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Genetic control of immune response to staphylococcal nuclease. XII: Analysis of nuclease antigenic determinants using anti-nuclease monoclonal antibodies

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Summary

SJL mice, which are high responders to Staphylococcal nuclease (nuclease), were immunized and used to produce hybridoma cell lines secreting anti-nuclease monoclonal antibodies (mAb). Ten stable clones were derived from a single fusion. Seven of these produced antibodies of the IgG_1,κ isotype and were more precisely characterized for antigenic specificity. Only one hybridoma cell line (54–10–4) produced anti-nuclease antibodies capable of inhibiting enzymatic activity of nuclease. Binding inhibition analyses strongly suggest that the other monoclonal antibodies, which failed to inhibit nuclease activity detect two different antigenic regions, or epitopes, of the molecule: epitope cluster 1 domain is defined by hybridomas 54–2–7, 54–5–2, 54–9–8, and 54–10–8; epitope cluster 2 by 54–5–1 and 54–1–9. Because of its capacity to inhibit nuclease enzymatic activity mAb 54–10–4 was considered specific for a third epitope of the nuclease molecule called epitope 3. Binding studies of these monoclonal antibodies were extended to peptide fragments of the nuclease molecule in order to examine possible cross-reactions with such fragments, as has previously been reported for antibodies purified from polyclonal antisera. Monoclonal antibodies specific for epitope cluster 1 on the native molecule also bound to the fragments 1–126 and 49–149 but failed to bind to fragment 99–149, suggesting that the corresponding epitope(s) is determined by amino acids localized between residues 49 and 99. The epitope clusters 2 and 3 appeared to be expressed only on the native molecule. Monoclonal antibodies of different clusters exhibited very different migration patterns on isoelectric focusing while monoclonal antibodies of the same cluster were indistinguishable, which suggests that they may have originated from the same B cell precursor. Taken together these data suggest that this panel of monoclonal antibodies detects at least three distinct epitopes of the nuclease molecule, one of which could be involved in the determination of the enzymatic site.

Introduction

Genetic control of the immune response by genes mapping in the major histocompatibility complex (Ir genes) has been reported for numerous antigens, including both synthetic polypeptides and natural proteins (1, 2). Because of its well-defined chemical properties, the protein enzyme Staphylococcal nuclease (nuclease) has been used for many immunological studies (3, 4, 5). Nuclease consists of a single polypeptide chain of 149 amino acids (6, 7), the three-dimensional structure of which is known (8). Moreover, numerous peptide fragments of nuclease can be produced by various cleavage procedures (9, 10), and because of the absence of disulfide bonds some of these fragments are capable of assuming the necessary conformation to bind anti-native antibodies (3). In inbred strains of mice, animals de-

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rived from strains of H-$2^{a,k,d,s}$ haplotypes are high responders to a single immunization of nuclease, while those of H-$2^{b,q}$ haplotypes are low responders, defining the Ir gene Ir-Nase (11). However, both high and low responder mice produce anti-nuclease antibodies when hyperimmunized (12). Non H-2 linked Ir genes have been shown to control this hyperimmune response to nuclease (13).

Idiotypic analysis of antibodies produced by different strains of mice in response to nuclease immunization has revealed extensive cross reactions (14). It is unknown, however, whether similar germline V gene(s) are responsible for the structural homologies on which such idiotypic cross-reactions depend. Recently, analyses of isoelectric focusing patterns have shown heterogeneity among the subpopulations of anti-nuclease antibodies expressing a common idiotypic marker (15). A potential limitation of these experiments resides in the polyclonal nature of the idiotypes analyzed and the lack of precise information on their specificity. The use of hybridoma technology might therefore provide appropriate reagents to extend these investigations at the clonal level. In addition, anti-nuclease monoclonal antibodies (mAb) could be advantageously used to analyze the anti-nuclease idiotypic repertoire.

The present investigation describes the characterization of seven such anti-nuclease mAbs. These mAb distinguish individual nuclease antigenic sites (epitopes). Certain of these epitopes appear to be expressed only on the native molecule while others are also found on some fragments of nuclease. Finally, binding to one of these epitopes inhibits nuclease enzymatic activity.

Experimental procedures

Animals

SJL/J mice were purchased from the Jackson laboratory (Bar Harbor, ME) and maintained in our animal facilities.

Immunization and fusion

Adult female SJL mice were injected i.p. with 100 μg of purified nuclease mixed 1:1 (v/v) with H37 RA Complete Freund Adjuvant (CFA, Difco, Detroit, MI) in a final volume of 0.3 ml. Mice were then boosted twice at 2 week intervals and 2 days after the second boost spleen cells from one animal were fused with Ig non secretor SP2/0 Ag14 myeloma cells in the presence of polyethylene glycol 1000 using methods previously described (17). Hybridomas were screened for production of anti-nuclease antibodies by an ELISA assay as described below, and cloned by limiting dilution in the presence of irradiated (3 000 rads) rat fibroblasts.

Isolation and purification of mAbs

Determination of immunoglobulin isotype was performed by Ouchterlony double diffusion in agar using Goat and Rabbit anti-Mouse class-specific antisera (Bionetics, Kensington, MD). All hybridoma cell lines derived from fusion 54 were found to secrete IgG_1,κ products. Despite the fact that IgG_1 murine antibodies are generally not thought to bind to protein A, all of these monoclonal antibodies were found to exhibit such binding. This may reflect the fact that these antibodies arose from SJL mice, while most previous studies of the binding of various immunoglobulin classes have been carried out on antibodies from BALB/c mice. Accordingly, these mAbs were readily purified from culture cell supernatant or ascitic fluid by protein A Sepharose (Pharmacia, Uppsala, Sweden) chromatography, followed by elution in 4 M guanidine hydrochloride and dialyzing against PBS. Anti-mouse MHC mAbs, 74.C (IgG_3), 10-2.16 (IgG_{2b}) and 11-4.1 (IgG_{2a}) used as negative contrtols, have been previously described (17, 18).

Antibody reagents

SJL (5 809) anti-nuclease antibodies to be used as positive binding controls in this study were obtained by repeated immunizations of mice with nuclease as previously described (12). Antisera were affinity purified by absorption to an elution from nuclease-Sepharose columns using 4 M guanidine hydrochloride as eluant and dialyzing against PBS. SJL anti-lysozyme antibodies (7 209) were purified on lysozyme-Sepharose. Covalent coupling of nuclease and lysozyme to Sepharose was performed as previously described (19). SJL nl Ig (6 132) was purified from sera by protein A-Sepharose chromatography, followed by elution of the IgG binding

fraction using 4 M guanidine hydrochloride and dialyzing against PBS. Alkaline phosphatase and peroxidase conjugated RAM IgG were purchased from Cappel Laboratories (Cappel, Cochranville, PA). Purified antibodies were biotinylated using biotin-N-hydroxy-succinimide ester according to published procedures (20).

Antibody-mediated nuclease inactivation assay

The spectrophotometric assay of anti-nuclease antibodies has been previously described (21). Briefly, 20 μl of a 5 μg/ml solution of nuclease was mixed with 20 μl of a 1 mg/ml solution of purified antibodies or mAb preparation and incubated 5 min at room temperature. 10 μl of this mixture was then added to a spectrophotometer cuvette containing excess of substrate (1.0 ml of heat-denatured DNA in 0.025 M Tris buffer pH 8.8 containing 0.01 M calcium), conditions under which the slope of OD_{260} is linear with time. Each sample was tested in triplicate. The change in OD_{260} was recorded on a multiple sample recording spectrophotometer, and the percentage of inhibition of enzymatic activity was calculated.

ELISA assays

Direct binding assay. Immunoplates (A/S Nunc, Roskilde, Denmark) were coated with 1 or 2 μg of nuclease or peptide fragments (in an equivalent molar amount) per well, incubated at 4 °C for 24 hr, washed 3× in PBS–0.05% Tween 20 and incubated for 1 hr at room temperature with varying amounts of monoclonal antibodies from 0.1–24 μg per well. After washing, the wells were incubated for an additional 1 hr with 200 μl of RAMIg-peroxidase (1:400 dilution, which represent an excess of developing reagent). After washing (4×) and incubation for 20 min with 200 μl of substrate solution (OPD 0.01% in H_2O containing methanol and H_2O_2) (excess of substrate), enzymatic reaction was stopped by adding 50 μl of 8 N H_2SO_4 solution per well. The binding was evaluated by measuring absorbance at 492 nm on an ELISA-plate reader. Hybridoma culture supernatants (100 μl per well) were screened for nuclease binding activity using the same assay. Some of these experiments were performed using biotin-labeled mAbs. In these assays 200 μl of 1:400 diluted commercial solution of avidin-alkaline phosphatase (Miles-Yeda LTD, Israel) was added to the wells containing the biotinylated mAb for 1 hr, the plate was washed, and then incubated with substrate: Sigma phosphatase substrate no. 104–40T 1 mg/ml in diethanolamine pH 9.8 (Sigma, St Louis, MO). The binding was evaluated by reading of absorbance at 405 nm. The majority of ELISA-assay data are scored on a zero to ten scale using Matrix analysis (Titertech Multiskan, Flow Laboratories, McLean, VA).

Inhibition of mAb binding assay. Various amounts of competitor mAb (titrated from 12–25 000 ng) were incubated for 1 hr in a nuclease coated plate. The plate was then washed 3× and incubated for an additional 1 hr with 200 ng of biotinylated mAb. After further washing binding was detected using avidin-alkaline phosphatase and substrate as above.

Nuclease and fragment preparation

Staphylococcal nuclease was prepared as previously described (22). Nuclease fragments 1–126, 127–149, 6–48, 49–149, 99–149 and 99–113 were prepared by trypsin or CNBr digestion of nuclease according to published methods (23, 24). Lysozyme was obtained from W.B.C. (Worthington Biochemical Corp, Freehold, NJ).

Isoelectric focusing

Analytic isoelectric focusing was performed as previously described (15) using a vertical slab gel apparatus (Hoeffer Scientific Instruments, San Francisco, CA). Briefly, 10 μl samples were loaded onto a 5% polyacrylamide gel containing 2% pH 5–9 carrier ampholytes (Pharmalyte, Pharmacia, Uppsala, Sweden). After 18 hr migration (1.5 MA constant current), focused proteins were fixed and labeled with ^{125}I radiolabeled nuclease, (4×10^6 cpm/gel). Autoradiograph was exposed for 3 days to Kodak X-O mat film.

Results

Derivation of hybrid cell lines producing anti-nuclease mAbs

Fusion of SJL anti-nuclease spleen cells with SP2/0 cells led to vigorous hybridoma growth in

116 of 960 wells after 2–3 weeks of culture. The corresponding culture supernatants were screened by direct binding to Staphylococcal nuclease using an ELISA assay. Anti-nuclease activity was exhibited in 13 wells. The anti-nuclease hybridomas were cloned by limiting dilution and 10 stable clones were obtained, 7 of which were more precisely characterized.

The results in Fig. 1A show the direct binding titration of biotinylated mAbs to nuclease coated plates. In this experiment most of the mAbs showed approximately the same binding activity with the exception of mAb 54–10–4, which showed a significantly weaker binding activity. Specificity of binding to nuclease is shown by the failure of these mAbs to bind to lysozyme, a protein of size and charge comparable to nuclease (Fig. 1B).

Inhibition of nuclease enzymatic activity

Inhibition of nuclease enzymatic activity assay has been used in the past to study the anti-nuclease activity of polyclonal antisera, and this assay was shown to be extremely sensitive. As shown in Table 1, antibodies purified from hyperimmune SJL anti-nuclease sera inactivated nuclease enzymatic activity as described previously (11). Normal SJL Ig was used as a negative control and showed no significant inhibition. Surprisingly, only one of the seven mAbs was able to inactivate nuclease enzymatic activity. In addition, the only inactivating mAb was 54–10–4 which had shown the lowest titer for nuclease binding by ELISA (3.1). The inhibition detected using 54–10–4 appeared to be real but was low compared to the inhibition obtained using the

Table 1. Inhibition of nuclease enzymatic activity by anti-nuclease mAbs.

Inhibitor sample added to nuclease	Nuclease activity $\Delta O.D._{260}$/min	Percentage of inhibition of nuclease enzymatic activity
saline	0.070 ± 0.006	0
54–1–9	0.074 ± 0.002	–5.7
54–2–7	0.073 ± 0.001	–4.3
54–5–1	0.072 ± 0.002	–2.7
54–5–2	0.071 ± 0.001	–1.4
54–9–8	0.069 ± 0.001	1.4
54–10–4	0.044 ± 0.001	37.1
54–10–8	0.068 ± 0.002	2.8
5 809[a]	<0.001	≃100
6 132[b]	0.071 ± 0.001	–1.4

[a] 5 809: SJL anti-nuclease Ig affinity purified on nuclease.
[b] 6 132: protein-A purified SJL N1 Ig.
Experiment was performed as described by incubating 20 μl of purified Ig (at a concentration of 1 mg/ml) with 20 μl of a 5 μg/ml solution of nuclease, then adding 10 μl of this mixture to DNA substrate solution, and recording the change in $O.D._{260}$.

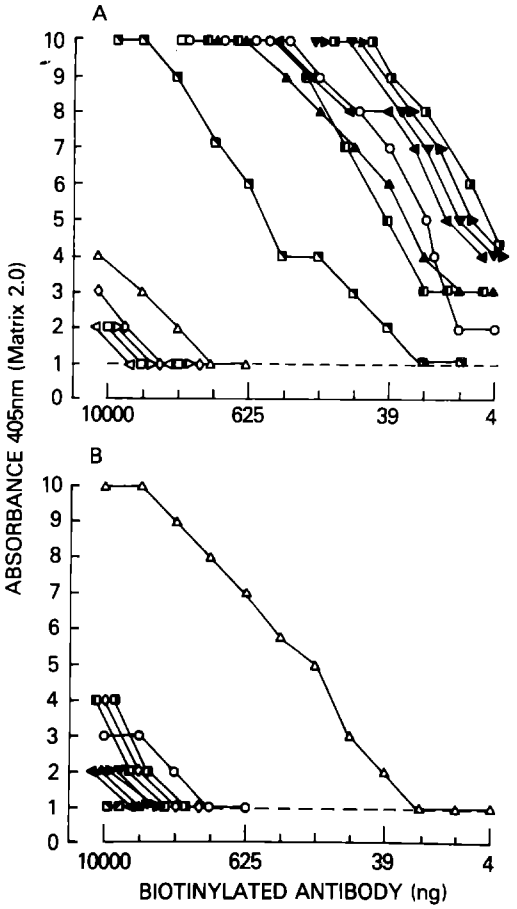

Fig. 1. Titration curves of direct binding of biotinylated anti-nuclease mAbs to nuclease (1.A) and lysozyme (1.B) coated ELISA-plate. Plates were coated with 2 μg of nuclease or lysozyme per well, then successively, biotinylated mAbs (in a range of 4–10 000 ng) and 200 μl of avidin-alkaline phosphatase (1:400) were added. Each step consists of a 1 hr incubation, and wells were washed between steps. The last step was a 5 min incubation with 200 μl alkaline phosphatase substrate (1 mg/ml) and absorbance reading at 405 nm. Symbols have been used as follows: 54–10–8 (▼), 54–9–8 (▲), 54–2–7 (▶), 54–5–2 (◀), 54–10–4 (◥), 54–1–9 (◨), and 54–5–1 (◧). Positive and negative antigen-binding controls [respectively SJL anti-nuclease Ig (○) affinity purified on nuclease, SJL N1 Ig (◇), SJL anti-lysozyme Ig affinity purified (△), and non anti-nuclease mAbs (anti-MHC mAbs) 10–2–16 (◁), 11–4–1 (▷), and 74C (□)], biotinylated in the same conditions were presented. The straight horizontal lines (– – –) show the background of absorbance when in the first step of incubation PBS was used instead of mAb. 10 on Matrix scale = 2.0 A_{405} units.

same concentration of SJL-anti-nuclease antibodies purified from anti-nuclease sera. These data suggest that the 54–10–4 mAb defines an epitope on nuclease distinct from the epitope(s) detected by the six other mAbs.

Topographic organization of epitopes detected by anti-nuclease mAbs

Binding inhibition studies (Figs. 2 and 3), showed that four mAbs, 54–5–2, 54–2–7, 54–10–8, and

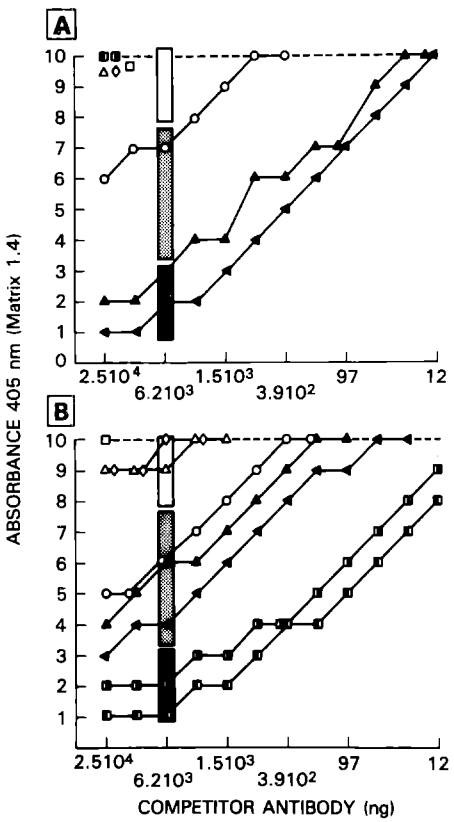

Fig. 2. Representative experiment of binding inhibition of anti-nuclease biotinylated mAbs (panel A: 54–5–2 mAb; panel B: 54–5–1 mAb) to nuclease by various anti-nuclease mAbs (respectively 54–5–1 mAb (■), 54–1–9 mAb (◨), 54–9–8 mAb (▲), and 54–5–2 mAb (◀) used as competitor. Binding inhibition using 74C (□), an anti-Ia^k mAb, was tested as negative control. Inhibition curves obtained using SJL anti-nuclease Ig (○), SJL NI Ig (◇), and SJL anti-lysozyme (△) as competitor were presented. Horizontal straight line shows the binding capacity to nuclease of A: 54–5–2 mAb and B: 54–5–1 mAb in the absence of inhibitor. The assay consisted of an 1 hr incubation of a nuclease coated plate (2 μg/well) with competitor mAb in a range of 12–25 000 ng), then after washing 100 ng of biotinylated mAb was added to the well for an additional 1 hr, and mAb binding was revealed as described in legend of Fig. 1. 10 on Matrix scale = 1.4 A_{405} units.

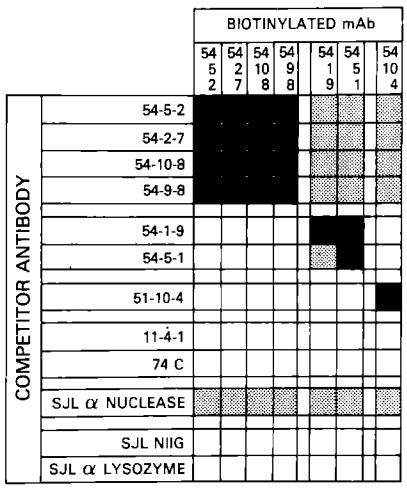

Fig. 3. Graphic summary of the competitive antibody binding inhibition analyses of 7 mAbs reacting with nuclease. A schematic representation shows the percent inhibition of binding of 100 ng of biotinylated mAb (except for 54–10–4 mAb tested at 1 μg because of low titer in direct binding assay), to nuclease using an excess of 6 200 ng (lower amount of homologous mAb giving 100% inhibition) of various anti-nuclease mAb as competitor. Assay was performed as described in legend of Fig. 2, and each representation has been deduced from repeated complete titration of a given competitor mAb. Controls have been determined as follows: the binding inhibition obtained using 6 200 ng of homologous competitor was considered as 100 percent inhibition. The binding obtained using the same concentration of non anti-nuclease mAbs 11–4–1 and 74C (anti-MHC mAbs), has been considered as 0% inhibition. □ less than 25% inhibition, ▨ partial inhibition (between 25 and 75% inhibition), ■ more than 75% inhibition.

54–9–8, all showed reciprocal inhibition of binding defining a cluster of antigenic determinants probably close to one another (called epitope cluster 1). Two other antibodies, 54–1–9 and 54–5–1, failed to inhibit the binding of any anti-cluster 1 mAb but inhibited each other reciprocally. These data suggest that 54–1–9 and 54–5–1 define a second region on the nuclease molecule, called epitope cluster 2, topographically distinct from cluster 1. As seen in Fig. 3, unidirectional blocking by anti-cluster 1 mAbs of anti-cluster 2 mAb binding was also observed. Such data could be interpreted on the basis of one epitope and different binding affinities (Ka mAb cluster 1 > Ka mAb cluster 2). However it is clear that 54–1–9 more efficiently inhibits 54–5–1 than do any of the cluster 1 mAb. Taken together such data could result from conformational changes of nuclease after interaction with anti-cluster 1 mAbs, could reflect differences in Ka of cluster 1

and 2 mAb when binding to partially overlapping epitopes, or could be dependent on other, as yet undetermined interactions.

54-10-4, as shown in Fig. 1 exhibited lower binding activity for nuclease than the other mAbs. For inhibition assays of this mAb it was not possible to work with the same amounts of biotinylated reagent as used for the other assays. Interpretable experiments were obtained using 1 μg of biotinylated mAb, under which conditions unidirectional partial binding inhibition by cluster 1 mAbs was observed. Since this mAb was the only one in the panel capable of inactivating nuclease (Table 1) it was considered to be specific for a third region of the nuclease molecule called epitope region 3.

Localization of epitopes detected on nuclease by mapping on fragments

Another appraoch to identification of molecular regions detected by the monoclonal antibodies involved the use of peptide fragments derived from the intact protein. This method requires the assumption that antigenic determinants on fragments are similar enough to the same determinants in the native conformation to permit cross-reactivity, an assumption which has previously been validated for several nuclease fragments using polyclonal antibodies. Fig. 4 shows the complete binding titration of several mAbs (cluster 1, 2, 3, and controls) to peptide fragments. As can be seen in the summary (Table 2), when immunoplates were coated with 1 μg of nuclease or nuclease peptide fragments (in equivalent molar amounts), and an excess (12 μg) of mAb was used for direct binding, epitope cluster 1 mAbs (54-2-7, 54-9-8, and 54-10-8) bound to the immunogen and also to peptide fragments 1-126 and 49-149. These mAbs failed to bind to fragments 6-48, 99-113, 99-149, and 127-149. These results suggested that epitope(s) 1 is determined by amino acids localized between residues 49 and 99. mAb 54-5-2 which was only tested on some of the fragments, was also shown to bind to a determinant expressed on the fragment 1-126 and not on fragment 99-149. Moreover, mAb 54-5-2 produced specific inhibition of binding of labeled 54-9-8 mAb to fragment 1-126 (data not shown). Epitope cluster 2 mAbs (54-1-9 and 54-5-1) failed to bind to any fragment tested, suggesting that the epitope(s) detected by these mAbs on the native molecule may be more conformation-dependent than epitope cluster 1. An alternative explanation is that cleavage results in loss of epitopes (cf Discussion). mAb 54-10-4 was only able to bind to the native molecule, which could reflect the fact that 54-10-4 reacts with an epitope located in the vicinity of the nuclease enzymatic site, a site only present on the native molecule.

Isoelectric focusing analysis

Each of the hybridoma cell lines described here was isolated from an independent fusion well. However, since they all were derived from a single

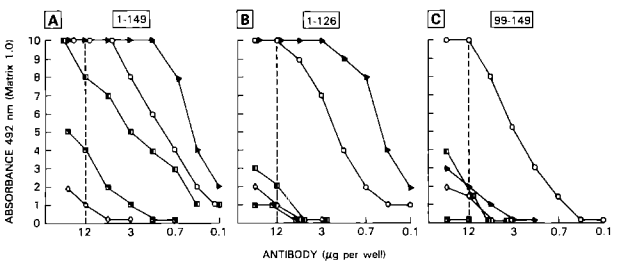

Fig. 4. Representative experiment showing the complete binding titration curves of anti-nuclease mAbs to some peptide fragments of nuclease. Experiment was performed as follows: plates were coated with 1 μg of nuclease (Fig. 4A) or an equivalent molar amount of peptide fragments 1-126 (Fig. 4B) or 99-149 (Fig. 4C), tested per well. After 1 hr incubation with antibodies, antigen binding molecules which still bind to antigen after washing were detected using RAM IgG-peroxidase/OPD enzymatic reaction. The conditions of this assay differ from assay presented in Fig. 1 since anti-nuclease antibodies are not directly biotinylated, the revealing reagent used is not the same, and plate is not coated with the same amount of antigen; in such condition all curves are shifted by several orders of magnitude as can be seen by comparing amount of reagent necessary to reach plateau binding in Figs. 1 and 3. Symbols meaning: 54-2-7 (▶), 54-5-1 (■), 54-10-4 (◥), SJL anti-nuclease Ig affinity purified (○), and SJL N1 Ig (◇). 10 on Matrix scale = 1.0 A_{492} units.

Table 2. Summary of binding of anti-nuclease mAbs to peptide fragments of the nuclease molecule.

Ig	O.D.$_{492} \times 10^3$							
	Plate coated with nuclease (1–149)	nuclease fragment						lyso-zyme
		6–48	1–126	99–149	127–149	99–113	49–149	
6 132[a]	119	265	186	139	286	173	236	252
5 809	1 251	622	1 306	1 299	1 059	1 128	1 122	183
7 209	182	272	170	169	173	146	175	547
54–10–4	356	86	93	24	91	69	48	87
54–9–8	1 395	182	1 371	259	219	173	1 526	192
54–10–8	1 362	272	1 364	218	251	237	1 541	158
54–2–7	1 565	140	1 426	186	263	255	1 680	144
54–5–2	1 492	NT	1 412	149	NT	NT	NT	155
54–1–9	756	259	158	221	162	139	268	234
54–5–1	838	244	139	199	171	147	264	213
Probe[b]	4	3	5	3	4	5	3	5

[a] 6 132: SJL N1 Ig; 5 809: SJL anti-nuclease Ig; 7 209: SJL anti-lysozyme Ig.

[b] Binding of RAM-IgG probe to antigen coated plate.

Experiments were performed as follows: plates were coated with 1 μg per well of native nuclease molecule, its fragments (in an equimolar ratio), or native lysozyme molecule. After 1 hr incubation with an excess (12 μg) of mAb or control antibodies, antigen binding molecules were detected using RAM IgG-peroxidase/OPD enzymatic reaction. Results are plotted in terms of Optical Density. Error deviation (2–4 replicates) was always lower than 15%.

fusion, and all were of the IgG_1,κ class, some of these hybridoma cell lines (those secreting antibodies mapped in the same cluster) could have been derived from a common B cell precursor. To help resolve this question, isoelectric focusing was performed (Fig. 5). Three different families of mAbs were distinguished with respect to pI values: mAbs 54–1–9 and 54–5–1 had identical patterns, and another group of 4 antibodies (54–2–7, 54–5–2, 54–9–8, and 54–10–8) all exhibited related patterns of migration, distinct from 54–1–9 and 54–5–1. The 54–10–4 mAb showed a third distinct pattern of migration. Each of these three families could therefore represent hybridomas originated from a common B cell precursor. Further analysis will be necessary to answer this question.

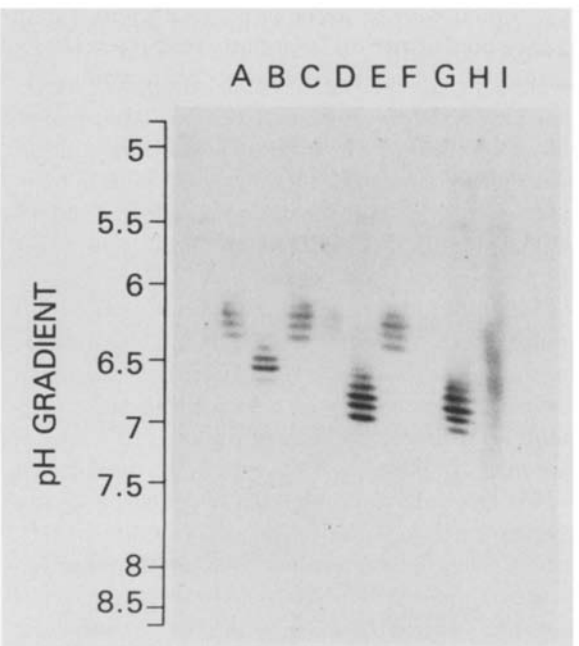

Fig. 5. Autoradiograph of spectrotypic patterns of SJL anti-nuclease mAbs. The following anti-nuclease mAbs were subjected to IEF and focused proteins were revealed by I^{125} labeled nuclease. A: 54–10–8; B: 54–10–4; C: 54–9–8; D: 54–5–2; E: 54–5–1; F: 54–2–7; G: was a negative control irrelevant for this study; H: 54–1–9; and I: 10–2–16 as negative control.

Discussion

Several monoclonal anti-nuclease antibodies were derived and characterized. Thus far, three families of antibodies could be identified on the basis of the following criteria: (1) antibodies could be clustered by binding inhibition assays as specific for one of three different epitope regions on the

nuclease molecule; (2) antibodies of the same cluster exhibited related isoelectric focusing patterns; and, (3) antibodies of the same cluster cross-reacted with nuclease fragments identically. However, studies of blocking patterns obtained using binding inhibition assays suggest a complex spatial organization for the three epitope clusters leading in some cases to unidirectional blocking. Similar asymmetric binding inhibition patterns have been described previously for anti-I-A^k monoclonal antibodies (25), although the explanation of this phenomenon remains unclear.

The panel of mAbs obtained and studied thus far does not appear to mimic a polyclonal SJL anti-nuclease response. Generally, SJL anti-nuclease sera exhibit high levels of enzyme inactivation (11) while only 1 of 7 mAbs in the panel was able to inactivate nuclease, and in this unique case inhibition was low compared to the inhibition obtained using the same concentration of heterogeneous SJL-anti-nuclease antibodies, which probably also contain non inactivating antibodies. Such differences could result from a necessity for antibodies with different fine specificities to cooperate in order to cause inhibition. Mixture of 54–10–4 with other anti-nuclease monoclonal antibodies from fusion 54 did not increase enzyme inactivation (data not shown). However cooperation may be efficient only when inactivating antibodies are mixed. Finally, it remains possible that single antibodies could inhibit enzymatic activity at the same level as polyclonal anti-nuclease antibodies, but that such antibodies are not represented in this panel.

One of the approaches used to determine whether or not these mAbs reflect the SJL anti-nuclease antibody repertoire consisted of the definition of a pattern of cross-reactivity with anti-nuclease fragments. SJL mice are high responders to nuclease fragment 99–149, very high responders to fragment 1–126, and low responders to fragment 6–48 immunization (14). In addition, the fraction of SJL anti-nuclease serum antibodies exhibiting reactivity with fragment 99–149 was shown to be lower than the fraction capable of binding to fragment 1–126 (26). Accordingly, some antibodies in this panel (anti-epitope cluster 1), were capable of cross-reacting with some peptide fragments of the nuclease molecule (i.e., 1–126 fragment). By contrast, other mAbs (anti-epitope clusters 2 and 3) which bound to the native protein, failed to bind to any of the peptide fragments tested. These results probably reflect the retention of sufficient conformation in peptide fragments to conserve certain antigen structures present on the native molecule. However some antigenic determinants may be lost after cleavage either because the cleavage site is located directly within a sequential epitope, or because the binding affinity to the altered conformation of the fragment is too low for detection. These data are in agreement with the previous demonstration that polyclonal populations of anti-nuclease antibodies can bind to peptide fragments of the nuclease molecule (27). Cross reactions of mAbs between nuclease and its fragments seem to be more common than has been observed in other immunological models in which natural proteins have been used as immunogens (reviewed in 28). This probably reflects the fact that nuclease consists of a single polypeptide chain without disulfide bonds.

Data presented here support the hypothesis that epitope cluster 1 is determined by amino acids localized in the region 49–99, a region which represents about 1/3 of the molecule by size. However the identification of amino acids involved in epitope 1 structure remains incomplete. Moreover, it is possible that some cleavage events (i.e., in position 99) could provoke a change in the three-dimensional structure of an epitope present on the native molecule (as discussed above). Such an epitope could be selectively expressed on a restricted number of peptide fragments (e.g., an epitope located in the region 99–126 might thus be detectable on fragments 1–126 and 49–149 but not on fragments 99–113 and 99–149). In addition no information concerning the location of epitope clusters 2 and 3 have been obtained by analysis of cross-reactivity with nuclease fragments. Further investigation will be necessary to complete the fine antigenic specificity definition of these mAbs.

In several model systems, proteins with high degrees of amino acid sequence homology have been purified from different species, permitting a comparative approach. For nuclease only a limited number of natural protein variants are available (Foggi, V8) (29). However, since the nuclease gene has recently been cloned (30, 31, 32), mutant molecules bearing changes in critical positions for mAb binding can now be obtained by site-directed mutation. Binding studies of the mAbs to such mutant proteins should permit precise localization of each

epitope, and such studies are now in progress. These investigations may also provide an understanding of the nature of recognition events between globular proteins through analysis of three-dimensional complementary structure interactions.

Finally, these mAbs may provide a new tool for analysis of the structural and genetic basis of idiotypic markers in the nuclease system. In addition to the anti-nuclease mAbs described here, we have also recently derived a panel of anti-nuclease mAbs from A/J and BALB/c strains. Idiotypic comparison of BALB/c (Igh-C^a; H-2^d), A/J (Igh-C^e; H-2^a) and SJL (Igh-C^b; H-2^s) derived mAbs may permit selection of mAbs expressing public markers. Corresponding hybridoma lines could be used as a source of rearranged DNA to study the organization of germ-lines genes coding for anti-nuclease immunoglobulin variable regions. Since certain of these genes have been previously mapped at a great distance (1–10 centiMorgans) from Igh-C by classical recombination studies (33, 34), they may be of considerable help in completing the map of V region genes.

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