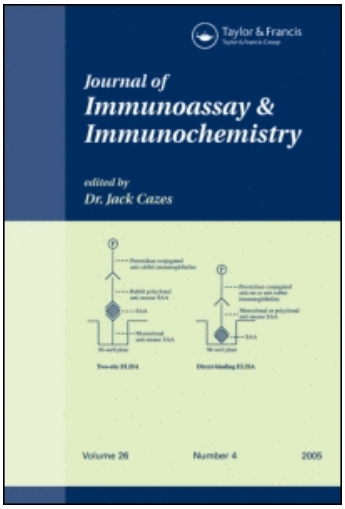

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Harry Towbin[a]; François Erard[a]; Jan van Oostrum[a]; Albert Schmitz[a]; Christiane Rordorf[a]
[a] Pharmaceuticals Research Laboratories, Ciba-Geigy Ltd., Basel, Switzerland

To cite this Article Towbin, Harry , Erard, François , van Oostrum, Jan , Schmitz, Albert and Rordorf, Christiane(1996) 'Neoepitope Immunoassay: An Assay for Human Interleukin 1β Based on an Antibody Induced Conformational Change', Journal of Immunoassay and Immunochemistry, 17: 4, 353 — 369
To link to this Article: DOI: 10.1080/01971529608005798
URL: http://dx.doi.org/10.1080/01971529608005798

JOURNAL OF IMMUNOASSAY, 17(4), 353-369 (1996)

Neoepitope Immunoassay: An Assay for Human Interleukin 1β Based on an Antibody Induced Conformational Change

Harry Towbin, François Erard, Jan van Oostrum,
Albert Schmitz, and Christiane Rordorf
Ciba-Geigy Ltd., Pharmaceuticals Research Laboratories, Basel,
Switzerland

ABSTRACT

A secondary monoclonal antibody (mAb2) was generated by immunization with immune complexes of human IL-1β and a primary monoclonal (mAb1). mAb2 bound to a neoepitope on the IL-1β/mAb1-complex with a dissociation constant (Kd) of 26 pM but not to uncomplexed IL-1β. As assessed by the binding of labeled IL-1β and neutralization of bioactivity, mAb2 enhanced the affinity of IL-1β to mAb1; Kd-values were 108 pM in absence and 5.4 pM in presence of mAb2. By analyzing a series of mutants of IL-1 where surface loops had been exchanged with the corresponding loops of human IL-1 receptor antagonist protein, a critical region responsible for mAb2 binding was localized to the C-terminal region. In addition to mAb1/IL-1β-complexes, mAb2 bound pro-IL-1β/mAb1 complexes as well as pro-IL-1β suggesting that mAb2 recognized a conformation of IL-1β resembling that of pro-IL-1β. Using this pair of mAbs, chemiluminescent and enzyme linked assays with detection limits of 2 pg/ml hIL-1β have been established.

(KEYWORDS: antigen-antibody complex, conformation, epitope mapping, immunization, chemiluminescence, interleukin 1)

INTRODUCTION

For constructing immunometric assays based on monoclonal antibodies (mAbs), it is often not trivial to identify a suitable partner because binding of the first mAb may affect the binding of the secondary mAb. Both enhancements and inhibitions by the second mAb are, for example, observed in checkerboard analysis where the binding of a labelled antigen to a solid phase mAb is analyzed. Inhibitions may be complete or partial and even enhancement is sometimes observed. Such interactions have often been interpretated as maps of the epitopes - sometimes with the implicit conclusion that the degree of inhibition was related to the physical distance of the epitopes on the protein. Another interpretation is that the binding of antibodies stabilizes particular conformations of the antigen which lead to changes of affinity towards the second mAb partner. Thus, the binding of an antibody to its antigen is known to be able to affect the conformations of both the antigen and of the antibody (1). These phenomena are best reconciled with a model postulating a shift of the conformational spectrum available to each binding partner as a result of the binding reaction.

In the course of the development of an immunoassay for human IL-1β, we first obtained a high affinity mAb (2). For this mAb we wished to produce a secondary mAb that would optimally collaborate with the existing mAb. To this end we used an approach which comprised immunization with a complex of mAb1 and IL-1β and tolerization with uncomplexed mAb1. The secondary mAb reported on here, represents an apparently extreme case of cooperation in a ternary system.

MATERIALS AND METHODS

Interleukins

Recombinant interleukin-1β (rhIL-1β) and interleukin-1 mutants were prepared in E.coli, purified and refolded by methods described in (3). Recombinant IL-1β precursor was obtained from Cistron (Pine Brook, NJ). mAb 2D8 has been described previously (2).

Immunization and Hybridoma Production

For preparing the immunogen, 12 ug rhIL-1β were incubated in PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH7) with 125 ug mAb 2D8 for 3 hours. To this solution 250 ug KLH (keyhole limpet hemocyanin) in PBS were added (final volume 125 ul), followed by 10 ul of 2.5% glutaraldehyde. After 15 min., the reaction was quenched by addition of 80 ul 1 M glycine (pH 6.6), followed by dilution with PBS to 500 ul. Female BALB/c mice were immunized with the conjugate (23 ug, containing approximately 0.75 ug IL-1β, emulsified with cFA) by subcutaneous injection. The animal was boosted after 5 months with a mixture of 40 ug conjugate and 20 ug unconjugated mAb 2D8 by intraperitoneal and intravenous injection. Four days later, the spleen cells were fused to the PAI myeloma cell line (4) according to standard methods (5,6). Cloning was done by limiting dilution. Isotypes were determined using the Biorad kit.

The test supernates were screened on microtiter plates coated with $F(ab')_2$ fragments (7) of mAb 2D8 at 3 ug/ml. The plates were sequentially

incubated (with intermediate washing steps) with 10 ng/ml rhIL-1β diluted in BP (1% bovine serum albumin, 0.05% Tween 20, 0.05% sodium azide, in PBS) for 2 h; with the test supernates for 16 h; and finally with a conjugate of anti-mouse IgG and alkaline phosphatase (Fc-specific, Sigma Cat.No. A-1418, 1:2000) for one hour. p-Nitrophenylphosphate was used a substrate.

Chemiluminescent Assays

IL-1β and antibodies were labelled with a chemiluminescent acridiniumester (2,6,-dimethyl-4-(N-succinimidyloxycarbonyl) phenyl 10-methylacridinium-9-carboxylatemethosulfate (DMAE, a gift of Dr. S.-J. Law, Ciba-Corning Diagnostics, Walpole, USA) following the general protocol of Weeks and Woodhead (1984) as described (8,9). Chemiluminescence was determined on a microtiter plate luminometer (MicroLumat 96P, Berthold AG, Regensdorf, Switzerland). The light reaction was started by injection of reagent A, followed by reagent B (obtained from Ciba Corning Diagnostics, CH-8305 Dietlikon, Switzerland). These reagents contain solutions of hydrogen peroxide (0.5%) in nitric acid (0.1 N) and of sodium hydroxide (0.25 N) with a detergent. Light collection was started immediately after injection of reagent B and the detection time per well was set to 2 s. The backgrounds of empty wells were approximately 50 counts.

Chemiluminescent assay for IL-1β: White microtiter plates (MaxiSorp, NUNC, Roskilde, Denmark) were coated with mAb 2D8 (5 ug/ml, 75 ul/well) and blocked with BP. Unknowns and standards (25 ul) were added to the emptied wells and 25 ul (4 ng) DMAE-labelled mAb 1437-96-

15 were added. After incubation at 4°C for 16 h, the plates were washed, and chemiluminescence was determined.

Enzyme Linked Assay for IL-1β

The assay for IL-1β was carried out as for the chemiluminescent assay with the following changes: transparent plates were used, the buffers were devoid of sodium azide, and the second antibody was mAb 1437.96.15 labeled (10) with peroxidase (final concentration 0.5 ug/ml). o-Phenylene diamine was used as substrate (Sigma, Cat.No. P4664).

Bioassays and Dissociation Constants

Bioassays for IL-1β in presence or absence of antibodies were carried out using an assay based on proliferation of the mouse T helper cell line D10S (11,12). Dissociation constants (Kd) for the (mAb1) / (mAb2.IL-1β) interaction were determined by equilibrating solutions of DMAE-IL-1β (5.8 pM) for 3 days at 4° with varying concentrations of mAb1 (2D8) in presence of 1 ug/ml mAb2 (96.15). Non-bound tracer was removed by precipitating with anti-mouse IgG coupled to paramagnetic particles and the bound fraction was determined on a Luminomat II luminometer (Ciba Corning Diagnostics). The Kd of the mAb1/IL-1β interaction was determined analogously except that an isotype matched control mAb was substituted for mAb2. The Kd of the interaction between mAb2 and the complex of mAb1 and IL-1β was determined by equilibrating 5.8 pM solutions of DMAE-IL-1β with varying concentrations of mAb2 in presence of 1 ug/ml mAb1 and determining the DMAE-IL-1β-mAb1 complex (non-bound) by a 10 minute incubation on microtiterplates

TABLE 1

Monoclonal Antibodies used in this Study

Designation	Short Designation	Sub-class	Immunogen	Specificity
2D8	2D8 (mAb1)	IgG1	IL-1β	• IL-1β • pro-IL-1β
1437.96.15	96.15 (mAb2)	IgG1	(IL-1β/2D8)-coupled to KLH	• IL-1β.2D8 (complex) • pro-IL-1β.2D8 (complex) • pro-IL-1β

coated with mAb2. The MW for all mAbs was taken as 160 000 and for calculations all reactions were assumed to proceed with two binding sites per IgG molecule.

RESULTS

Generation of Secondary mAb

With the goal of generating a suitable partner for a previously established antibody against IL-1β (2) we injected BALB/c mice with an immune complex consisting of recombinant IL-1β and the first mAb (mAb1). For enhancing the immunogenicity in this syngeneic system, the complex was conjugated to KLH. Furthermore, in order to suppress the generation of anti-idiotypic antibodies against mAb1, mAb1 was injected intraperitoneally in unconjugated form, simultaneously with the conjugate. The fusion was screened on microtiter plates coated with $F(ab')_2$ fragments of mAb1 in presence or absence of IL-1β. One positive clone

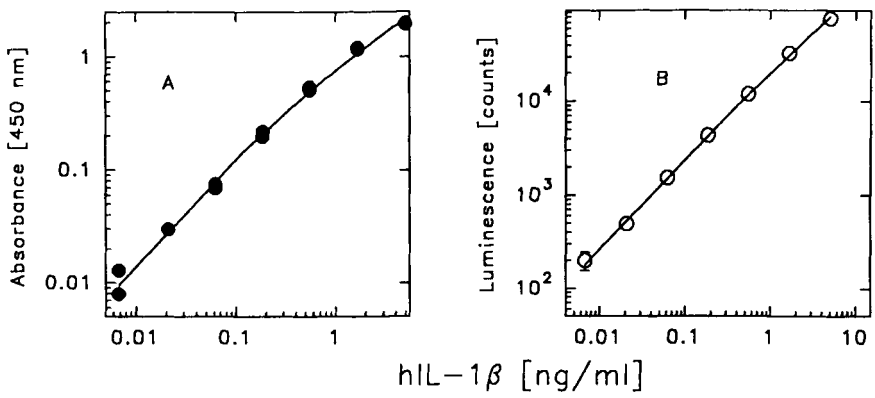

FIGURE 1. Standard curves for enzyme linked (panel A) and chemiluminescent (panel B) two-site immunometric assays for hIL-1β. mAb1 (2D8) was used for coating and mAb2 was labelled with peroxidase or DMAE, respectively. The samples were incubated in presence of the secondary antibodies. Error bars indicate standard errors of triplicates.

which bound IL-1β specifically (mAb2, clone 1437.96.15) was expanded, its antibody purified, and labelled with a chemiluminescent tracer. For a summary of the properties of the antibodies used in this study see table 1.

Suitability for Immunoassay

As anticipated from the way the antibody was selected, mAb2 was suitable for constructing a two-site immunometric assay. In both configurations - with either mAb bound to the plate - the limit of detection was below 2 pg/ml (Fig. 1). Natural IL-1β, as secreted *in vitro* by peripheral blood mononuclear cells upon stimulation, was readily detected using chemiluminescent or enzyme linked versions of the assays. However, if mAb2 was used for coating it was essential that mAb1 was present during incubation with IL-1β (Fig. 2). With mAb2 coating and an intermediate

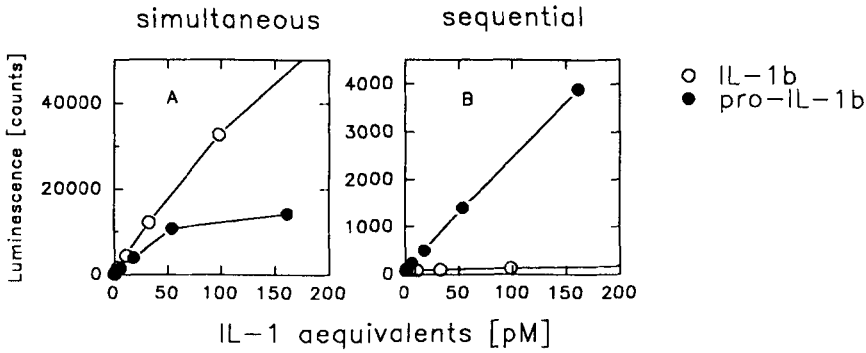

FIGURE 2. Differential recognition of IL-1β and pro-IL1β by mAb2 (96.15) in two-site immunometric assays. In panel A, plates coated with mAb 96.15 were incubated simultaneously with recombinant hIL-1β or pro-IL-1β and DMAE-labelled mAb 2D8. In panel B the incubations were carried out sequentially. Note that both forms of IL-1β are recognized by simultaneous incubations, whereas only pro-IL-1β is recognized under conditions of sequential incubation.

washing step before addition of labelled mAb1, virtually no signal (0.5% of the simultaneous system) was obtained even at the highest concentration of IL-1β tested (10 ug/ml). Crossreactions with hIL-1α or IL-1ra were below 0.05%.

Reaction of mAb1 and mAb2 with pro-IL1b

Pro-IL1β was recognized in the sandwich assay with an efficiency of 60% compared to rhIL-1β on a molar basis. In contrast to IL-1β, pro-IL-1β was recognized by mAb2 in the absence of mAb1, albeit with an affinity approximately 10-fold lower than in the presence of mAb1 (Fig. 2). These data suggested that a neoepitope on IL-1β was recognized which was created by complexation with mAb1 and which was also present in pro-IL-1β.

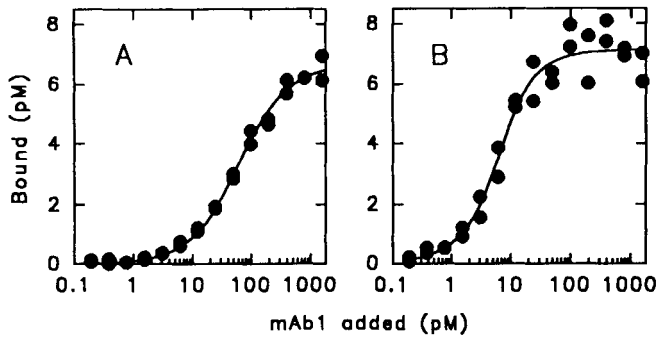

FIGURE 3. Binding of DMAE-labelled IL-1β to mAb1 or mixtures of mAb1 and mAb2. Constant concentrations of DMAE-labelled IL-1β were incubated with varying concentrations of mAb1 in presence of 1 ug/ml of a nonrelated mAb (panel A) or 1 ug/ml of mAb2 (panel B). For details see Methods section.

Dissociation Constants and Neutralisation of IL-1β Bioactivity

For a closer analysis of the cooperativity phenomenon we determined the affinities of this system consisting of mAb1, mAb2, and IL-1β. In the experiment of Fig. 3, the dissociation constants were derived from antibody dilution curves using a constant concentration of labelled IL-1β (cf. table 2). It is apparent that the Kd of mAb1 (108 pM) was reduced (5.4 pM) due to the presence of mAb2. The Kd of mAb2 towards a preformed complex of IL-1β and mAb1 was 26 pM (Fig. 4).

The stabilizing effect of mAb2 was also reflected by the ability of the mAb pair to neutralize the bioactivity of IL-1β in a proliferation assay (Fig. 5). In the combination of the two mAbs, mAb2 distinctly improved the ability of mAb1 to neutralize IL-1β. mAb2 alone had a slight antiproliferative effect only at high mAb concentrations (10 ug/ml).

TABLE 2

Dissociation constants

Interaction analyzed	Kd [pM]
2D8 / IL-1β	108
96.15 / IL-1β	not detected
2D8 / IL-1β in presence of excess 96.15	5.4
96.15 / IL-1β in presence of excess 2D8	26

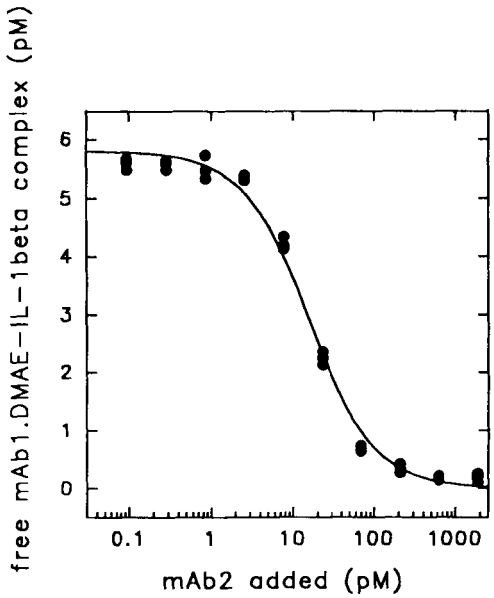

FIGURE 4. Estimation of affinity of mAb2 binding to a complex of DMAE-labelled IL-1β. Complexes formed between labelled IL-1β and an excess of mAb1 were preincubated with varying concentrations of mAb2 and the nonbound binary complex measured by a brief exposure to a plate coated with mAb2. For details see Methods section.

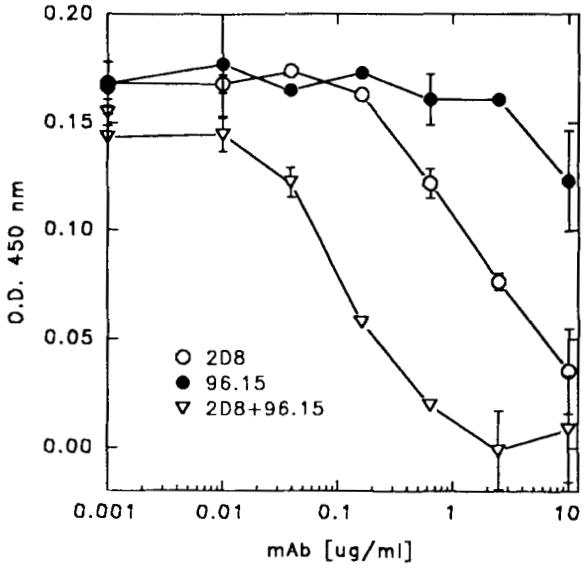

FIGURE 5. Inhibition of IL-1β dependent proliferation of D10S cells by combinations of mAbs against IL-1β. Dilutions of the antibodies or aequimolar mixtures were added to the cell cultures. In the case of antibody mixtures the concentrations refer to one of the components. The IL-1β concentration was 10 pg/ml.

Recognition of Loop Exchange Mutants

In order to delineate the sites of interaction of mAb2, the reactivity of a range of muteins of IL-1β was tested in two-site immunometric assays. The muteins consisted of hybrids of hIL-1β and hIL-1 receptor antagonist protein (hIL-1ra) where loops of hIL-1ra had been exchanged on hIL-1β. For all the muteins evidence for correct folding had been obtained by X-ray and CD (circular dichroism) analysis.

The effects of the muteins on the capturing antibody (mAb1, 2D8) were analyzed by competition assays using labelled (wild type) rhIL-1β as well as by a two-site immunometric assay. In the two-site assay, 2D8 was

TABLE 3

Relative Activities of Loop Exchange Muteins in Competitive and 2-Site Immunometric Assays.

Loop exchange mutein			Assay type			
			2D8	2D8 + poly-clonal	2D8 + DMAE-96.15	Interpre-tation
No.	Sequence of IL-1β deleted	Sequence of IL-1ra inserted	CLIA compe-titive[1)]	ELISA 2-site[2)]	CLIA 2-site[2)]	Peptide impor-tant for binding of
wt	-	-	1	1	1	
1[3)]	A1-R11	R1-W16	0.017	0.24	6	2D8
2	V19-A28	Y24-A32	1.7	4.4	9	
3	A28-F42	A32-I46	0.005	0.023	0.01	2D8
4	V47-V58	I51-L56	0.017	0.043	0.075	2D8
5	L62-L69	L58-L67	0.24	1.17	3	
6	V72-L82	V70-L80	0.68	2.9	1	
7	E83-E96	E81-D95	0.85	5	10	
8	I104-L110	S103-T109	0.567	3.5	10	
9	E113-W120	E112-W119	0.739	4.4	10	
10	T124-P131	T123-P130	<0.005	0.007	0.03	2D8
11	G135-I143	T134-V143	0.57	1.6	8	
12[3)]	D145-S153	F145-E152	0.49	3.2	0.00001	96.15

[1)] Chemiluminescent immunoassay (CLIA), activity relative to wildtype (wt)
[2)] Relative activity
[3)] Muteins 1 and 12 correspond to the N- and C-termini, respectively

used as a coating antibody for capturing IL-1β (or the muteins) and a rabbit anti-IL-1β serum for detection. The two assays gave qualitatively similar results (Table 3). This allowed the interpretation that the rabbit serum was not sensitive to the mutations introduced - probably because multiple epitopes were recognized. Several muteins (numbered 3,4,10, cf. table 3) exhibited greatly reduced reactivity. Nevertheless, the residual activity was clearly measurable.

In the following experiment, the reactivities of the 2D8 bound muteins towards mAb2 were analyzed by the two-site immunoassay. Here, the signals generated depended on the binding abilities of both, 2D8 as well as mAb2. In order to compensate for reduced reactivity towards 2D8 we calculated the ratios between binding activities of the 2D8/mAb2 over the 2D8/polyclonal systems. Using this compensation it became clear that the muteins effecting mAb 2D8 had little effect on mAb2. However, mutein 12 where the C-terminal of IL-1β was replaced by the C-terminus of IL-1ra, as the only mutein, dramatically reduced the reactivity towards mAb2 whereas that of 2D8 had not been affected.

An analogous analysis was also carried out with 19 single aminoacid exchange muteins. Here, the effects were less striking. Muteins E128K and Q34E reduced the binding of mAb1 to 0.8% and 21% of control, respectively. Mutein Q141E (in presence of mAb1) bound to mAb2 with an activity of 10% whereas its binding to mAb1 alone remained at 78%. For the remaining muteins - including D145E and V151D in the C-terminal region which had been shown above to affect mAb2 binding - the differences to the wild type control were not considered significant (above 50% of control).

DISCUSSION

The secondary antibody we obtained by immunization with an immune complex had the unexpected property of only reacting with IL-1β in the immune complex. The data is best reconciled with a model where IL-1β changes conformation upon binding to the primary mAb and where this changed conformation exposes an epitope on IL-1β recognizable by

mAb2. The conformation of IL-1β stabilized by mAb1 and recognized by mAb2 appears to bear similarity to that of the IL-1β precursor because mAb2 is able to bind this precursor - though with reduced affinity - in the absence of mAb1. This observation makes it unlikely that a neoepitope on mAb1 markedly contributed to the binding of mAb2 as reported for anti-metatypic antibodies (13,14).

Although our own data caution against overinterpreting binding data on conformationally sensitive molecules, a reasonable conclusion of the epitope mapping study is that the C-terminal sequence of IL-1β is important for the binding of mAb2. The conformational changes of IL-1β postulated by the model appear compatible with the flexibility of C- and N-termini of many proteins. Note that it is the C-terminal part of the IL-1 precursor molecule that gives rise to mature IL-1β and that the mature IL-1β and the precursor therefore have identical C-termini.

In terms of assay technology, the immune complex specific mAb2 as secondary mAbs have the advantage of avoiding the hook effect observed in simultaneous incubations at antigen excess. In addition, depending on an intermediate washing step, our pair of mAbs offers the curious possibility of determining either pro-IL-1β or the sum of pro-IL-1β and IL-1β with same reagents. This is achieved with mAb2 as coating mAb. Only pro-IL-1β is retained when the plate is washed before adding labelled mAb1. By contrast, the signal generated by adding mAb1 during incubation with the sample will be sensitive to both, IL-1β and the precursor.

The determinations of the affinities of the various reactions leading to the formation of the ternary complex clearly show the stabilizing effect of mAb2 with respect to IL-1β binding. mAb1 displayed a Kd of 108 pM; in

the presence of excess mAb2 the Kd decreased to 5.4 pM. Such stabilizing antibodies have the potential to improve two-site immunometric assays, particularly in the case of simultaneous incubations. In addition, they may prove to be useful in competitive assay formats.

Immunizations with immune complexes have previously been advocated for directing an immune response (15,16). Our immunization with the additional tolerization support this general concept. However, at present, it is difficult to predict whether antibodies specific for neoepitopes will be found at useful frequencies for other antigen / mAb1 systems.

Do antibodies specific for immune complexes occur naturally? Because they are difficult to detect by current assay systems this question is hard to answer. Such antibodies may for example help to stabilize immune complexes and provide explanations for the superiority sometimes claimed for polyclonal antibodies.

Address correspondence to: Dr. H. Towbin, Ciba-Geigy Ltd., K-681.546, CH-4002 Basel, Switzerland. FAX ++41 61 / 696 93 01.

ACKOWLEDGEMENT

We thank J. Motz, V. Saderi, and O. Zingel for their excellent technical assisstance.

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