 Virus

The fifth passage of ALV-J field strain NX0101 was used at the beginning. The virus was isolated from a meat-type parent breeder farm in Ningxia in 2001[12].

1.3 Preparation of anti-ALV-J chicken serum and its titer

SPF chickens were raised in an isolator with filtered air of positive pressure and then inoculated with NX0101 strain ALV-J of 8×10^3 TCID50 in 0.2 mL of CEF culture medium for each bird at the age of 7 d. Serum samples were collected from each inoculated chicken at the age of 42 d and kept at −20℃ before use. NX0101-infected CEF in 96-well plates were prepared, and cultured for more than 5 d. Then the infected CEF in plates were fixed with mixtures of acetone and alcohol (6∶4) for 10 min. The plates were used for tittering of chicken serum samples by indirect fluorescence antibody test (IFA). Serum samples from each chickens above were serially diluted as 1∶10, 1∶50, 1∶100, 1∶200 and 1∶400 in PBS, then 100 μL of diluted sera of each sample at different dilution was added into 96-well plates with ALV-J infected CEF. Plates were kept at 37℃ for 45 min, and washed 3 times with PBS. Then 100 μL of FITC-labeled anti-chicken IgG rabbit serum (Sigma product, used in 1∶160 in PBS) was added, kept at 37℃ for 45 min, and washed 3 times with PBS. At the same time, normal serum from un-infected SPF chickens was used as the negative control, and monoclonal antibody JE9[13] was used as the positive control in IFA. Antibody titers to ALV-J were determined by the highest dilutions, which gave positive reactions in IFA, for each serum sample. Among many serum samples, in this study, only one serum sample with the highest titer was chosen and used in the whole experiment. This is to guarantee antigenic homogeneity of mono-specific serum samples used in the serial passages of ALV-J in CEF

with antiserum, since antigenic reactions in different individuals could be different, and even serum samples collected from the same bird but at different time may have specific preference to different epitopes.

1.4 Determination of virus neutralizing titer of the selected mono-specific serum against ALV-J

CEF in bottles were inoculated with ALV-J strain NX0101 and incubated for 2 d, then trypsinized. CEF suspensions were re-inoculated into a 12-well plate. After incubation for 12 h, culture media in 6 wells were supplemented with the selected serum to the final dilution of 1∶25, 1∶50, 1∶100, 1∶200, 1∶500 and 1∶1000. Another 6 wells were supplemented with normal SPF chicken serum to the same dilutions as anti-ALV-J serum. Every 36 h, each well was replaced with fresh medium with the same chicken serum sample at the same dilutions to keep the antibody activity in the medium. The culture medium was changed for 3 times, then CEF in 12-well plate continued to be cultured for 3 more days. The supernatants from each well were inoculated into another 12-well plate with normal CEF monolayers. Culture medium was treated as above for 2 more times. When the 3rd passages were finished, the plate was fixed with the mixture of aceton and alcohol for IFA to determine virus neutralizing titer of the serum.

1.5 Serial passages of ALV-J strain NX0101 in CEF with mono-specific chicken serum

One bottle of CEF monolayer was inoculated with NX0101 of 5th passage, at which the viral genome was sequenced, and cultured for 2 d. Then NX0101 infected CEF were trypsinized and cell suspensions were inoculated into a 6-well plate and cultured for 12 h. The six well were divided into groups A and B. In the further serial passages, 3 wells in A as A1, A2 and A3 lineages were passed separately without anti-NX0101 mono-specific serum in medium as controls. Another 3 wells in B as B1, B2 and B3 lineages were passed separately with the same anti-NX0101 serum sample at certain concentration (the highest concentration, with which virus replication would not be inhibited as determined in subsec. 1.4). Medium in each well was changed every 36 h, CEF monolayers were kept for another 3 d after the 3rd change of medium, then the supernatant from each well was transferred into another 6-well plates with fresh CEF monolayers. Such steps were repeated for 30 passages. Every 5 passages, the cells in each of 6 lineages were harvested and kept at −70℃.

1.6 Amplification, cloning and sequencing of env genes

The genomic DNA was extracted from CEF in each well of A1, A2, A3, and B1, B2, B3 lineages at 10th, 20th and 30th passages. To amplify a 2.2 kb fragment with the whole *env* gene of ALV-J, a pair of primers were used as: 5′-gctgccatcgagaggttact-3′ (forward) and 5′-agttgtcagggaatcgac-3′ (reverse)[9,14 − 16]. The PCR products were cloned into pMD18-T and sequenced by commercial services.

2 Results

2.1 Titer of anti-NX0101 mono-specific chicken serum

From 10 NX0101-inoculated SPF chickens, one serum sample with the highest titer was chosen for use in this study. When IFA was conducted with the antiserum at 1∶10, 1∶50, 1∶100 and 1∶200, very bright greenish fluorescence was demonstrated in cytoplasm but not in nucleus in infected CEF. In NX0101-CEF with the serum in dilution of 1∶400, the fluorescence was weak. IFA titer of the serum sample was determined as 1∶200.

2.2 Determination of highest concentration of anti-NX0101 serum for not complete inhibition of virus replication

Virus neutralization indicated that the virus replication was completely inhibited in medium with anti-NX0101 chicken serum at 1∶25 dilution but viruses could still replicate at different levels in medium with the same serum at 1∶50—1∶1000. So, the 1∶50 dilution of anti-NX0101 chicken serum was used in medium in further 30 serial passages in B1, B2 and B3 lineages.

2.3 Changes of antibody titers during incubation at 37℃ for 36h

During the incubation period at 37℃, antibody activity may be decreased. Results indicated that

1∶2 dilutions of medium with 1∶50 anti-NX0101 serum gave clear positive to NX0101-CEF in IFA even 36h after incubation at 37℃. Compared to the original IFA titer of 1∶200 as in subsec. 2.1., it still maintained 37.5%—50% of original activity.

2.4 Variations of ALV-J gp85 genes during the serial passages without specific antibody

Table 1 demonstrates homogeneity of gp85 at the amino acid level between the original NX0101 and its derivatives at different passages in the group A. The average homologies between the original NX0101 and its derivatives at 10th, 20th, and 30th passages were 98.37%, 98.3% and 98.5% respectively, and the lowest homology was 97.7% (Table 1). Although there were some variations among 3 separated lineages A1, A2 and A3, they were located on the whole gp85 gene randomly. The phylogenetic tree also indicated such random mutations in 3 lineages of group A (Fig. 1).

2.5 Variations of ALV-J gp85 gene during the serial passages with specific antibodies

Table 2 demonstrates homogeneity of gp85 at the amino acid level between the original NX0101 and its derivatives at different passages in group B. The result indicated that the homologies of gp85 at the amino acid level between the original NX0101 and its derivatives at different passages in thegroup B were in the range of 93.8%—96.1%, being much lower than that between the original NX0101 and its derivatives in group A. The average homologies between the original NX0101 and its derivatives at the 10th, 20th and 30th in group B were 96%, 94.7% and 94.36%, and trended to gradually decrease (Table 2). However, the mutation frequency became smaller with the passages. As indicated by the phylogenetic tree in Fig. 1, the gp85 of

Table 1 Comparisons of gp85 homology between the original ALV-J NX01010 strain and its derivatives of different lineages and passages in group A without antibody

	NX-0	A1-10	A1-20	A1-30	A2-10	A2-20	A2-30	A3-10	A3-20	A3-30
NX-0		97.7	98.7	99.7	98.7	98.1	97.7	98.7	98.1	98.1
A1-10	2.3		98.7	97.4	96.8	96.1	95.8	96.8	96.4	96.4
A1-20	1.3	1.3		98.7	97.7	97.1	96.8	97.7	97.1	97.1
A1-30	0.3	2.6	1.3		98.4	97.7	97.4	98.4	97.7	97.7
A2-10	1.3	3.2	2.3	1.6		98.7	97.1	97.7	98.4	97.7
A2-20	1.9	3.9	2.9	2.3	1.3		97.1	97.1	97.1	97.1
A2-30	2.3	4.2	3.2	2.6	2.9	2.9		96.8	96.1	96.1
A3-10	1.3	3.2	2.3	1.6	2.3	2.9	3.2		98.7	98.1
A3-20	1.9	3.6	2.9	2.3	1.6	2.9	3.9	1.3		98.1
A3-30	1.9	3.4	2.9	2.3	2.3	2.2	3.9	1.9	1.9	
$\bar{X}$ ±SD	1.61±0.633				2.59±0.81					

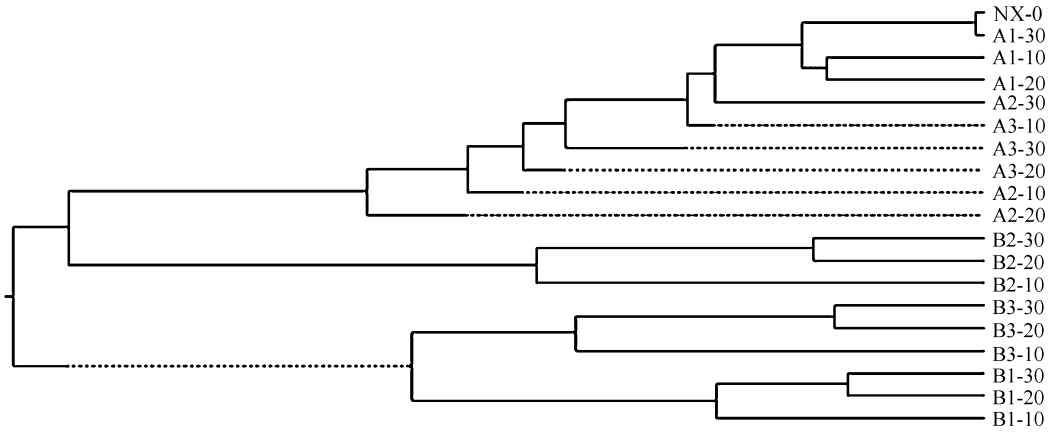

Fig. 1. Phylogenetic tree of gp85 amino acid sequences of original NX0101 strain and its derivatives of different lineages and passages in groups A and B.

20th passages was close to gp85 of 30th passages than gp85 of 10th passages in each lineage of group B. But gp85 of different passages in the same lineages was always located in the same branches in the phylogenetic tree (Fig. 1). Besides, homologies of gp85 between some different lineages in the group B were smaller than their homologies with the originals. For example, homologies of gp85 between B1-30 and B2-30 (30th passages of B1 and B2 lineages) or B2-30 and B3-30 (30th passages of B2 and B3 lineages) were only 93.2% or 92.2%, and lower than 94.5%, 95.5% and 93.8% for homologies between the original and its derivatives at 30th passage in 3 lineages of the group B (Table 2), indicating variability in mutations of gp85 under the immune pressure. Compared to group A without specific antibodies during the serial passages, derivatives in group B with antibodies gave more amino acid changes in the 3 hr-regions in gp85. Also, amino acid replacement happened at all 4 sites of aa#144－147 in all 3 lineages in group B (Fig. 2).

2.6 Comparisons of gp85 mutation frequencies in the passages with or without specific antibodies

Variability of gp85 amino acid during the passages with or without antibodies is listed in the left parts of Tables 1 (without antibody) and 2 (with antibody). When compared with their original virus, average variability of viruses at different passages of 3 lineages in the group A was (1.61±0.63)% and significantly smaller than (4.87±0.87)% for the group B (p<0.01) (Table 3). The group comparison showed that the average variability of viruses at different passages

Table 2 Comparisons of gp85 homology between the original ALV-J NX01010 strain and its derivatives of different lineages and passages in group B with antibody

	NX-0	B1-10	B1-20	B1-30	B2-10	B2-20	B2-30	B3-10	B3-20	B3-30
NX-0		96.1	95.8	94.5	96.1	95.2	95.5	94.8	94.1	93.8
B1-10	3.9		98.1	96.8	95.8	93.5	94.8	95.5	96.1	95.1
B1-20	4.2	1.9		98.1	95.8	93.2	94.5	95.5	95.5	93.8
B1-30	5.5	3.2	1.9		93.8	93.5	93.2	93.5	94.8	93.5
B2-10	3.9	4.2	4.2	6.2		94.8	96.8	95.8	95.1	93.5
B2-20	4.8	6.5	6.8	6.5	5.2		96.8	94.2	93.5	92.2
B2-30	5.2	5.2	5.5	6.8	3.2	3.2		95.5	95.1	94.5
B3-10	4.2	4.5	4.5	6.5	4.2	5.8	4.5		96.8	95.8
B3-20	5.9	3.9	4.5	5.2	4.9	6.5	4.9	3.2		98.4
B3-30	6.2	4.9	6.2	6.5	6.5	7.8	5.5	4.2	1.6	
$\bar{X}$ ±SD	4.87±0.87			4.94±1.52						

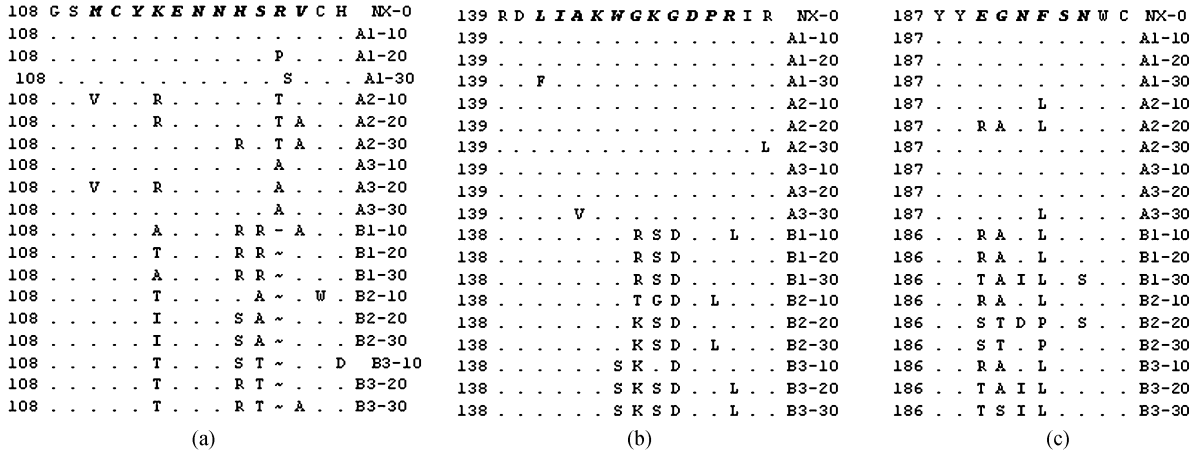

Fig. 2. Comparisons of amino acid sequences of hr-regions on gp85 between the original NX0101 and its derivatives in different lineages and passages of groups A and B. (a) Subregion of hr1-region at aa #108－122; (b) subregion of hr1-region at aa #139－154; (c) a subregion of hr2-region at aa #187－196. Because of a deletion at #119 for all viruses in group B, the aa numbers changed from that site. The bold letters in each regions represent the high variable sites speculated according to NS/S ratios.

of 3 lineages in group A was 2.59%±0.81%,being significantly smaller than (4.94±1.52)% in group B (p<0.01). This indicated that the specific antibody could increase the mutation frequency of gp85 during the passages of the virus.

Table 3 Comparisons of gp85 mutation frequencies in passages between groups with or without specific antibodies ($\bar{x}$ ±SD)

Groups	Diversity relative to original NX0101 (%)	Diversity within a group (%)
A without antibody	1.61±0.633	2.59±0.81
B with antibody	4.87±0.87	4.94±1.52
p value	p<0.01	p<0.01

2.7 gp85 mutation sites common to different lineages in the group B

Fig. 2 demonstrates 3 hr-regions at aa#110－120 (a), #141－151 (b) and #189－194 (c) in gp85. In these 3 hr-regions, there were much more amino acid changes at different passages in group B with antibody than in group A without antibody. In group B, most changes, which appeared in the low passages, still remained continuously, indicating that these changes were not random mutations but influenced by antibody selection. In 3 hr-regions of group A, there were totally 11 sites with changed amino acids. Among them, however, 6 changed sites appeared only in 1 of 3 lineages, changes in another 4 sites could not be maintained during the continued passages, and the left site was changed into different amino acids in 3 different lineages, indicating that most of the amino acid changes were due to random mutations.

The results also demonstrated that the changes in group B with antibody mainly happened in the first 10 passages. Among 13 sites with amino acid mutations in 3 hr-regions of the lineage B-1, for example, 8 changes had already happened in the 10th passage and still remained in the 20th and 30th passages. Some changes happened during the 10th to 20th passages. The 3 hr-regions of gp85 trended to be stable by 20th passages. Few changes happened after 20th passage even still under the influence of the same specific antibodies.

2.8 The ratios of NS/S mutations of gp85 and its 3 hr-regions

High ratios of NS/S of hr-regions on gp85 were recognized as important indicators for the roles of immune selective pressures. The ratios of NS/S in the whole gp85 and its 3 hr-regions of aa#110－120, #141－151 and #189－194 in both groups A and B are listed in Table 4. On the whole gp85, the ratios of NS/S were 1.53 (49/32) and 0.93 (28/30) respectively for groups B and A with or without antibodies in the culture medium. However, it was quite different between groups A and B if NS/S was compared for their hr-regions. The ratios of NS/S were only 2 (8/4), 1 (3/3) and 1.3 (4/3) in 3 hr-regions in the group A without antibody, but they were as high as 4.1 (13/3), 4.7 (14/3) and 3.3 (11/3) in the same 3 hr-regions for the group B with antibody. The above analysis indicated that antibody-mediated immune selective pressures gave high ratios of NS/S only to hr-regions but not the whole gp85, and it implied that these hr-regions on gp85 were the sites influenced by specific antibodies or specific antigenic epitopes related to virus neutralization.

3 Discussions

Subtypes of ALV were determined based on their envelope glycoprotein gp85. There were about 70%－80% homologies among subgroups A, B, C and D, but the subgroup J had homology of only 40% with each of 4 subgroups A, B, C, and D. Since ALV-J was first recognized in 1989, more studies were given to its mutations. Usually, subgroup J could be more easily changed in its hr1, hr2 and vr3 regions on gp85 than

Table 4 Comparisons of NS/S ratios on the whole gp85 and 3 variable regions between groups with and without antibody

Regions	Group A without antibody			Group B with antibody		
	NS	S	ratios	NS	S	ratios
Total gp85	28	30	0.93	49	32	1.53
110－120 aa(hr1)	8	4	2	13	3	4.33
141－151 aa(hr1)	3	3	1	14	3	4.70
189－194 aa(hr2)	4	3	1.3	11	3	3.67

other subgroups, especially in hr1 and hr2. In these studies, hr1, hr2 and vr3 regions were speculated as major regions influenced by immune pressures according to their NS/S ratios and antigenic determinants related to virus neutralization[8−12]. All these studies were made by analysis of gp85 genes in large numbers of ALV-J field strains. This paper is the first try to study the role of immune selective pressures on ALV-J gp85 evolution *in vitro* by specific antiserum supplemented in the cell culture medium.

In the primary test, a suitable concentration of antiserum to ALV-J at 1∶50 in the culture medium was determined. With such concentration, antibody-mediated selective effect was high enough but virus replication was not completely inhibited. Considering decreased antibody activity during the culture at 37℃, culture medium was changed every 36 h for supplement of fresh antiserum to keep antibody activity at a certain level. Actually, IFA titers to ALV-J in medium 36 h after culture at 37℃ were still kept at about 37.5%−50% relative to its original activity, indicating that the viruses released from infected cells were always influenced by the antibody during the culture.

It was recognized that all field ALV-J strains were originally from one recombination event between an exogenous ALV and an endogenous ALV-J but not from multiple recombination events[17]. After the original exogenous ALV-J emerged, it gradually mutated during the spread in birds under the influences of some selective pressures, especially immune pressures. Mutations in gp85 may happen randomly, which could explain why the homology among different lineages in group B with antibody was smaller than that between the original virus and its derivatives in 3 lineages of the same group B.

The most amino acid changes happening at low passages could be the same at the sites in the further passages in group B with antibody (Fig. 2), indicating that they were not random mutations but the changes under the influence of immune pressures. Virions with such mutations became a dominant population and formed a quanspecies. In group A without antibody there were also some amino acids changed, however, such changes were demonstrated only at a certain passage of a specific lineage and they were random mutations mostlikely. The deletion at aa #118 and changed amino acids in the region of aa #145−148 seem to be genetic markers which differentiated viruses produced during the passages between groups A and B. In group B with antibody, 4 amino acids changed at aa #145−148 when compared to the original virus or derivatives in group A. It may be speculated that this is the major site involved in virus neutralization.

Analysis of genetic phelogenetic relationships of viruses in different passages indicated that genetic homology among viruses in group A did not have a strict relations to their passages or lineages but such close relationships were showed in group B with antibody. As Fig. 1 demonstrated, viruses in group B were regularly divided into 3 subgroups based on their lineages in the phelogenetic tree, and viruses at 20th passages in all 3 lineages were always closer to viruses at 30th passages than to viruses at 10th passages in the same lineage. It may suggest that some quanspecies of the original virus gradually lost its epitopes recognized by the antibodies and became the dominant population in the culture with the selection of specific antibodies. But with the epitope changed, the role of immune pressures became less and less, and finally virus population became stable.

To compare and analyze changes of epitopes on gp85 under the influence of specific monoserum, ALV-J strain NX0101 was passed for 30 passages in only 3 independent lineages for both groups A (without antibody) and B (with antibody) respectively. This was due to the limited amount of specific monoserum. It was considered that antibody responses of different chicken individuals to different epitopes may not be the same and even the same individual could give different antibody responses to the same antigen in different periods. To guarantee the epitope homogeneity of the monoserum during the period, only one serum sample with the highest IFA titer was used in the experiment. With the limited monoserum sample, we had two choices for the experiment design: to use less lineages or less passages. Since we could not predict how many passages with the serum are necessary for the changed epitopes to become relatively stable, less lineages were designed.

Both ALV and human immunodeficiency virus (HIV) belong to retroviruses, their genome structures

are very similar and include LTR on the both sides of the genomes and *gag, pol* and *env* genes. They are easy to mutate due to shortage of correction mechanism of RNA polymerase and under various natural selective pressures, especially the immune selective pressure from the hosts. The most common mutations happened in *env* genes. It is obvious that evolution of avian retroviruses under the immune selective pressure could be a model for the study of mutations or evolutions of HIV under the similar immune pressures from the host.

So far, evolutions of viruses under the immune selective pressures have been studied only based on comparisons of field strains isolated from patients or sick animals. Since the phylogenetic relationships among field virus strains were not clear, it is difficult to exactly identify epitope-related nucleic acid mutations. By using ALV-J as a model, this study is the first try to analyze evolutions of ALV-J under a controlled condition in serial cell cultures starting from a known viral population, which could clearly demonstrate the selective roles of antibody-mediated pressures. Both cell-mediated immunity and humeral immunity play roles in the host immune reactions against viral infection, but only changes in epitopes on the envelope glycoprotein of ALV-J under the influence of antibody selective pressures were mimicked in this study. Further investigation in chickens is needed to see if the mechanism in such *in vitro* studies is similar to viral evolutions under the real immune selective pressures *in vivo*.

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