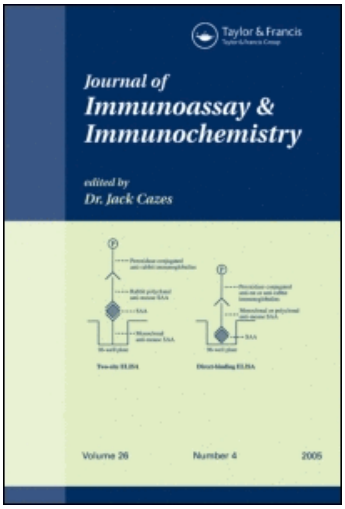

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 04 July 2004

To cite this Article Andersen, Ditte C. , Jensen, Charlotte H. , Gregersen, Annemette , Brandt, Jette , Kliem, Anette , Skjødt, Karsten , Koch, Claus and Teisner, Børge(2005) 'Screening for Epitope Specificity Directly on Culture Supernatants in the Early Phase of Monoclonal Antibody Production by an ELISA with Biotin-Labeled Antigen', Journal of Immunoassay and Immunochemistry, 25: 2, 147 — 157

To link to this Article: DOI: 10.1081/IAS-120030524

URL: http://dx.doi.org/10.1081/IAS-120030524

JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 25, No. 2, pp. 147–157, 2004

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ABSTRACT

This report describes an assay for comparison of epitope specificity in groups of monoclonal antibodies against a given antigen. The only prerequisite is the biotin-labeled antigen. One of the monoclonal antibodies is captured onto a plastic surface via a rabbit anti-mouse Ig, and the other preincubated with biotinylated antigen. When the two antibodies react with the same epitope subsequent binding of the biotin-labeled antigen is abolished (inhibition). In the cases where no inhibition was observed,

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DOI: 10.1081/IAS-120030524 1532-1819 (Print); 1532-4230 (Online)

the two antibodies were considered to react with distinct, independent epitopes. The obvious advantages using this assay, are that it can be performed directly on culture supernatants in the early phase of monoclonal antibody production, and also works for antigens with repetitive epitopes. Moreover, the bonus effect, i.e., a signal in excess of the reference signal when sets of monoclonal antibodies with different epitope specificity are compared, gives a relative measure of affinity.

Key Words: Epitope specificity assay; Culture supernatants; Monoclonal antibodies; Biotin-labeled antigen; ELISA.

INTRODUCTION

The binding of an antibody to its antigen is restricted to external accessible areas on the antigen molecule. However, the same antigen may possess several structures (epitopes or antigenic determinants)[1] to which the antibody can bind. Furthermore, individual antibodies can interact with the same epitope with different affinities and the specific interaction may involve differences in contributions from the various forces involved in the non-covalent interactions. The overall representation of structures present on the antigen that may participate in interaction with antibodies is often referred to as the antigenicity of the antigen.[2]

Previously, epitopes on an antigen were regarded as discrete areas, and several investigations were aimed to determine the exact number of epitopes on an antigen.[3] However, in recent years the concept of an epitope has changed and, very much so, due to the application of monoclonal antibodies. Epitopes are now regarded merely as a continuum of accessible structures on the outer surface of the antigen. This concept has also emphasized the phenomenon referred to as overlapping epitopes, which is defined as epitopes where binding of one antibody interferes with the binding of another, although the sites of contact can be demonstrated to be different. On the other hand, it must be emphasized that certain structural entities on an antigen appear to be dominant, as a substantial proportion of a polyclonal antibody raised in response to the antigen has specificity for these epitopes (immunodominant structures).[4]

Monoclonal antibodies are often produced with the aim to develop assays utilizing sandwich techniques for quantification of an antigen. Such assays will require two antibodies, one immobilized on a solid phase (the capture antibody), and one used to detect binding of antigen to the capture antibody (the signal antibody). The capture and the signal antibody, therefore, must interact with the antigen without interference from other individual binding sites (epitopes). Thus, when deriving monoclonal antibodies, it is important

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to obtain information about the epitope specificities of individual antibodies at an early phase of production to avoid time- and cost-consuming procedures.

The aim of the present study was to develop a simple and inexpensive ELISA technique to compare the epitope specificity of monoclonal antibodies, using the hybridoma culture supernatant directly in the early phase of production.

EXPERIMENTAL

Antigens

Fetal antigen 1 (FA1), the C-terminal propeptide of human procollagen type I (PICP) and the endometrial protein referred to as PP14 were purified as described previously.[5–7] The purified antigens were used for production of monoclonal antibodies and for the inhibition assay as described below. Biotinylation of the antigens was performed by gently mixing a solution of biotinsuccimid ester (BNHS; 40 mg/mL in dimethylsulfoxide) with antigen preparations at room temperature for 4 hr. The amount of BNHS in mg was 1/6 of the amount of antigen in mg, and excess of BNHS was removed by over-night dialysis at 4°C.

Monoclonal Antibodies

For each antigen, 3–5 CF1 × Balb/c female mice were injected subcutaneously with 5–25 μg of purified antigen. Injections were repeated at intervals of 14 days until the antibody titers were high. Animals with the highest titers were selected for fusion and boosted in the tail vein with 5–10 μg purified protein. Fusions were performed according to Köhler and Milstein,[8] as modified by Reading,[9] with the myeloma cell line SP2/0-Ag14 using PEG 4000 as fusogen. Positive clones were identified as described below. Cells from wells containing antigen-specific antibodies were recloned three times using the limiting dilution method.[10] The resulting hybridomas were further propagated in Nunclon Multidishes (Nunc, Denmark) until tested in the inhibition assay.

ELISA Techniques

Antigen-Specific ELISA

Maxisorp® flatbottom microtiter plates (Nunc, Denmark), in which the antigens were immobilized indirectly via their respective polyclonal rabbit antibodies, were used for testing antibody titers in mouse serum prior to fusion, and for the

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selection of positive clones. Microtiter plates were coated (overnight, 4°C) with affinity purified rabbit anti-FA1, anti-PICP, or anti-PP14 preparations diluted in carbonate buffer pH 9.6. Following wash (PBS pH 7.4, 0.05% Tween 20), the plates were saturated with antigen by incubation with amniotic fluid (diluted 1 : 100 in washing buffer) overnight at 4°C. After washing, a dilution series of serum samples from the immunized mice were applied (100 μL/well) and incubated at 4°C overnight in order to determine the antibody titers. Plates were further processed by incubation (30 min, room temperature) with HRP labeled rabbit anti-mouse Ig, (P260, DAKO, Denmark; diluted 1 : 1000), followed by the addition of substrate and chromogen (H_2O_2, OPD). The enzyme-catalyzed color development was stopped after 15 min by the addition of 1 M H_2SO_4, and plates read at 492 nm.

Assay for Epitope Specificity (Inhibition Assay)

Plate 1: Maxisorp® flat bottom microtiter plates (96-well) were coated with 100 μL rabbit anti-mouse immunoglobulin (Z109, DAKO, Denmark) diluted to 2 μg/mL in carbonate buffer (pH 9.6) overnight at 4°C. Plates were subsequently washed and each row (A-H: one supernatant per row) received 100 μL supernatants (diluted 1 : 2), from the hybridomas to be analyzed (overnight at 4°C; separate plates for separate antigens). The following day plates were washed and residual mouse immunoglobulin binding sites saturated with 100 μL normal mouse serum (diluted 1 : 100) for 2 hr at room temperature.

Plate 2: In non-absorbing round bottom microtiter plates (96-well, Nunc, Denmark), 100 μL of the same hybridoma supernatants were incubated with 100 μL biotinylated antigen overnight at 4°C (column 1–8: one supernatant per column). The number of columns for each antigen and monoclonal antibody was equal to the total number of rows in plate 1. Column number 9 and 10 received only biotinylated antigen (100 μL) and growth medium (100 μL) to determine the maximal signal (i.e., the signal from which inhibition was calculated), and column number 11 and 12 received 100 μL growth medium and 100 μL dilution buffer (assay blank).

One hundred microliter from each well in plate 2 was transferred to plate 1 and incubated at room temperature for 2 hr. After washing 100 μL HRP-streptavidin (diluted 1 : 4000, Zymed Lab., CA) was added to each well and plates were further processed as described above, using H_2O_2 and OPD as substrate and chromogen, respectively.

RESULTS

The results of analysis of 11 anti-FA1 producing hybridomas in the inhibition assay for epitope specificity are shown in Table 1. The anti-FA1

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Table 1. The epitope specificity assay performed on culture supernatants with monoclonal antibodies against FA1.

Solid phase MAb (1st MAb)	Liquid phase MAb (2nd MAb)										
	F12	F15	F30	F59	F31	F32	F33	F38	F54	142-1	142-2
F12	**23***	226	344	226	117	112	120	117	104	275	243
F15	106	**17***	**13**	**31**	79	81	79	79	78	121	113
F30	89	**17**	**12***	**30**	92	76	74	73	74	104	98
F59	102	**14**	**12**	**25***	78	79	76	77	75	126	123
F31	114	109	133	96	**18***	**19**	**18**	**18**	**15**	145	137
F32	114	115	139	101	**17**	**18***	**16**	**17**	**14**	151	142
F33	116	109	127	95	**22**	**23**	**22***	**21**	**20**	144	137
F38	116	107	126	93	**21**	**20**	**20**	**20***	**18**	142	132
F54	115	107	126	93	**21**	**22**	**20**	**21**	**18***	144	136
142-1	173	256	312	215	109	107	106	104	91	**21***	**18**
142-2	183	277	335	233	114	113	111	109	96	**21**	**20***
	Gr. 1	Gr. 2			Gr. 3					Gr. 4	

Note: The results are listed as percentage of the reference signals. Asterisks show results where solid and liquid phase antibodies were identical, and bold values represent results where antibodies were considered to have identical epitope specificity.

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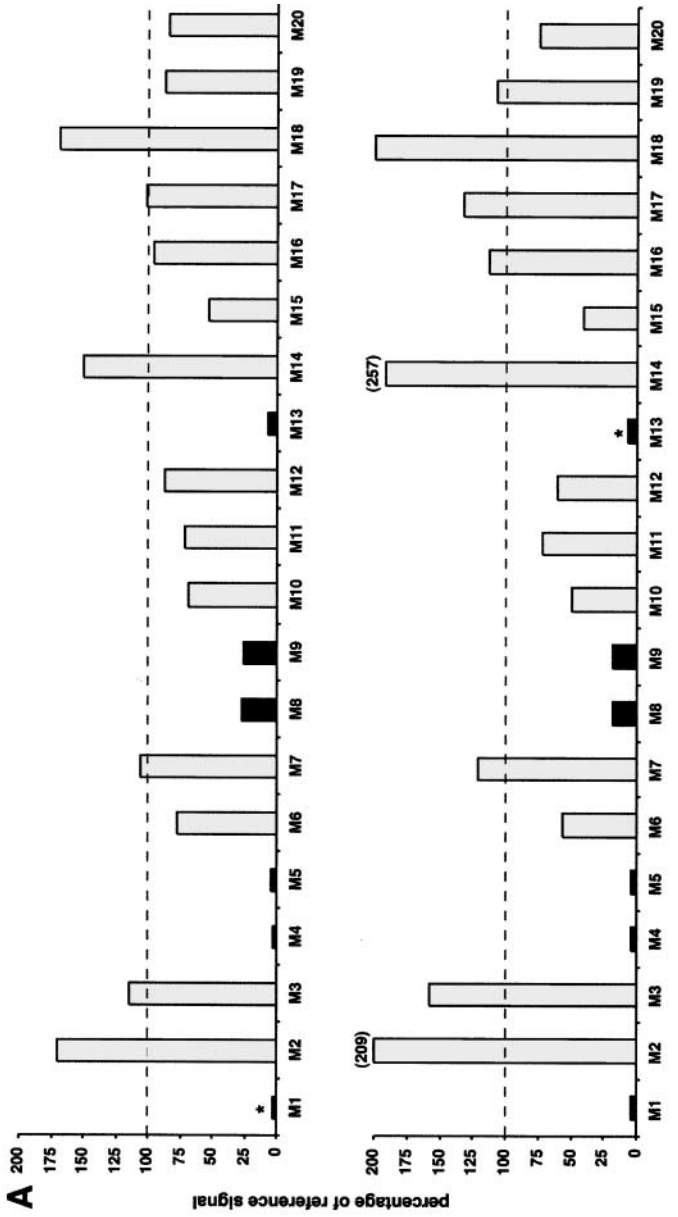

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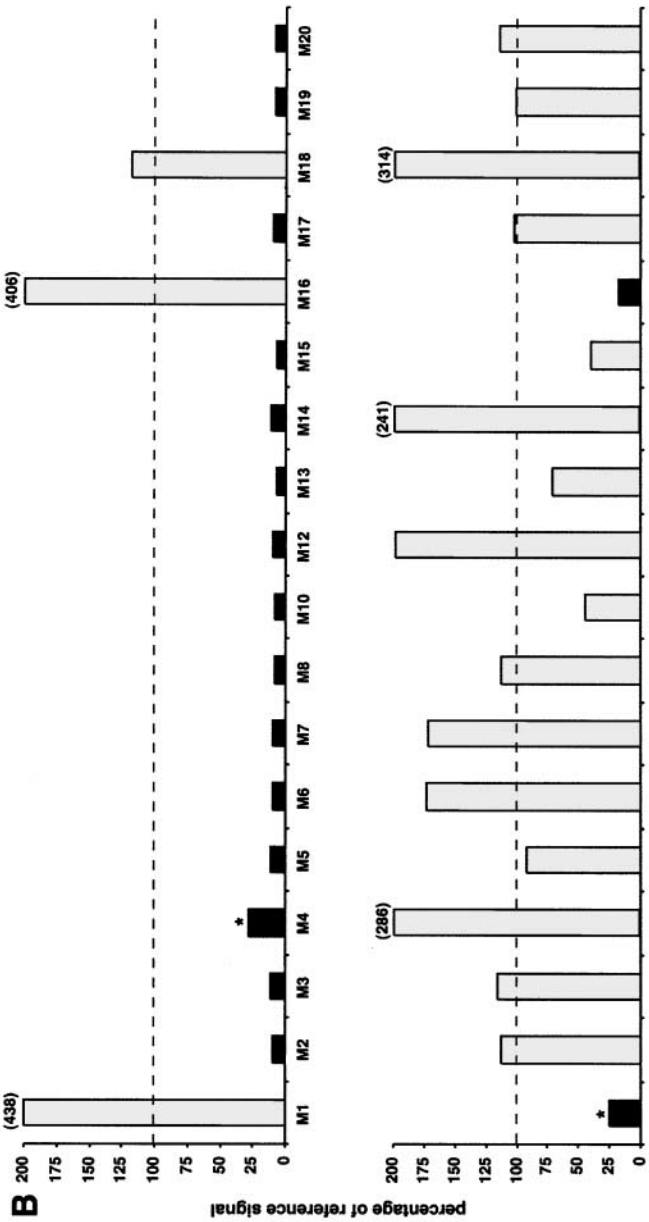

Figure 1. The inhibition assay applied on 20 culture supernatants (M1–M20) with antibodies against PICP (A) and 18 supernatants (M1–M18) with antibodies against PP14 (B). In A results are shown using two antibodies with identical epitope specificity (top: M1; bottom: M13) whereas the 2 antibodies (top: M4; bottom: M1) used in B are directed against different epitopes. The results are given as percentage of the reference signal and ■ indicates results $< 30\%$ of the reference signal and ■ $> 130\%$ i.e., the bonus effect.

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producing hybridomas clearly fell into four groups with respect to epitope specificity, containing one (F12), two (142-1 and 142-2; from an earlier production and succesfully applied on immunhistochemistry),[5] three (F15, F30, and F59), and five (F31, F32, F33, F38, and F54) hybridomas, respectively.

As seen in Table 1, the liquid phase binding of a MAb to biotinylated FA1 prevented subsequent binding of the complex to the same MAb in the solid phase as expected (indicated by asterisk), and also, to the MAbs within the same group (bold). For some MAb pairs belonging to different groups, the opposite effect was observed, i.e., the resulting signal produced by the binding of biotinylated Ag was 2–3 times higher than the reference signal, i.e., the signal observed with biotinylated FA1 and growth medium only (bonus effect).

FA1 is a single chain molecule, but the inhibition assay was also analyzed using molecules containing two identical polypeptide chains, i.e., the C-terminal propeptide of human procollagen type I (PICP); (two α1-chains and one α2-chain with interchain S–S bonds; the antibodies are directed against the α1-chain) and the endometrial protein, PP14, which is a non-covalently mediated homodimer.[7] Results of the analysis are shown in Fig. 1. For PICP (Fig. 1A), the results are demonstrated using two antibodies with identical specificity (i.e., M1 and M13) as the solid phase antibodies, whereas, the epitope specificity analysis of 20 monoclonal antibodies against PP14 is illustrated using 2 monoclonal antibodies with different epitope specificity (Mab 4 and Mab 14) as the solid phase antibodies. The results shown in Fig. 1 demonstrate that the inhibition assay also works for antigens with repetitive epitopes.

DISCUSSION

In this study, we have developed a simple method to identify monoclonal antibodies, which can react simultaneously and independently with the same antigen, a requirement in two-site immunoassays. Moreover, the method is applicable during the early phase of production of monoclonal antibodies and directly on the culture supernatant.

The only prerequisite for the resulting method is access to a labeled antigen, and in the model systems described here, the antigens were labeled with the hapten biotin. The assay compares two culture supernatants in sets, which during the initial screening after fusion were found positive for specific antibody reactivity against the antigen in question. One antibody (A) is incubated with biotin-labeled antigen, and the complex subsequently transferred to an ELISA plate, where the other antibody (B) has been bound to the plastic surface via an antibody bridge (rabbit anti-mouse Ig). The preincubation of

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biotinylated antigen with antibody A will block (or diminsh) the possibility for antibody B to bind to the epitope, but, when antibody A and B react with different and distinct epitopes, the subsequent binding of the labeled antigen to antibody B will ideally be identical to the reference signal. However, for some combinations of antibody pairs in which the monoclonal antibodies recognize distinct epitopes, we observed what is referred to as the bonus effect, i.e., an OD-signal in excess of the reference signal (Table 1; Fig. 1.) The bonus effect is most likely due to a higher affinity for the epitope recognized by the test antibody compared to the affinity between the capture antibody and the corresponding epitope. As seen from Table 1, the signal using 142-1 or 142-2 (identical epitope specificity) as capture antibodies was two to three times higher than the reference signal when F30 was applied as a test antibody. This indicates that F30 has a functional affinity, which is higher than those of 142-1 and 142-2. This explanation is further substantiated by the fact that using F30 as capture antibody and 142-1 (or 142-2) as test antibody, the signal did not exceed the reference signal (Table 1).

Several assays have been described to determine epitope specificity of monoclonal antibodies, but most of these can only be used later in the process of monoclonal antibody development, because they require at least one antibody being available in a purified form and labeled with a signal molecule.[11,12] Other methods, which can be used in direct connection with the initial screening after fusion, comprise assays that will detect (or modulate) a biological function, e.g., neutralization of a toxin molecule, or binding to a receptor.[13–15] Such methods do, however, only discriminate between two groups of antibodies, i.e., neutralizing (or blocking) and non-neutralizing. Other methods are based on the reactivity pattern in Western blot after SDS-PAGE separation of enzyme fragmented antigen or antigen fragments.[16] Such assays will detect groups of antibodies, which recognize individual sets of fragments depending on their epitope specificity. However, a difference in reactivity pattern, as detected by this method, does not always correspond to reactivity to non-overlapping epitopes. Monoclonal antibodies intended for use as serological markers are often characterized by comparing their reactivities with, e.g., bacteria with similar antigenic structures.[17]

Würzner et al.[18] have described a method in which one antibody is also captured with an anti-mouse Ig, and remaining binding sites were blocked with normal mouse serum. The next step was incubation with free antigen, followed by premade complexes of the second monoclonal antibody complexed with enzyme labeled anti-mouse Ig and saturation with normal mouse serum. However, in contrast to our method, the technique has one major drawback, namely, that it will not function if the antigen presents repetitive epitopes or if it is present in the preparation as polymers/aggregates.

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In conclusion, our method to determine relative epitope specificity is easy to perform, has a high capacity, can be perfomed early during production of monoclonal antibodies directly on culture supernatants, and works on molecules with single, as well as, repetitive epitope patterns. Moreover, the bonus effect identifies antibodies with high affinity in sets of monoclonals being compared.

ACKNOWLEDGMENTS

This work was supported by The Danish Medical Research Council, The Novo-Nordisk Foundation, The Carlsberg Foundation, The Danish Foundation for Advancement in Medical Science, and Arvid Nilssons Fond, Denmark.

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Received May 22, 2003
Accepted June 30, 2003
Manuscript 3104

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