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CHARACTERIZATION OF ANTIGENIC REGION SITES ON 32 KD CAPSID PROTEIN OF INFECTIOUS BURSAL DISEASE VIRUS USING MONOCLONAL ANTIBODY

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Five monoclonal antibodies (Mabs) directed against infectious bursal disease virus strain P3009 were produced and characterized. All five Mabs were found to immunoprecipitate 32 Kd capsid protein, however, none of them neutralized virus infectivity. Antibody competitve binding assays demonstrated the existence of at least three antigenic regions, A, B, and C, of which the regions A and B are overlapping. The binding of Mabs to this protein did not be affected after the viral proteins were denatured, indicating that the binding reaction to 32 Kd protein with all five Mabs prepared in this study was not conformation-dependent.

Key Words: **Infectious bursal disease virus (IBDV), 32 Kd capsid protein, monoclonal antibodies, antibody binding site.**

Infectious bursal disease virus (IBDV) has been shown to be the causative agent of young chickens with severe inflammatory changes in cloacal bursae followed by immunosuppression [1,17]. The virus consisted of two-segmented double-stranded RNA and 4 capsid proteins (90Kd, 40Kd, 32 Kd and 28 Kd) and a protein precurosr (47 Kd)[3,9,13]. The 90 Kd protein associated with virus genone and could be a RNA dependent RNA polymerase[12]. Monoclonal antibody (Mab) with neutralizing activities has been shown to bind with 40 Kd protein[2,7], and therefore, this protein was considered to be a major host protective immunogen. The antibody to 32 Kd protein did not have any protective effect in chicken[2]. In this paper we described the production of Mabs directed against 32 Kd protein of IBDV strain P3009 isolated in Taiwan. The antibody binding site on this prorein was mapped by the competitive binding assays. The effect of denaturation of 32 Kd capsid protein on the capacity for antibody attachment was analyzed using these antibodies.

MATERIALS AND METHODS

Virus and Virus Purification

IBD virus strain P3009 used in this study was originally isolated from bursa of chicken exhibiting severe inflammatory lesions using chicken embryo (CE) cells as described previously[8]. Virus was partially

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purified by fluorocarbon extraction followed by isopycnic ultracentrifugation in CsCl density gradient as described elsewhere[9]. The viruses banding at a buoyant density of 1.33 g/ml were withdrawn for further studies.

Production and Characterization of Monoclonal Antibody

IBD virus strian P 3009 was used to immunize mice for Mab production. Each of Balb/C mice was primed, intraperitoneally, with 100 ug of purified virions emulsified with complete Freund's adjuvant and boosted at 4 th week with the same amount of antigens and 4 days before the mice were sacrificed, they were boosted again, intravenously, with 0.1 ml of purified intact virions (100 ug). The methods for the preparation and subcloning of hybridoma lines were followed the procedures described by Hadas and Theilen[5]. The ascites fluids were made in Balb/C mice primed with pristane. Hybridoma culture supernatants and mouse ascites fluids were screened for antibody in an ELISA. Further confirmation for virus specific binding Mabs was performed by indirect fluorescent antibody (IFA) staining. Isotype determination of hybridoma antibodies present in the individual ascites fluids of mouse was performed by ELISA with a ZYMED-Mab kit[7].

Serology and Immunoprecipitation

Virus neutralization (VN) of Mabs was performed in microtiter plates based on the methods described by Lee and Lukert [10]. Specific bindings of Mab to viral protein were carried out by immunoprecipitation of viral proteins labeled with (35 S) mehionine[13].

Competitive Binding Assay

Mabs were purified from ascites fluids using an affinity column of protein-A Sepharose CL-4B (Pharmacia, Uppsala, Sweeden) as preuiously described [4]. Immunoglobulin fractions were coupled to horse-radish peroxidase (HRPO) by periodate method[16]. Eventually, the HRPO-coupled antibodies were isolated by a Sephadex G-200 column. Antibody binding and titers of ascites fluids were determined by ELISA following the procedures described by Lee[7]. The endpoint titers of Mabs were determined from the point at which the antibody-binding curves crossed the average absorbance attributed to the non-specific binding of the conjugated antibody for each plate and the level of maximal binding was obtained on the resulting antibody-binding curve.

The procedures for monoclonal antibody copetitive binding assays (CBA) was followed the procedures as described preiously[7]. Both HRPO-conjugated Mab and the unconjugated competing antibody were used for competiton assay. The percentages of competition were calculated using the formula 100 (A-n)/(A-B) described by Kimura-Kurado and Yasui[6]. Competition at the plateau was rated as strong (+) if greater than 70%, significant (±) if between 30 and 50% and negative (-) if less than 30%.

Immuno-Dot Binding Assay

Antigens used for this assay were prepared from density gradient-purified virions either by dilution with TNE buffer (0.01 M Tris, 0.1 M NaCL, 0.01 M EDTA; pH, 7.6) or by boiling for 3 min in a solution contanining 2% SDS and 5% 2mercaptoeth-

anol (2-ME) or one of them. Antigens with approximately lug spotted onto nitrocellulose strips were probed with Mab and the binding reaction was detected by HRPO-labeled goat anti-mouse IgG (H+L) serum and substrate as previously described[14].

RESULTS

Production and General Characterization of Monoclonal Antibodies

Culture fluids from 34 of 173 wells of microtiter plates exhibited positive reactivities in ELISA at 10 to 14 days after cell fusion. The hybridomas from 37 of the 43 ELISA-positive wells were stabilized by 3 to 4 passages and were subcloned 2 to 3 times in 96-well microtiter plates at the limiting dilution. Five Mabs, 3C1, 1E9, 1A9, 4C5 and 2G10, were determined by immunoprecipitation of (35 S) methionine-labeled viral specific proteins. When CE cells infected with IBDV-P3009 for 2 h and then labeled with (35 S)methionine for 4 h were lysed, one protein with molecular weights of 32 Kd was precipitated by all selected five Mabs as indicated in Fig. 1. Examination of the isotypes of Mabs showed that all Mabs were of IgG cloass. Four Mabs (3C1, 1A9, 4C5 and 2G10) were IgG2a, whereas only one Mb (1E9) was from the IgG2b subclass (Table 1). All of them were unable to neutralize virus-P3009 infectivity. Their ELISA titers of ascites fluids ranged from $10^{5.0}$ to $10^{6.6}$ (Table 1).

Nature of Antibody Binding Site on 32 Kd Protein

The binding of Mabs tested in immuno-dot binding assays was made to determine if the need for a native structure of 32Kd protein for antibody attachment. Fig. 4. showed that all Mabs bound to antigens of IBDV-P3009 in their native structure, i. e., antigens prepared in TNE buffer. After denaturation of antigens by boiling in both SDS and 2-ME or in one of them, the Mabs retained their binding properties to the proteins.

Avidity of Monoclonal Antibodies to 32 Kd Protein and Mapping of Antibody Binding Sites

An approximate measure of relative avidity of mabs for their respsctive proteins could be obtained as the absorbances

Table 1. General Characteristics of Monclonal Antibodies Directed against 32 Kd Capsid Protein

Hybridoma line	Isotype Specificity	Ascites IgG (mg/ml)[a]	ELISA titer (log 10)[b]	Neurtalization titer (log 10)[c]
3C1	IgG 2a	8	5.3	0.3
1E9	IgG 2b	10	6.0	0
1A9	IgG 2a	6	5.6	0
4C5	IgG 2a	9	6.6	0.6
2G10	IgG 2a	7	5.0	0

[a] Total IgG as determined by BIO-RAD standard assay procedure.
[b] Ascites fluids.
[c] Reciprocal of the highest dilution of Mab (Ascites fluids) which neutralize 200 TCID50/ml of IBDV.

reached at plateau reflected amount of Mabs bound to IBDV[11,15]. Binding curves of Mabs used in competitve binding assay were made according to the parellel ELISA titrations of the binding characteristics of 32 Kd Mabs to IBDV. All Mabs apparently saturated IBDV at dilutions from $1:10^{2.2}$ to $1:10^{3.4}$ as shown in Fig. 2. To determine whether the Mabs could be grouped into clusters recognizing the same antigenic region of the 32 Kd capsid protein all Mabs listed in Table 1 were conjugated to HRPO and used as probes in CBA. Conjugation had no apparent adverse effect on their binding capacity. Their end point titers ranged from approximately $10^{2.2}$ to $10^{3.4}$ (data not shown). Binding of HRPO-conjugated Mabs was inhibited approximately greater than 87%in the presence of saturating unlabeled homologous antibody. Each antibody was used both as a competitor and as a HRPO-conjugated probes. Growth

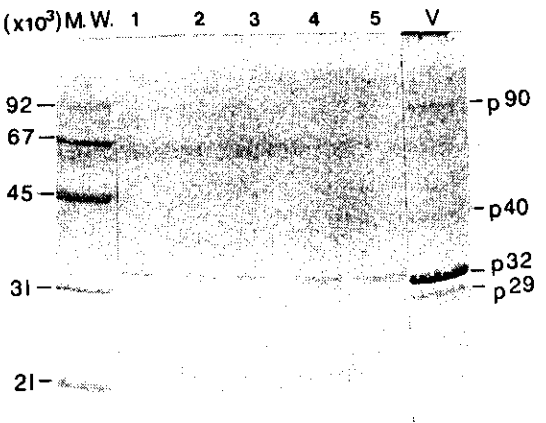

Fig. 1. Specificity of IBDV-P3009 monocloncl antibody for the capsid protein. Protein was immunoprecippitated with ascites fluids of Mads from the lysates of CE cells infected with IBDV-P3009 and labeled with (35 S) methionine (10 uci/ml) for 4 h starting at 2 h after infection. The immunoprecipitates were electrophoresed on SDS-PAGE and protein bands were fluorographed. Lanes 1 to 5 represented the momoclones used 3C1, 1E9, 4C5 and 2G10, respectively. Lane V represented virion capsid proteins. Viral capsid proteins precipitated as designated by "P" followed by molcular size in kilodaltons (arrows).

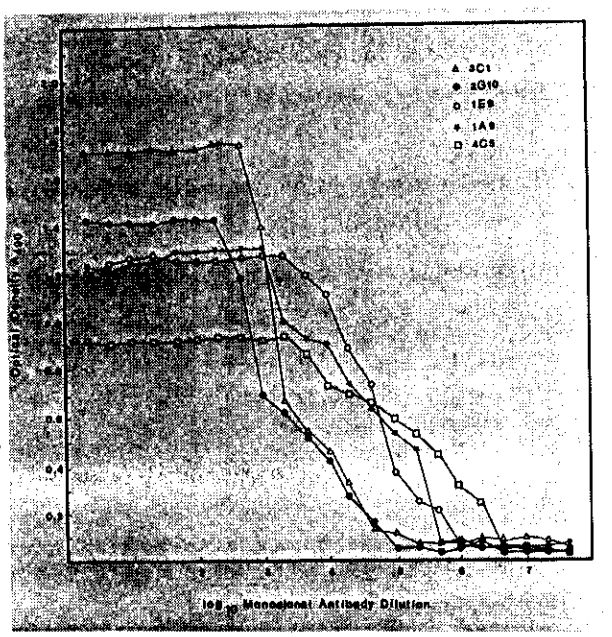

Fig. 2. Avidity assay of monoclonal antibodies to the IBDV-P3009 capsid protein. Serial two fold dilutions of monoclonal antibodies were allowed to react with antigen (IBDV-P3009) absorbed on the wells of microtiter plates. After removal of unbound antibodies the bindings of antibodies were detected by addition of HRPO-labeled rabbit anti-mouse IgG (H+L). The resulting optical density A490 was read and used to rate Mabs for their avidity.

medium concentrated by ammonium sulfate precipitation was used as control. Three antigenic regions on 32 Kd capsid protein could be defined by CBA (Fig. 3 and Table 2). Mabs, 3C1, 1E9 exhibited reciprocal bining competition and delineated one region which was designated A. Mabs 4C5 defined a second region which was named B and was overlapping with region A. Another region C could only be bound with Mab 2G10 and was separared from other two regions.

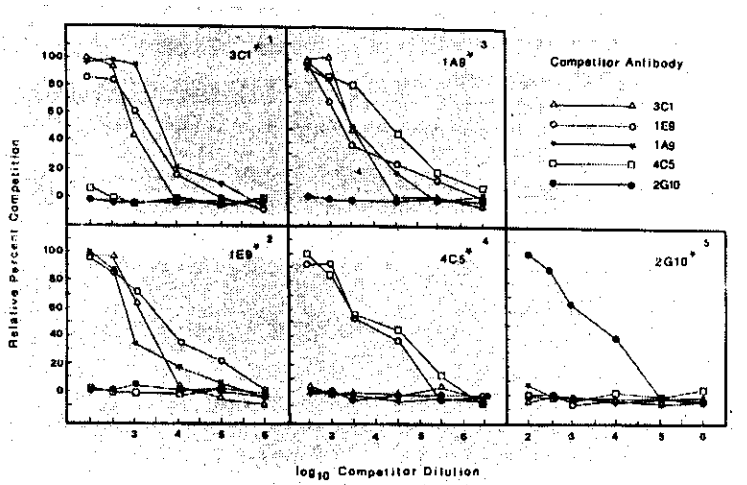

Fig. 3. Competitive binding assay with HRPO-conjugated monoclonal antibodies to the 32 Kd protein of IBDV-P3009. Purified virions were absorbed in the well of microtiter plates. The plates were incubated with HRPO-labeled Mabs to which various dilutions of the competing antibodies had been added. The exent of blocking by competitor antibody of conjugated probe was calculated and expressed as relative percentage competition as described in text. The percentage of competition was estimated from OD490 in the presence of competitor compared with that in the absence of competitor as described in text. HRPO-conjuated (•) antibodies were Mabs 3C1 (1), 1E9 (2), 1A9 (3), 4C5 (4), and 2G10 (5).

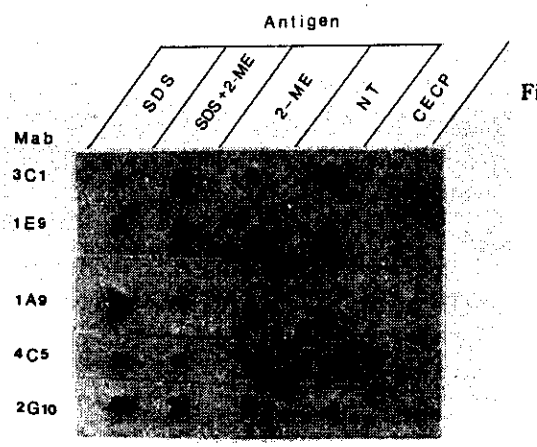

Fig. 4. Immuno-dot binding assay of monoclonal antibodies to IBD virions in their native (NT) or denaturated states (SD-2ME). Approximately lug purified virions were dotted on nitrocellulose strips and probed with an amount of Mab giving twice maximal binding. The binding was visualized with a peroxidase-labeled second antibody as described in text. Chicken embryo cell preparation (CECP) was applied for negative control.

Table 2. Results of Competitive Binding Assay between Monoclonal Antibodies to 32 Kd Capsid Protein

Competitor	Peroxidase -labeled monoclonal antibody					Epitope
	3C1	1E9	1A9	4C5	2G10	
3C1	+	+	+	−	−	
1E9	+	+	+	−	−	A
1A9	+	+	+	+	−	
4C5	−	−	+	+	−	B
2G10	−	−	−	−	+	C

competition more than 70%, + ; competition between 30 and 70, ±;competition less than 30%, −.

DISCUSSION

The results in this paper showed that the purified intact virions of IBDV P3009 could induce the production of Mabs. Five Mabs were isolated for further studies. They were determined by ELISA and IFA followed by VN tests (Table 1.). All Mabs did not exhibit virus neutralization activites. This result was similar to the findings described by Bacht et al.[2],that the capsid protein with molecular weights of 32 Kd was bound by Mabs which did not neutralize virus infectivity. The specific binding of Mabs to viral capsid protein was determined by immunoprecipitation of virus-infected cell lysates. All Mabs bound to a 32 Kd protein in its native conformation as shown in Fig. 1. The effect of denaturation of capsid prtein treated with SDS and 2-ME on the antibody attachment was also characterized. When protein conformation was altered by SDS and 2-ME or either one of them and then carried out in immuno-dot binding assays, the binding of Mab to denaturated proteins was observed (Fig. 4). In fact, similar results were obtained when proteins was treated with SDS and 2-ME, electrophoresed on PAGE and probed with Mabs (data not shown). Therefore, the antigenic sites recognized by these Mabs was apparently not dependent on the conformation of the protein.

Five Mabs were used in CBA to analyze the numbers of epitopes on 32 Kd protein of IBDV-P3009. Epitopes defined by CBA were based on the assumption that if two epitopes were close to each other, the binding of a Mab to one of the epitopes will sterically hinder the binding of another Mab to the other epitope. Two Mabs bound to the same epitope would completely inhibit binding activites each other in a manner related to their relative antibodies and concentration [11,15]. Five Mabs directed to 32 Kd protein clearly delineated three antigenic regions, A, B and C of which regions A and B were overlapping. No competition was observed between Mab 2G10 and others, suggesting that region C was far from regions A and B. In conclusion, at least 3 antigenic regions exist in 32 Kd capsid protein of IBDV-P3009 strain. All Five Mabs prepared in this study bound to this protein do not neutralize IBD virus and

their binding reactivities are not conformation-dependent. In addition, previous studies[7,2] have demonstrated that Mabs against 40 Kd capsid protein of IBD virus could neutralize virus infectivity. Hence, development of a subunit vaccine of IBD contatining 40 Kd capsid protein which may induce protective antibodies in chickens seems to be possible.

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以單源抗體鑑定家禽傳染性華氏囊病毒32 Kd蛋白衣之抗體結合位

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以細胞融合技術生產5株抗家禽傳染性華氏囊病病毒P 3009株之單源抗體，其中4株屬IgG 2a，另一株則爲IgG2b。所有5株單源抗體均能沈澱32 Kd蛋白衣，但均無中和病毒之能力，以此5株單源抗體做抗體競爭結合試驗，結果顯示32 Kd蛋白衣至少含有3個抗體結合位，分別以A、B及C示之。而結合位A及B則有部份重疊。當病毒蛋白衣以變性化學試劑加以處理，該等抗體仍能與之結合，顯示本試驗所分離之5株單源抗體和32 Kd蛋白衣之結合並不因蛋白衣3度空間結構之改變而失去與之結合之能力。

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