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Production of Monoclonal Antibody to Infectious Laryngotracheitis Virus by Cell Fusion

Chung Ok Choi, Chung Gil Lee, Sung Man Cho,
Soo Hwan An* and Joon Hun Kwon*
College of Veterinary Medicine, Chonnam National University

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닭 傳染性 喉頭氣管炎 바이러스에 대한 단크론성 抗體生産

崔晶鈺·李政吉·趙成萬·安壽煥*·權俊憲*
全南大學校 獸醫科大學

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摘 要

국내에서 분리한 강독전염성후두기관염 바이러스 (ILTV)에 대한 세포융합방법에 의해 단크론성 항체(MCA) 생산을 시도한 결과 총 8회의 세포융합을 통하여 총 1017개의 융합세포가 생산되었으며 그중 ILTV와 특이적으로 작용하는 항체를 생산하는 3주의 Hybridoma를 작성하였다. 이 3주의 MCA는 모두 IgG 형에 속하였으며 마우스 복강내접종하여 생산된 복수항체의 형광항체가는 10^5-10^6에 달하였고 약독 및 강독 ILTV에 차이가 없이 작용하였으며 중화능력은 인정되지 않았다. 이 MCA를 이용하여 간접형광항체법으로 인공감염계에서 ILTV 검출을 시도한 결과, 기관 및 안점막의 도말표본에서 감염후 10일 까지 진단이 가능하였으며 표준 양성혈청을 이용한 형광항체법이나 핵내봉입체 검출방법에 비해서 진단효율이 높았다.

I. INTRODUCTION

Infectious laryngotracheitis (ILT) is an acute respiratory disease caused by Gallid herpesvirus 3 belonging to subfamily alpaherpesviridae (Russell and Edington 1985). The disease was not recognized until February 1982 in Korea, when outbreaks of the disease spread rapidly throughout the country within 6 months (Choi et al. 1985).

Main clinical symptoms include sneezing, coughing, gasping, sometimes eye and nasal discharges, expectoration of blood, diarrhea, anorexia, and weight and egg production drop. In acute or peracute form the desease spreads very fast and within 2 to 5 days all birds in the flock will show symptoms if a few brids once get infected. However, in the chronic form it could take more than a month to infect all birds in the flock. The

* 家畜衛生研究所 (Institute of Veterinary Research)
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disease affects birds of any age and mortality varies greatly depending on the pathogenicity of the virus, being 7 to 15 percent on the average. Eradication of the disease is difficult since some birds tend to remain as carrier after they recover and sporadically disseminate virus to the environment for nearly life long.

Like other respiratory diseases ILT tends to prevail in winter and at the change of season in other countries (Jordan, 1966). However, in Korea the disease occurs regardless of season and the first great epidemic recorded was in summer 1982 (Choi et al. 1985). At present in Korea the disease became endemic and seems to cause economic losses more in broiler than in layer birds since the hygienic condition of most broiler farms in this country is still required a great improvement.

The disease often occurs in a complicated form with other virus and/or bacteria causing respiratory diseases such as Newcastle disease, infectious bronchitis and mycoplasmosis. Economic losses are often severe due to the mixed infection especially in flocks under suboptimal hygienic condition. To take proper measures it is required diagnosis as early as possible.

There are several methods available for the rapid diagnosios, namely, rapid smear technique, immunofluorescent antibody test (IFA) (Hitchner et al. 1977, and enzyme linked immunosorbent assay (ELISA) (Meulemans and Halen, 1982). Smear technique, involving tracheal smear, staining and observing intranuclear inclusion body, although fast for the diagnosis, has some limit, because of the short duration of the inclusion body after infection and cofusion with other intranuclear inclusion body forming viruses. For the IFA and ELISA tests, although sensitive, fast and reliable, is required highly purified monospecific antibody to detect ILTV.

This experiment was carried out to produce monoclonal antibody (MCA) against ILTV so that the MCA can be used for the rapid detection of ILT antigen from suspected chicken tissue by immunological methods such as IFA or ELISA. The antibody can be further utilized in analysing and purifying antigen of ILTV of varying pathogenicity.

II. MATERIALS AND METHODS

◦ Viruses : ILTVs used were a virulent strain, 82-33-7, isolated from the case of the first outbreak in Korea, and a mild vaccine strain from Arthur Webster (Australia) and locally produced vaccine. Other viruses used were Newcastle disease virus (Kyojungwon, B_1), infectious bronchitis virus (Massachusetts type), infectious bovine rhinotracheitis virus (Colorado), Marek's disease virus (MDV-19 from Australia), Herpesvirus of turkeys (FC 126) and Japanese encephalitis virus (Anyang).

◦ Propagation and purification of the virus : Virulent and vaccine ILTV were propagated in chicken embryo kidney (CEK), chicken kidney (CK) cells and also in chorioallantoic membrane (CAM). Briefly, CEK cells from 18-day-old SPF embryos were prerared by overnight trypsinization in 0.125% trypsin at 4℃ and cultured in plastic plates or roller bottles containing medium 199 supplemented with 10% calf serum (CS) and 10% tryptose phosphate broth (TPB). When cultures became confluent, the concentration of CS was reduced to 2% for maintenance of CEK growth (Choi, 1980). The CK cells were cultured as described by Churchill (1965) and Choi (1980). For largescale production of virus roller bottle cultures were used. The CEK or CK cell cultures inoculated with the ILTV were incubated for 3 days at 37℃. Cells were harvested with rubber policeman after culture fluid was discarded and cells were washed three times with phosphated buffered saline (PBS pH 7.4). The infected cells were frozen and thawed three times and centrifuged for 10 minutes at 8,000rpm. The supernatant was used as it is as a crude virus of used after purification. The crude virus was concentrated by ultracentrifuge for two hours at 27,000rpm using Beckman type 30 rotor. The virus resuspended in TNE buffer (0.01M Tris-

HCl, 0.1M NaCl, 0.002M EDTA) was ultracentrifuged in cecium chloride density gradient with type 41 Ti SW rotor.

The virus band was collected and ultracentrifuged once again as before and dialysed against PBS. Antigenic titer of the purified virus was measured against the standard postive serum by agar gel preciptiation test.

The virus was also propagated in chicken embryo by dropping the virus on CAM of 10-day-old SPF embryos. After five days only the area of CAM where pocks were formed, was harvested, pooled and washed twice with PBS. Then, 30 percant emulsion with PBS was made and virus was purified as described for the virus propagated in cell cultures.

◦ Production of standard antiserum to ILTV : The purified ILTV was inoculated into 3-month-old SPF chicken via intramuscular route 3 times at 2 weeks intervals. Whole blood was collected one week after final immunization. The antiserum containing polyclonal antibody (PCA) produced clear arc against purified ILTV in agar gel precipitation and neutralizing antibody titer of the serum was 400.

◦ Immunization of mice : A group of ten 6 to 8-week-old BALB/c mice was immunized with the purified ILTV (Wood, 1984a, An and Kim 1983). Each mouse was given two injections of intraperitoneal (i.p.) and subcutaneous route in 0.2ml of virus mixed with an equal volume of freund's complete adjuvant. Three weeks after the first injection, a second injection was given, except that incomplete adjuvant was substituted for complete adjuvant. A final i.p. injection without adjuvant followed 14 days later. Blood was obtained by orbital bleeding, and sera were tested for the presence of antibody in an indirect immunofluorescent antibody (IFA) test.

◦ Hybridization and selection : Fusions were performed according to published procedure (An and Kim 1983, Wood 1984a, b). Briefly, spleen cells collected from 3 to 4 days after final immunization were fused with SP2/0 myeloma cells at a ratio of 10 : 1. The cell mixtures were pelleted at 1000xg for 10 minutes and gently resuspended in 0.5ml of 50% polyethylene glycol (PEG) in Dulbecco's modified Eagle's medium (DMEM) for 1 minute. During the ensuing 5 minute, the fusion mixture was diluted with 5ml of serum-free DMEM, then the fusion mixture was further diluted with 5ml of DMEM containg 15% fetal calf serum. Finally the cells were collected by centrifugation at 1000xg for 5 minutes, washed twice with DMEM contating 15% fetal calf serum by centrifucgation. The cells were then resuspended in HAT selective medium (DMEM containing 10% fetal calf serum, 2mM glutamine, 100μM hypoxanthine, 16 μM aminopterin, 100iu penicilin/ml, and 100μg streptomycin/ml), and dispensed into 96-well Costar tissue culture plates. Half of the medium was replaced with fresh HAT medium 3 to 4 days later. After about 10 days, hybridized cells were maintained in medium without aminopterin.

Beginning about days 10 to 14, the medium from wells showing cell growth was tested for antibody activity against ILTV by IFA. Cultures producing antibody postive for the virus were transferred to 24-well plates for cell expansion. Hybridomas secreting specific antibody were cloned by limiting dilution in 96 well plates with feeder layers of peritoneal washings from 4 to 6 week-old BALB/c mice. Hybridomas producing specific antibody to ILTV were stored in liquid nitrogen and some were also injected into BALB/c mice via I.P route at 2 x 10^6 cells per mice. Ascitic fluid was harvested, clarified by centrifugation and tested titers by end point dilution in IFA test.

◦ IFA test : Chicken embryo kidney cells grown on coverglass were infected with ILTV of 100 median tissue culture infective dose ($TCID_{50}$). One to 2 days after incubation at 37℃ at a humidified atmosphere of 5% CO_2, the coverslips were removed, washed twice with PBS and fixed with cold acetone for 5 to 10 minutes, then the coverslips were kept at 20℃ until used. Normal CEK cultures for the control were treated as same. Chicken embryo fibroblast grown on cove-

rslip was used for the antigen preparation of Newcastle disease, infectious bovine rhinotracheitis and black goat kidney cells for Japanese encephalitis virus. To determine the specificity of MCA, coverslip cultures fixed with acetone were treated for 30 minutes with fluid collected from the well of hybridoma culture and washed 3 times with PBS. Then the coverslip was treated with FITC conjugated rabbit anti-mouse Ig G+M+A (Capple) for 30 minutes and washed. The stained coverslip was examined under the fluorescent microseope.

◦ Classification of isotype of MCA : Isotype of MCA was determined by AGP test. The slide glass was overlaid with 1.5% agar and wells of 3mm in diameter was punched, one in center and 6 in surround at 3mm distance. The center well was filled with anti-mouse IgG, IgA or IgM and the surrounding wells with lysed hybridoma cells and hybridoma cells producing specific arc formation was examined 24 hours after at room temperature.

◦ Detection of ILTV antigen in various organs from chickens : Twenty-five 6-week-old SPF chickens were injected with virulent ILTV via in trachea route in 0.1ml of 100 EID_{50} per bird. Five organs including trachea, conjunctiva, lung, liver and spleen were collected from 3 birds each at one to three day intervals beginning from day 0 to 10 days postinoculation (PI). Slide smears were prepared with each organ. Trachea taken from the chicken was opened longitudinally and mucous membrane was scraped with scalpel after mucus was gently removed with cotton. The scraped material was smeared on slide and fixed with cold acetone for 5 minutes and kept at 20℃ until IFA test using MCA. Conjunctival scrapings and smears were similarly prepared as for trachea. In case of lung, liver, and spleen stamp smears were made from the cut surface made with sharp blades and processed as for the trachea. The IFA test was conducted using MCA as described before.

◦ Comparision of diagnostic efficiency : Three diagnostic methods for ILT; IFA with MCA, IFA with PCA, and hematoxylin and eosin (HE) staining for the intranuclear inclusion body, were compared in slide smears made with scrapings of trachea removed from experimentally infected chickens. Thirty-five 5-week-old SPF birds were divided into 3 groups. One group of 20 birds was received 1000 median TCID of virulent ILTV in 0.1ml volume via intra-trachea route. A group of 10 birds was given 10^5 median embryo lethal dose of virulent NDV in 0.5ml via intramuscular route. The rest of 5 birds was used as control. The three groups were accommodated separately. Tracheal scrapings were made at 4 and 7 days PI for the control group. The samples were fixed with acetone and kept at-20℃ for IFA test.

For the HE staining, the scrapings were fixed with a modified Shaudinn fixative (Jackson and Sinkovic 1969), and examined under microscope for intranuclear inclusion bodies after staining.

III. RESULTS

◦ Growth and purification of ILTV : To produce high titered virus, growth rate of the virulent ILTV was investigated in CEK, CEL and CAM. As shown in Table 1, virus growth rate was highest on CAM and followed by CEL in the order. Virus growth on CAM was peaked at 5 days PI and decreased thereafter while peaks reached at 3 days PI in CEK and in CEL. Although growth rate of the virus was better in CEK than in CEL, the number of CPE produced by the same inoculum showed no obvious difference between the two cell types.

The virus purified by cesium chloride density gradient ultra-centrifugation was belonging to the density beteen 1.26 and 1.29, and up to 1 : 4 dilutions formed band against positive antiserum in AGP test (Table 2).

◦ Cell fusion and specificity of MCA : Out of the initial 1017 hybridomas produced in 8 separate fusions, only 3 were found to be positive (Tables 3 and 4). The fusion rate was improved when mouse ascitic macrophage was laid a day before cell fusion. Three hybridomas secreting MCA

Table 1. Growth rate* of infectious laryngotracheitis virus(82-33-7) in different cells

Cell	Dose $TCID_{50}$	Material	Titer in days(Log $TCID_{50}$/ml)* 1	2	3	4	5
CEK	5.0	Cell	ND	3.4	4.2	3.2	4.2
		Fluid	ND	3.2	4.2	3.3	4.2
CEL	5.0	Cell	0	0	0	2.1	2.1
		Fluid	0	0	0	2.1	2.1
CAM	5.0	CAM(20%)	0	1	4.9	4.6	6.6
		AF	ND	ND	2.9	3.4	5.2

*Cell culture fluid was removed and cells harvested with tryptose phosphate broth in 1ml per bottle(2^{oz}) were kept at -20℃ until assay was made, when the stored cells were thawed and sonic treated for 2 minutes and used as it is for assay.

ND : Not done

Table 2. Density and agar gel precipitation (AGP) titer of purified infectious laryngotracheitis virus

Lot No	Material used	Density of purified virus	AGP titers
1	CEK	1.26 - 1.28	2
2	CAM	1.27 - 1.29	4
3	CAM	1.26 - 1.28	4

Table 3. Results of cell fusion experiments

No. of fusion	Feeder cells	Spleen cells	Wells growing hybridoma	No. of hybridomas producing anti-ILTV antibody
4	−	1×10^{8}	405	0
4	+	1×10^{8}	612	3

specific to ILTV were all produced in microplates with feeder cells.

Ascitic fluid obtatined from mouse injected with MCA producing hybridoma cells was found to contain antibody titer fo 10^{5} to 10^{6} in IFA. All the MCA produced had no neutralizing ability against any ILTV tested (Table 5). The MCAs were all classified as IgG isotype. When CEK cells infected with ILTV were stained with IFA using the MCA, bright mass fluorescence was seen mainly in the nucleus of the syncytium cells around the CPE area. In the cytoplasm of some cells, bright fluorescence was also occasio-

Table 4. Specificity of monoclonal antibodies to infectious laryngotracheitis virus(ILTV)

Virus	Cells cultured	Specificity of clones 1	2	3
ILT(AW)	CEK	+	+	+
ILT(82-33-7)	CEK	+	+	+
NDV(KJW)	CEF	−	−	−
NDV(B_1)	CEF	−	−	−
IBV(Mass)	CEF	−	−	−
IBRV(Colorado)	MDBK	−	−	−
MDV(MDV 19)	CEF	−	−	−
HVT(FC 126)	CEF	−	−	−
JEV(Anyang)	BGK	−	−	−
None	CEK	−	−	−
None	CEF	−	−	−

NDV : Newcastle disease virus
IBV : Infectious bronchitis virus
IBRV : Infectious bovine rhinotracheitis virus
MDV : Marek's disease virus
HVT : Herpesvirus of turkeys
JEV : Japanese encephalitis virus
CEK : Chicken embryo kidney
CEF : Chicken embryo fibroblast
MDBK : Madin-Darby bovine kidney cell
BGK : Black goat kidney cell
( ) : Name of strain

Table 5. Characterization of monoclonal antibody to infectious laryngotracheitis virus

Clones	IFA titer	Neutralizing	Area of antigen detected	Isotype
1	10^{5}	0	Intranucleus and cytoplasm	IgG
2	10^{6}	0	〃	IgG
3	10^{5}	0	〃	IgG

IFA : Indirect fluorescent antibody

nally noticed mainly around the nucleus (Fig. 1). Occasionally nucleus of single cells scattered away from the area of CPE was also seen stained with IFA.

Figure 1. Chicken embryo kidney cells infected with ILTV, stained by indirect, fluorescent antibody(IFA) method using monoclonal antibody to ILTV(100X)

◦ Detection of viral antigen from chickens by IFA test with MCA : Viral antigen was detected in IFA with MCA beginning from 3 days to 10 days PI from tracheas (Fig. 2) of experimental chickens (Table 6). From conjunctiva, antigen could be detected from 3 to 7 days (Fig. 3). From other organs tested ILTV antigen was not detected during the period of 10 days PI except for lung from which antigen was detected at 3

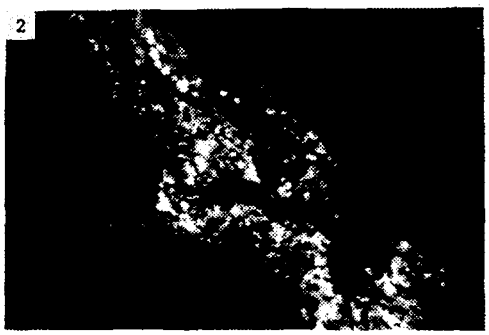

Figure 2. Tracheal smears stained by IFA. The trachea was taken from the chicken 7 days after infection with ILTV(50X)

Table 6. Detections of infectious laryngotracheitis virus infection from various organic smears by indirest fluorescent antibody test using monoclonal antibody

Organs	Days Post-infection						
	0	1	2	3	5	7	10
Trachea	−	−	−	+	+	+	+
Conjunctiva	−	−	−	+	+	+	±
Lung	−	−	−	+	+	−	−
Liver	−	−	−	−	−	−	−
Spleen	−	−	−	−	−	−	−

− : Negative + : Positive
± : Weak Positive

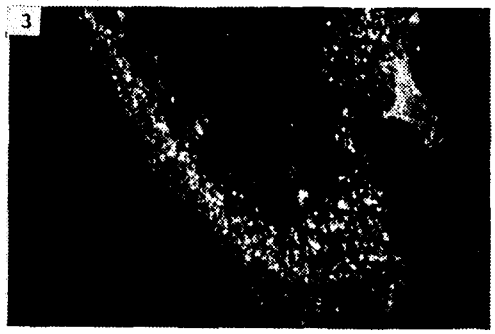

Figure 3. Conjunctival epithelial cell smear(50 X)

and 5 days PI.

◦ Comparison of diagnositc efficiency : Detection rate of ILT in ILTV infected chickens was higher in IFA test with MCA than in IFA test with PCA or in HE staining for inclusion body. The difference in detection rate was more marked at 7 days PI than at 4 days PI (Table 7). All the 3 methods did not detect any positive cases to ILTV in chickens infected with NDV in control groups.

Table 7. Comparison of diagnostic efficiency among the test methods for the detection of infectious laryngotracheitis(ILT) from tracheal smears

Virus infected	No. of birds	Days PI	Methods	No. of birds +	±	−
ILTV	10	4	IFA(MCA)	10	0	0
	10	4	IFA(PCA)	7	2	1
	10	4	HE	6	2	2
ILTV	10	7	IFA(MCA)	8	1	1
	10	7	IFA(PCA)	4	2	4
	10	7	HE	1	2	7
NDV	10	4	IFA(MCA)	0	0	10
	10	4	IFA(PCA)	0	0	10
	10	4	HE	0	0	10

* ILTV used was a virulent strain, 82-33-7 and NDV strain was Kyojungwon.
PI : Post infection
IFA : Indirect fluorescent antibody staining
MCA: Monoclonal antibody
PCA : Polyclonal antibody(Positive chicken serum)
HE : Hematoxylin Eosin stain

Ⅳ. DISCUSSION

Primary aim of this experiment was to improve the diagnostic techniques presently used by introducing MCA against ILTV and 2nd was to search a way of differentiation between pathogenic and apathogenic or low pathogenic strains. Although the second aim has not been achieved the first aim can be met with the 3 hybridomas. Ascitic fluid obtained from mice injected intraperitoneally with these hybridoma cells contains antibody titers high enough to be utilized in IFA or indirect ELISA. As shown in comparative tests for the detection of ILTV from simple tracheal smears, the conventional IFA test with PCA was imporved considerably by usig MCA to ILTV. When MCA was used not only the detection rate was improved but also the picture of staining become much clearer and nonspecific staining was much less than that seen in IFA with PCA.

Recently ELISA method has been developed to detect antibody to ILTV (Meulemans and Halen 1982). The measurement of antibody level will help estimating vaccine response or infection history in areas free of ILT. However, antibody detection alone can not be used as a diagnostic method since both vaccine and field virus produce similar type and level of antibody in chickens. Therefore, to improve diagnosis by ELISA, antigen detection rather than antibody should be able to carry out. Regadless of virulence of the viruses once viral antigens are detected during the course of clinical respiratory signs the birds can be considered as positive to ILT provided that the abscence of other agents causing respiratory diseases are checked. For this purpose this MCA can be utilized.

Although HE staining for inclusion body has certainly some merits such as rapid and requiring no expensive equipment like fluorescent microscope or ELISA reader, as far as sensitivity and accuracy concern it is not comparable to IFA or ELISA as demonstrated in the present study.

To develop a method by which strain differences in pathogenicity could easily be determined further works are needed.

Ⅴ. SUMMARY

A total of 3 hybridoma clones producing monoclonal antibody (MCA) against infectious laryngotracheitis virus (ILTV) was established by somatic cell hybridization between mouse myeloma cells and spleen cells from mice immunized with ILTV.

The MCAs were screened by the indirect flourescent antibody (IFA) staining and the specific hybridomas were cloned by limiting dilution method. The MCAs produced by the 3 hybriomas were all classified as immunogloblin G and found to be reacting against common antigen(s) of high and low pathogenic ILTV examined. The titer of these antibodies in mouse ascitic fluid

was from 10^5 to 10^6. Indirect fluorescent antibody test using these antibodies was found to be quite effective for the detection of ILTV from infected chickens being the most sensitive among the test methods adopted.

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