Comparison of Four Distinct Idiotypes of Monoclonal Antibodies against Hen's Egg Ovomucoid

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Two monoclonal antibodies (*mAb 41B3* and *mAb 51B3*), directed against hen's egg ovomucoid (OM) and different from those we previously reported (*mAb 23E5* and *mAb 32A8*), were prepared and purified. A competitive radioimmunoassay using ^{125}I -labeled OM showed that both *mAb 41B3* and *mAb 51B3* could bind all three homologous domains (domains I (DI), II (DII) and III (DIII)) of OM and that they reacted most efficiently with DIII. To analyze the paratope specificities of these four mAbs, a sandwich assay and a competitive radioimmunoassay were done. Only the pair *mAb 41B3* and *mAb 51B3* could not simultaneously bind the OM or domains in the sandwich assay. Only *mAb 41B3* inhibited the binding of *mAb 51B3* to antigens and *vice versa* in the competitive radioimmunoassay. These results suggest that *mAb 41B3* and *mAb 51B3* recognized a closely related site distinct from the epitopes for *mAb 23E5* or *mAb 32A8*. These mAbs may be useful for general studies on epitopes of protein antigens as well as for analyses of the antigenic determinants of OM.

Although hen's eggs are considered a good food, they contain proteins that cause allergic reactions in some humans, particularly in children.¹⁾ Ovomucoid (OM), a glycoprotein in egg white, is a major allergen of eggs.²⁾ The primary structure of OM is known³⁾; it consists of three homologous domains (domain I (DI), domain II (DII), and domain III (DIII)) that have heterogenous sugar chains.⁴⁾

To study the antigenic sites of OM, we had prepared, purified, and characterized two monoclonal antibodies (*mAb 23E5* and *mAb 32A8*) against OM.⁵⁾ Both mAbs could bind to every domain of OM, but their affinity was not equal. *MAb 23E5* bound more efficiently to fragments containing DIII than to fragments without DIII. *MAb 32A8* reacted most efficiently with DIII-2 (with carbohydrates) and least efficiently with DIII-1 (free from carbohydrates) in all the domains. These results suggested that *mAb 23E5* mainly recognized a region on the polypeptide chain of DIII and that *mAb 32A8* might have recognized a region located on the carbohydrate moiety of

OM.

However, these two mAbs cannot by themselves reveal all the antigenic structures of OM. For this paper, we have prepared, purified, and characterized two new mAbs (*mAb 41B3* and *mAb 51B3*) distinct from the previous two. To test whether the mAbs recognized different epitopes on OM, a sandwich assay and solid-phase competitive radioimmunoassay (RIA) were used. These assays could determine whether the two mAbs could simultaneously bind to one ovomucoid molecule (*i.e.*, whether they recognized the distinct epitopes) or not (*i.e.*, whether they could recognize the same epitope or two close sites). We found that *mAb 41B3* and *mAb 51B3* could bind to closely related sites (containing the same site) different from the epitopes for *mAb 23E5* or *mAb 32A8*.

In this paper, we describe the preparation of the new mAbs specific for OM, their reactions with ovomucoid and its domains, and their paratope specificity.

MATERIALS AND METHODS

Mice. Mice (BALB/c) were purchased from Charles River Japan Inc.

Antigens. Eggs of White Leghorn hens were obtained from the National Institute of the Animal Industry, Ibaraki, Japan. OM was prepared from the eggs by the method of Fredericq and Deutsch,⁶ and purified by DEAE-Sephacel ion-exchange chromatography. Domains (DI, II, III-1, III-2, I-II, II-III) were prepared by combining enzymic digestion (using *Staphylococcus aureus* V.8 protease from Miles Laboratories)⁷ and chemical cleavage of the peptide bonds (using cyanogen bromide),⁸ and were then purified by gel filtration on Bio-Gel P-30 or affinity chromatography on trypsin-Sepharose 4B.⁹ Reduced and carboxymethylated OM (RCM-OM) was obtained by the method of Konigsberg.¹⁰

Immunization. Four- to six-week-old female BALB/c mice were immunized intraperitoneally with 100 µg of purified OM in complete Freund's adjuvant (CFA). They were boosted with a similar dose in incomplete Freund's adjuvant (IFA) after 2 weeks, and then intravenously injected without any adjuvant 3 days before cell fusion.

Cell fusion. Cells were fused by the general method of Köhler and Milstein.¹¹ Spleen cells of immunized BALB/c mice were harvested and hybridized with X63-Ag.8-6.5.3 mouse myeloma cells (1/10 of the number of spleen cells), using 45% polyethyleneglycol (M.W. 3350) at 37°C. The cells were seeded in 24-well culture plates (1.0×10^6 spleen cells/well) and were fed 14 days after hybridization in HAT medium for HAT selection. The supernatants from the growing culture were tested for antibody activity by an enzyme-linked immunosorbent assay (ELISA)¹² with OM as the coated antigen. Hybridomas producing anti-OM antibodies were cloned twice by limiting dilution with normal thymocytes as feeders. The cloned anti-OM antibody-producing lines were grown by tissue culture, and some were grown as a tumor in BALB/c mice to obtain antibody-enriched ascites fluids. The ascites fluids were stored frozen at -80°C, after they had been centrifuged to remove precipitates.

Purification of monoclonal antibodies. Monoclonal antibodies were purified from mouse ascites fluids using an Affi-Gel protein A monoclonal antibody purification system (Bio-Rad Laboratories).

Properties of monoclonal antibodies. Each mAb was isotyped by immunodiffusion¹³ using several anti-mouse immunoglobulin sera; their pIs were found by isoelectric focusing (IEF).¹⁴

Specificity of monoclonal antibodies. The affinity of the mAbs for the domains or RCM-OM was compared with that for OM by solid-phase competitive RIA using ¹²⁵I-labeled OM as previously reported.⁵

Paratope specificity of monoclonal antibodies. To test whether the mAbs recognized different epitopes on OM or not, a sandwich assay and solid-phase competitive RIA were used. The second antibodies in both assay (10 µg of mAb 32A8, 41B3 and 51B3) were iodinated with 0.2 mCi ¹²⁵I using chloramine T as the oxidant.¹⁵

Sandwich assay. The wells of flexible microtiter plates (Falcon) were filled with 100 µl of a suitable concentration of one mAb in phosphate-buffered saline (PBS) for 2 hr. The plates were then rinsed three times with PBS containing 0.05% Tween-20 (PBS-Tween) and treated with 125 µl of 1% bovine serum albumin (BSA) in PBS for 2 hr to avoid non-specific binding. After washing with PBS-Tween, 100 µl of OM or the domains at saturating concentration in PBS were allowed to bind to the coated mAb. The plates were washed, and finally, 100 µl of a second antibody labeled with ¹²⁵I in PBS containing 0.1% gelatin (Gel-PBS) was added and incubated for 2 hr. Finally, the plates were rinsed, and the radioactivity of the second antibody bound to each well was measured by a γ-counter (Aloka).

Solid-phase competitive radioimmunoassay. Plates were coated with 100 µl of antigens (100 µg/ml) and treated with BSA as already described. Ninety µl of non-labeled mAb (competitor) serially diluted in Gel-PBS was added and mixed with 10 µl of ¹²⁵I-mAb, before being incubated for 2 hr. The plates were rinsed with PBS-Tween, and the radioactivity of each well was measured. Relative values of the bound counts (B/B_0) were calculated with the equation: $B/B_0 = B' \cdot BKG / B_0' \cdot BKG$ where B' is the ¹²⁵I-mAb (cpm) bound to coated antigens in the presence of a competitor; B_0' is the ¹²⁵I-mAb (cpm) bound to coated antigens in the absence of a competitor; and BKG is the ¹²⁵I-mAb (cpm) bound in the absence of coated antigens.

RESULTS

Characterization of monoclonal antibodies

Two monoclonal antibodies (mAb 23E5 and mAb 32A8) directed against OM had already been prepared, purified, and characterized as previously reported.⁵ To prepare new anti-OM mAbs different from these, lymphocytes from the OM-immunized BALB/c mice were hybridized, and some stable clones with specificity for OM were found. Two clones (41B3 and 51B3) were selected for further characteri-

TABLE I. PROPERTIES OF *mAb 41B3* AND *mAb 51B3*

Each mAb was isotypized by immunodiffusion using various anti-mouse immunoglobulin sera, the pIs being measured by isoelectric focusing.

mAb	41B3	51B3
Class	IgG ₁	IgG ₁
Type	κ	κ
pI	7.1	5.8

FIG. 1. Binding Curves of ^{125}I -OM to *mAb 41B3* in the Presence of Domains.

a) —●—, OM; —▲—, DI; —△—, DII; —○—, DIII-1; —□—, DIII-2. b) ●, OM; ▲, DI-II; △, DII-III; ○, RCM-OM. B/B_0 = bound ^{125}I -OM to mAb in the presence of unlabeled antigens (competitors)/bound ^{125}I -OM to mAb in the absence of competitors.

zation. Table I summarizes the properties of *mAb 41B3* (produced by clone 41B3) and *mAb 51B3* (produced by clone 51B3). Isotypic analysis showed that both mAbs belonged to the IgG₁ subclass with a κ chain. It was shown by IEF that the pI of *mAb 41B3* was 7.1, and that of *mAb 51B3* was 5.8.

Specificity of monoclonal antibodies

The specificity of both mAbs was studied by solid-phase competitive RIA using various

FIG. 2. Binding Curves of ^{125}I -OM to *mAb 51B3* in the Presence of Domains.

a) —●—, OM; —▲—, DI; —△—, DII; —○—, DIII-1; —□—, DIII-2. b) ●, OM; ▲, DI-II; △, DII-III; ○, RCM-OM. B/B_0 = bound ^{125}I -OM to mAb in the presence of unlabeled antigens (competitors)/bound ^{125}I -OM to mAb in the absence of competitors.

concentrations of OM and the domains to inhibit the binding of ^{125}I -labeled OM to each mAb (Figs. 1 and 2). An increasing concentration of each unlabeled domain resulted in initially decreased binding, and finally fully inhibited the binding of ^{125}I -OM to each mAb. Thus, both mAbs could react with every domain of OM, but the affinities of the antibodies for the domains were not equal. Table II shows the values of I_{50} calculated from the ratio of the concentration of each domain needed to inhibit 50% of the binding to ^{125}I -OM to the concentration of unlabeled OM required to give the same inhibition. Lower values of I_{50} mean higher affinities of mAb for the domain. The values of I_{50} for three mAbs (23E5, 41B3, and 51B3) resembled each other and, particularly, I_{50} of *mAb 41B3* for each domain was very similar to that of *mAb 51B3*. Either *mAb 41B3* or *mAb 51B3* bound to fragments containing DIII (DIII-1 (free from carbohy-

TABLE II. I_{50} OF THE DOMAINS

The values of I_{50} were calculated from the ratio of the molar concentration of each domain needed to inhibit 50% of the binding to ^{125}I -OM to the molar concentration of unlabeled OM required to give 50% inhibition of the binding to ^{125}I -OM. The I_{50} values of *mAb 23E5* and *mAb 32A8* are cited from our previous report.

mAb	23E5	32A8	41B3	51B3
OM	1	1	1	1
DI	25.0	813	139	130
DII	14.7	39.8	24.7	25.9
DIII-1	0.28	2140	0.49	0.70
DIII-2	1.00	9.33	0.75	0.75
DI-II	24.2	1.07	3.80	3.86
DII-III	0.59	78.6	0.70	0.60

drates), DIII-2 (with carbohydrates), and DII-III) more efficiently than to fragments without DIII (DI, DII, and DI-II). These results raise the possibility that both *mAb 41B3* and *mAb 51B3* may mainly be directed against the polypeptide chain of DIII, and that these two mAbs recognize the same antigenic region. Neither *mAb 41B3* nor *mAb 51B3* could bind much to RCM-OM (Figs. 1 and 2) and it was found that they recognized the conformation of the antigen as well as did *mAb 23E5* and *mAb 32A8*.

Association constants of monoclonal antibodies

The association constants (K_a) for the two mAbs for OM were found from Scatchard plots (Fig. 3); the K_a for *mAb 41B3* was 1.26×10^8 l/mol and for *mAb 51B3*, 2.29×10^8 l/mol. These values are higher than that for *mAb 23E5* ($K_a = 2.20 \times 10^7$ l/mol). The K_a for *mAb 32A8* could not be calculated as the Scatchard plot did not give a straight line.

Sandwich assay

To find whether the four mAbs (*23E5*, *32A8*, *41B3*, and *51B3*) recognized the same epitope on the ovomucoid molecule, a sandwich assay and solid-phase competitive RIA were done. In the sandwich assay, one of the four mAbs was fixed in the wells at first. When exposed to antigens (OM, DI, DII, DIII-1, DIII-2, DI-II, or DII-III), the coated mAb would bind to

FIG. 3. Scatchard Plots of Two Monoclonal Antibodies against OM.

●, *mAb 41B3* $Y = -1.26 \times 10^8 X + 0.316$; ○, *mAb 51B3* $Y = -2.29 \times 10^8 X + 0.561$; B/F = bound/free ^{125}I -OM; b = amount of bound OM.

restricted regions on the antigen molecule. Then, a ^{125}I -labeled mAb (the second mAb) was added, and this would only bind to the antigen molecules that were fixed on the solid phase (bound to the coating mAb) and had the epitope specific for the second mAb but different from the coating mAb. Thus, the radioactivity remaining in each well allowed detection of the simultaneous binding of two antibodies on the antigen molecules. Low radioactivity would generally mean that the antibodies did not simultaneously bind because the epitopes on the antigen molecule directed against two antibodies were the same or near. On the other hand, high radioactivity would indicate that the antibodies could simultaneously bind because the epitopes directed against the antibodies were distinct. Table III demonstrates the results of the sandwich assay using ^{125}I -labeled *mAb 32A8*, *41B3*, and *51B3*. When the solid phase antibody and the second antibody were the same, or the two antibodies were *mAb 41B3* and *mAb 51B3*, or *mAb 32A8* was used as the solid phase or the second antibody in presence of DIII (DIII-1 or DIII-2), the radioactivity was low.

Solid-phase competitive radioimmunoassay

The results of a competitive analysis of the four anti-OM mAb by RIA using ^{125}I -labeled mAbs (*32A8*, *41B3*, and *51B3*) are presented in

TABLE III. SANDWICH ASSAY OF FOUR MONOCLONAL ANTIBODIES

Plates coated with one of the four mAbs (solid phase mAb) were incubated with each domain. (a), OM; b), DI; c), DII; d), DIII-1; e), DIII-2; f), DI-II; g), DII-III) Then, a 125 I-labeled mAb (one of *mAb 32A8*, *mAb 41B3* and *mAb 51B3*) was added and incubated. The radioactivity remaining in each well was measured.

a)	Solid phase Ab			
	23E5	32A8	41B3	51B3
Labeled Ab				
32A8	2971	9	10967	10188
41B3	8427	9871	130	27
51B3	11247	8892	16	0

b)	Solid phase Ab			
	23E5	32A8	41B3	51B3
Labeled Ab				
32A8	4020	2	6720	8895
41B3	8899	7093	45	37
51B3	14081	9673	9	0

c)	Solid phase Ab			
	23E5	32A8	41B3	51B3
Labeled Ab				
32A8	4779	9	7235	7224
41B3	9037	4986	18	35
51B3	12891	7399	11	39

d)	Solid phase Ab			
	23E5	32A8	41B3	51B3
Labeled Ab				
32A8	0	4	8	0
41B3	9029	58	0	80
51B3	13163	0	12	32

e)	Solid phase Ab			
	23E5	32A8	41B3	51B3
Labeled Ab				
32A8	0	0	0	0
41B3	10434	115	53	52
51B3	13847	20	6	0

f)	Solid phase Ab			
	23E5	32A8	41B3	51B3
Labeled Ab				
32A8	2101	0	10122	6851
41B3	11958	9611	51	27
51B3	11684	4263	0	0

g)	Solid phase Ab			
	23E5	32A8	41B3	51B3
Labeled Ab				
32A8	5100	0	4392	4902
41B3	9045	8842	297	52
51B3	14264	12612	26	0

FIG. 4. Solid-phase Radioimmunoassay of the Paratope Specificity of Anti-OM Monoclonal Antibodies.

OM coated plates were incubated with serial dilutions of each mAb (Δ , *mAb 23E5*; $\circ$, *mAb 32A8*; $\blacktriangle$, *mAb 41B3*; $\bullet$, *mAb 51B3*) and constant amounts of 125 I-mAb (a), *mAb 32A8*; b), *mAb 41B3*; c), *mAb 51B3*). When 1) the labeled and coated mAbs were the same and 2) the two antibodies were *mAb 41B3* and *mAb 51B3*, higher inhibitions were shown.

FIG. 5. Solid-phase Radioimmunoassay of the Paratope Specificity of Anti-OM Monoclonal Antibodies. Plates coated with one of the domains (a), DI; b), DII; c), DIII-1; d), DIII-2; e), DI-II; f), DII-III) were incubated with serial dilutions of each mAb (Δ , *mAb 23E5*; $\circ$, *mAb 32A8*; $\blacktriangle$, *mAb 41B3*; $\bullet$, *mAb 51B3*) and constant amount of ^{125}I -*mAb 41B3*. Both *mAb 41B3* and *mAb 51B3* inhibited the binding of ^{125}I -*mAb 41B3* to antigens.

FIG. 6. Solid-phase Radioimmunoassay of the Paratope Specificity of Anti-OM Monoclonal Antibodies. Plates coated with one of the domains (a), DI; b), DII; c), DIII-1; d), DIII-2; e), DI-II; f), DII-III) were incubated with serial dilutions of each mAb (Δ , *mAb 23E5*; $\circ$, *mAb 32A8*; $\blacktriangle$, *mAb 41B3*; $\bullet$, *mAb 51B3*) and constant amount of ^{125}I -*mAb 51B3*. Both *mAb 41B3* and *mAb 51B3* inhibited the binding of ^{125}I -*mAb 51B3* to antigens.

Figs. 4, 5, and 6. When the labeled and unlabeled mAbs were the same, increasing the quantity of a competitor allowed 100% of the binding activity of labeled mAb to the antigens to be inhibited. When they were not the same, various degrees of inhibition were found. Both *mAb 41B3* and *mAb 51B3* caused high inhibition, but little inhibition was shown by other pairs. It is generally concluded that a high inhibition means the same or resembling paratope specificities of two mAbs, and that a low inhibition means distinct paratope specificity. These results are consistent with those of the sandwich assay.

DISCUSSION

The antigenic structures of OM have already been studied using rabbit antiserum and mouse mAb (23E5 and 32A8) directed against OM.^{5,16-19} Ovomucoid is a glycoprotein with heterogenous carbohydrates and consists of three homologous domains that each have three intramolecular disulfide bonds. The epitope structures of OM are closely related to these features. From our previous work, we concluded that 1) one antibody specific for OM can bind all three domains, although the affinities are not equal, that is, each domain has common epitopes because of their homology; 2) almost all the anti-OM antibodies recognize the conformation of antigens; 3) the specific anti-OM antibodies have generally the highest association constant for DIII, then DII > DI; and 4) the sugar chains are important in binding OM to some of the antibodies, that is, it is likely that some of them recognize the region on OM containing the carbohydrates.

For this paper, we prepared, purified, and characterized two mAbs (41B3 and 51B3) different from those previously reported. Both *mAb 41B3* and *mAb 51B3* reacted most efficiently with DIII in the three domains and also had slightly more affinity for DIII-1 (free from carbohydrates) than for DIII-2 (with carbohydrates). These results suggest that they mainly recognize a region on the polypeptide

chain of DIII.

From Scatchard plots we calculated the *K_a* of each mAb for OM, with *K_a* values for *mAb 23E5*, *41B3*, and *51B3* of 2.20×10^7 , 1.26×10^8 , and 2.29×10^8 l/mol, respectively. These three mAbs were similar in their binding to OM and the domains, but not identical. The Scatchard plot for *mAb 32A8* did not give a straight line, probably because the sugar chains of OM were concerned in the binding of *mAb 32A8* to OM, which consisted of heterogenous carbohydrates.

To analyze the paratope specificity of the four mAbs directed against OM, the sandwich assay and solid-phase competitive RIA were done. These two quite different assays gave similar results. Only the mAb pair of *41B3* and *51B3* could not simultaneously bind the antigens in the sandwich assay. Only *mAb 41B3* inhibited the binding of *mAb 51B3* to OM or the domains and *vice versa* in the solid-phase competitive RIA. However, these two assays did not give similar results when DIII was used as the antigen. An excess of *mAb 32A8* did not inhibit the binding of DIII to *mAb 41B3* or *mAb 51B3* in the solid-phase competitive RIA. These results indicate that *mAb 41B3* and *mAb 51B3* recognized regions distinct from the binding site to *mAb 32A8*, although the sandwich assay showed that the mAb pair of *32A8* and *41B3*, or that of *mAb 32A8* and *mAb 51B3*, did not simultaneously bind DIII. According to the analysis of paratope specificity, the four mAbs can be classified into three groups that are directed against distinct epitopes of OM; that is, *mAb 41B3* and *mAb 51B3* belong to the same group distinct from *mAb 23E5* or *mAb 32A8*. However, it is not clear by these two assays whether *mAb 41B3* and *mAb 51B3* recognized exactly the same antigenic site, overlapping sites, two close sites, or a conformational-dependent distinct site. Ovomucoid consists of three homologous domains, and each domain has common epitopes. Therefore, it is possible that two of the same mAbs could bind to one ovomucoid molecule simultaneously, but this was not shown by these assays.

Protein antigens generally have several antigenic sites on one molecule. At present, it is not clear whether one antigenic site is correlated to another antigenic site on the same molecule or not. For example, studies using mAb against distinct regions of the protein antigens insulin²⁰⁾ and myoglobin²¹⁾ showed that an anti-idiotypic antiserum to one mAb inhibited antigen binding by another mAb. Thus, these mAbs binding to distinct sites of one protein antigen have been found to express shared idiotypes. These results suggest that these epitopes on one antigen are related to each other. A mAb specific for horse cytochrome *c*,²²⁾ however, did not express shared idiotypes. It is said from this result that these epitopes are completely distinct as well as those of other protein antigens in their immune response.

We have now prepared four mAbs specific for OM that recognize at least three distinct epitopes of OM as just described. These mAbs may be useful for general studies on epitopes of protein antigens as well as for analyses of the antigenic determinants of OM.

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