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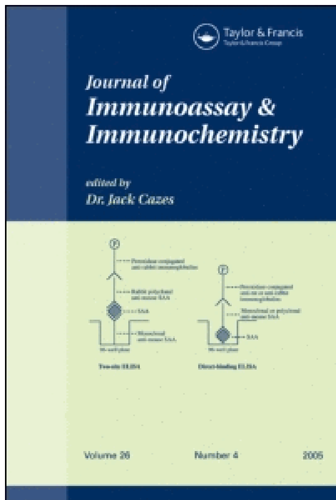


**Journal of Immunoassay and Immunochemistry**
Publication details, including instructions for authors and subscription information:
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**To cite this Article** Dimitrijević, Lj. , Radulović, M. , Ćirić, B. , Odrljin, T. , Jankov, R. M. and Marzari, R.(1992) 'Immunochemical Characterisation of a Murine Monoclonal Anti-Idiotypic Antibody', Journal of Immunoassay and Immunochemistry, 13: 2, 181 — 196

**To link to this Article: DOI:** 10.1080/15321819208021226

**URL:** http://dx.doi.org/10.1080/15321819208021226

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JOURNAL OF IMMUNOASSAY, 13(2), 181-196 (1992)

# IMMUNOCHEMICAL CHARACTERISATION OF A MURINE MONOCLONAL ANTI-IDIOTYPIC ANTIBODY

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## ABSTRACT

Y7, a murine monoclonal IgG1 κ antibody against a human monoclonal IgM λ DJ molecule, was affinity purified on an IgM λ immunoaffinity column. As detected by enzyme-linked immunosorbent assay (ELISA) the isolated Y7 monoclonal antibody was shown to be not cross-reactive with human IgG, human secretory IgA, μ chain, λ+κ chains and another human monoclonal IgM λ BR. Binding to the polyclonal human IgM standard in the same assay was about 30 percent. The epitope specificity of affinity purified and biotinylated Y7 MoAb was localized only in the nonreduced pepsin Fab fragments of IgM λ DJ immunogen. As the immunogen was determined to be a specific antibody to phosphorylcholine, the specificity of Y7 MoAb was further ascertained in its capacity to induce 95% inhibition of immunogen binding for phosphorylcholine.

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## INTRODUCTION

It has been known for thirty years, that the idiotypes are the most powerful markers of Ig connected antigen specificity. However their exact involvement in immune network regulation is still not completely understood. The classification of the idiotypes in the presence of anti-idiotypic antibodies (a-Ids) has been made according to immunochemical, clonal and network criteria.

The development of the hybridoma technology gave rise to a great number of monoclonal a-Ids which have played a very important role: 1/in studying the relation of paratopic and idiotypic determinants „(1); 2/ in explaining internal image functioning (2); and 3/ in discovering shared or regulatory (3, 4, 5) idiotypic determinants on Ig molecules and T cell receptor structures which seem to be a prerequisite for immune network functioning.

Although the application of monoclonal a-Ids in therapy is still unsuccessful, the possibility of using monoclonal a-Ids as therapeutic agents in growing B and T cell tumors (6, 7, 8) has attracted additional interest for this field of research. Recent studies indicate the importance of natural (network) idiotypes (9, 4) in network regulation of the immune system.

Hypervariable regions of IgMs are representative markers of the dominant idiotypes most commonly found among "natural" antibodies. Therefore, the a-Ids against human IgM idiotypes, may be of great importance in elucidating of idiotype/anti-idiotype interactions in human autoimmunity, lymphoproliferation and/or immunodeficiency disorders.

Having in mind the difficulties in producing and characterization of a-Id as "natural", we produced a murine monoclonal a-Id against a human IgM molecule derived from a patient with Waldenstrom macroglobulinaemia. The immunochemical characterization of such an antibody is presented in this article.

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## MATERIALS AND METHODS

### Human Ig Antigens

Human IgM λ DJ immunogen was isolated from the serum of a patient (DJ) with Waldenstrom macroglobulinaemia by euglobulin precipitation with 10-fold excess of distilled water. Preparative 1% agarose gel electrophoresis of euglobulin precipitate applied to Sephadex G-200 was further performed. Human IgM λ BR, which was used as a control monoclonal IgM λ, was isolated from another patient, (BR), with Waldenstrom macroglobulinaemia. Only the ammonium-sulfate precipitate was used as starting material for agarose gel electrophoresis. Polyclonal human IgM, μ chain, κ and λ chains were all supplied by Calbiochem. Polyclonal human IgG (ICN) was also used. The IgM pepsin fragments used in this study were produced as described by Schrohenloherer & Bennett (10). Both the solution of immunogen and polyclonal human IgM (10 mg/ml in 0.1 M Na-acetate buffer pH 4.1) were treated at an enzyme/substrate ratio of 2:100 (w/w). Digestion was terminated by neutralization with a saturated solution of Tris base.

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### Isolation of monoclonal antibody Y7

The Y7 monoclonal antibody was isolated by immunoaffinity chromatography (11) from cell culture supernatants or ascites. Ascites were induced in BALB/c mice pretreated with incomplete Freund's adjuvant (12) or pristane. The immunoaffinity column was made by coupling purified IgM λ DJ immunogen at a total concentration of 50 mg to 10 ml of cyanogen bromide-activated Sepharose CL-4B (Pharmacia) (13). Ascites were diluted in phosphate-buffered saline (PBS), pH 7.3, and passed through the column which was then extensively washed. The Y7 MoAb was eluted

with 0.1 M glycine-HCl, pH 2.5, dialyzed against PBS, pH 7.3, diluted 1:5 and lyophilized. The purity of the monoclonal antibody was checked by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) as described by Jergil & Ohlsson (14) and silver staining (15). Radial immunodiffusion for subclass determination was done using monospecific anti-mouse Ig anti-sera (Litton, Bionetics).

**ELISA**

Activated polyvinyl-chloride (PVC) microtiter wells (Flow) were coated overnight at $4^{0}$C or for one hour at $37^{0}$C, with 50 µl of immunogen (20 µg/ml), and the previously described human Ig antigens in PBS. After saturation with 2% bovine serum albumin (BSA) and extensive washing in PBS-0.05% Tween 20, different concentrations of isolated Y7 antibody were added and the plates further incubated for one hour. Binding of Y7 was detected by adding 50 µl of rabbit anti-mouse Ig peroxidase conjugated antibody (Serolab). The enzyme reaction was developed for 20 min using ortho-phenylene diamine (OPD) as the substrate and the optical density (O.D.) at 492 nm was recorded in a Titertek ELISA reader (Flow Laboratories).

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**Immunoblotting**

Western blotting was done essentially as described by Tobwin et al. (16). IgM λ DJ pepsin fragments were electrophoretically separated in 5% the SDS-PAG electophoretical system of Weber & Osborn (17) and transferred to nitrocellulose. The binding of MoAb was detected in the presence of a streptavidin-peroxidase complex which was visualized by soaking nitrocellulose papers in 1.4 mM 3,3'-diaminobenzidine (DAB) and 1 mM hydrogen peroxide in PBS at room temperature for 10-15 min.

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**Anti-idiotypic specificity of Y7 MoAb**

IgM λ DJ paratopic specificity was detected by solid phase ELISA. Activated PVC microplate wells (Flow) were coated with 50 μl of 3% BSA in PBS during an overnight incubation. Phosphorylcholine chloride (PC) in deionized water (15 mg/ml) (milli-Q) was further added to albumin precoated plates and incubated for one hour at room temperature. Washings were done as described previously. Binding of increasing concentrations of IgM λ DJ in 1% BSA/PBS, as well as IgM λ BR was visualized after subsequent incubation with rabbit anti-human IgM (μ-chain specific) antibody conjugated with peroxidase (Sigma) and OPD. Competitive ELISA was carried out as previously described, except that concentrations of 3-12 μg/ml IgM λ DJ were preincubated for one hour at room temperature with increasing concentrations of isolated Y7 MoAb as well as with mouse IgG1 standard (Litton Bionetics, USA). Percent inhibition was calculated as follows:

$$\%\text{Inhibition} = 100 \times \left(1 - \frac{\text{binding in the presence of inhibitor}}{\text{binding in the absence of inhibitor}}\right)$$

## RESULTS

**Isolation of Y7 MoAb**

The isolation was performed on an immunoaffinity column, with IgM λ DJ immunogen coupled to agarose. Most of the proteins from the ascites, which where not retained by the column passed through as the first peak. Nonspecifically adsorbed proteins were mostly desorbed with water and 1M NaCl in PBS, while the monoclonal antibody Y7 was eluted as the second peak with 0.1 M glycine-HCl pH 2.5 (Fig. 1.A). The antibody was isolated at a high purity (Fig. 1.B), which was confirmed by 10% SDS-PAGE and silver

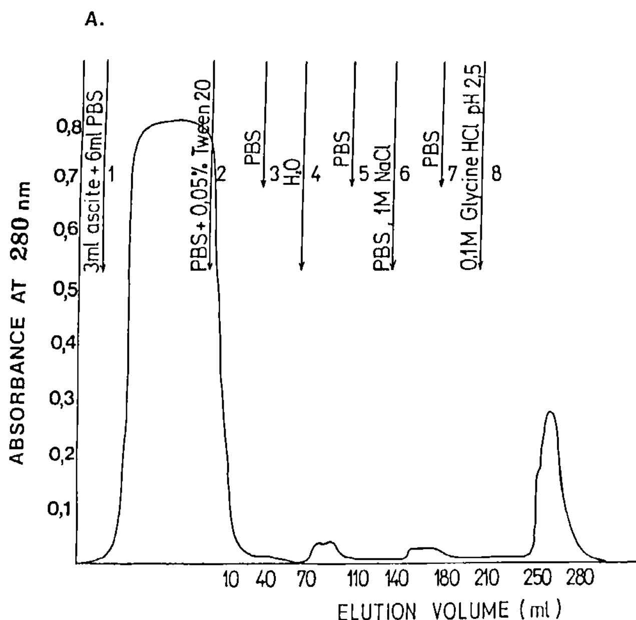


Figure 1.A/ Elution pattern of Y7 MoAb produced in ascites fluid, from IgM λ DJ immunoadsorbent column with the glycine-HCl buffer. B/ 10% SDS-PAGE under reducing conditions: of immunoaffinity isolated Y7 MoAb (lane 1), IgM λ DJ immunogen (lane 2) and control mouse IgG1 (lane 3). Gel was silver stained.

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staining. Y7 MoAb belonged to the IgG1 subclass with kappa light chains as determined by radial immunodiffusion.

## Binding of the Isolated Y7 MoAb to Human Ig Proteins

The binding of isolated MoAb to the immunogen was tested over a range of concentrations from 10 to 1000 ng/ml. A plateau region of binding with immunogen was achieved at a concentration of 250 ng. Estimation of the Kd of Y7 MoAb from the antibody dilution curve (18), as a reciprocal value of the molar concentration

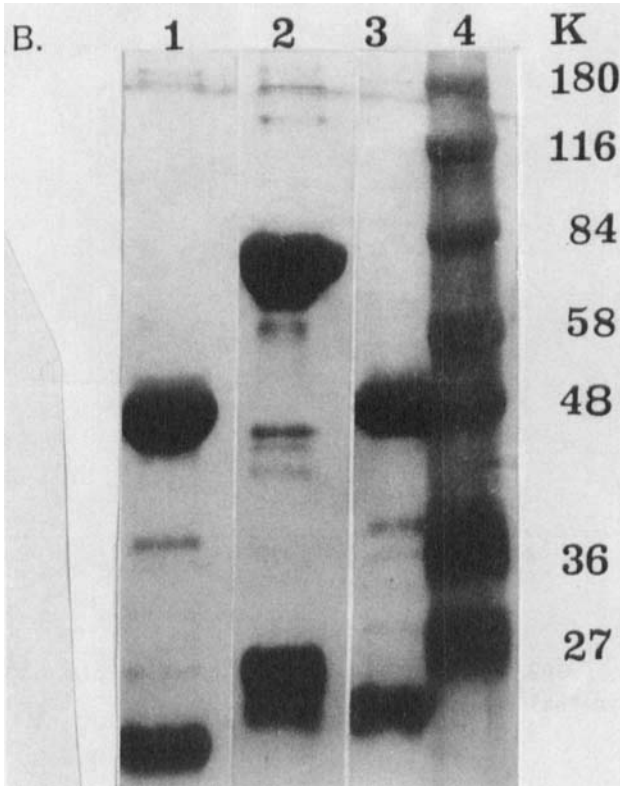


Figure 1 Continued

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necessary for 50% saturation would suggest a Kd value of around $10^{-9}$M. Under the same experimental conditions three times less binding and 10 times less binding were detected for the human polyclonal IgM standard and human monoclonal IgM λ BR respectively. No binding to human μ chain, κ and λ chains, human IgA and human IgG was detected. In Fig.2 representative binding curves are shown as the O.D. versus different concentration of monoclonal antibody Y7 added to polyclonal human IgM, IgM λ DJ immunogen and monoclonal IgM λ BR. Based on these results the IgM λ DJ specific epitope was detected in the presence of Y7 MoAb.

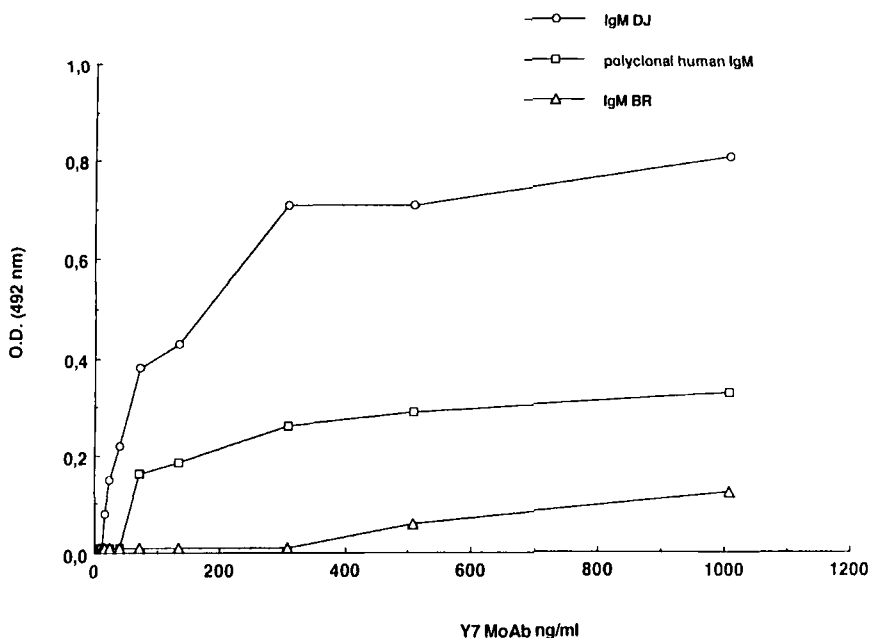


Figure 2. Binding of Y7 MoAb to IgMs coated ELISA plates: IgM λ DJ (o-o), polyclonal human IgM (□-□), IgM λ BR (Δ-Δ)

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## Localization of IgM λ DJ Specific Epitope

The enzyme pepsin in 0.1 M, Na-acetate buffer pH 4.1, produced incomplete fragmentation. Fragments of different size were detected. The most pronounced electrophoretical bands of IgM λ DJ under nonreducing conditions had relative molecular masses of 180, 140, 119, 84 and 56 kDa and were all detected in the presence of Y7 MoAb (Fig. 3). No binding was detected in the presence of reduced electrophoretically separated IgM λ DJ pepsin fragments. Binding of Y7 for pepsin fragments of polyclonal human IgM either under nonreducing or reducing conditions was at the level of nonspecific binding. The specificity of Y7 MoAb was in this way localized in the Fab fragment of IgM λ DJ which was further defined as a conformational determinant.

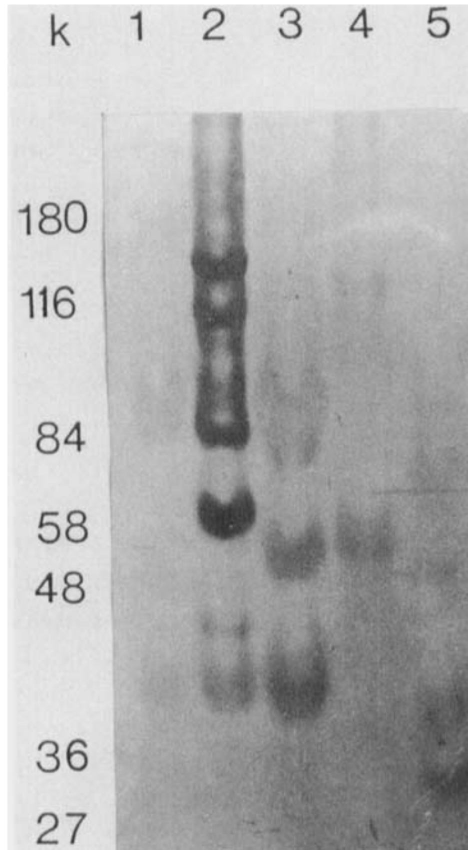


Figure 3. Western blot of IgM fragments under reducing and nonreducing conditions: IgM λ DJ prior to fragmentation under reducing conditions (lane 1), IgM λ DJ pepsin fragments under nonreducing conditions (lane 2), IgM λ DJ pepsin fragments under reducing conditions (lane 3), polyclonal human IgM pepsin fragments under nonreducing conditions (lane 4) and polyclonal human IgM pepsin fragments under reducing conditions (lane 5).

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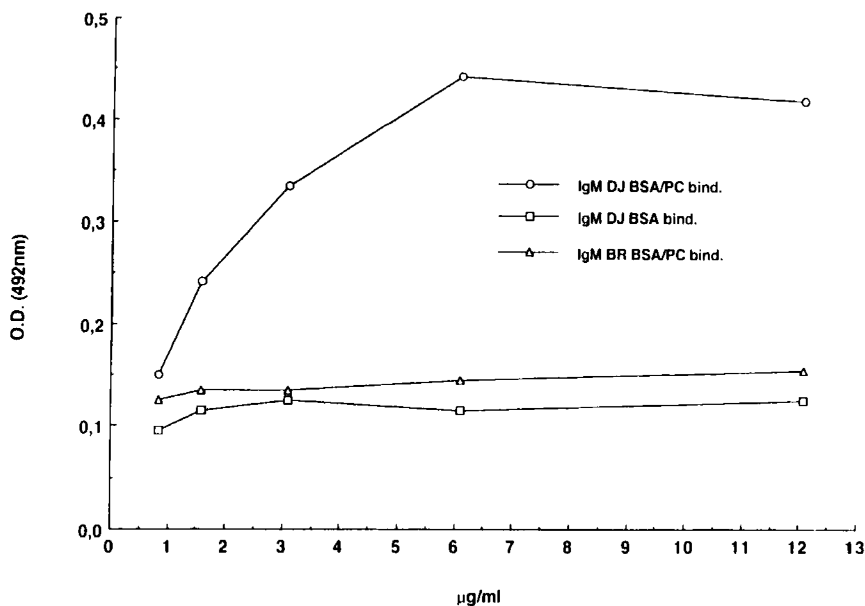


Figure 4. Dose-dependant binding of IgM λ DJ to PC/BSA (o-o). Binding of IgM λ DJ to albumin alone (□-□) and binding of human monoclonal IgM λ BR (Δ-Δ) to PC/BSA are presented as controls.

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## Anti-idiotypic Property of Y7 MoAb

After localization of the Y7 specific epitope in the Fab fragment of IgM λ DJ immunogen, the anti-idiotypic property of Y7 MoAb was further characterized by its ability to inhibit IgM λ DJ immunogen binding to PC/BSA. It was shown that IgM immunogen exhibited dose-dependent binding to albumin/PC with saturation achieved at a concentration of 6 μg of immunogen (Fig.4). More than 95% inhibition with Y7 MoAb was achieved with all concentrations of immunogen tested. 50% inhibition was achieved

with Y7 MoAb (Fig. 5) in concentrations between 100 and 1000 ng/ml. Under the same experimental conditions no inhibition occurred in the presence of the mouse monoclonal IgG1 used as the control.

## DISCUSSION

The characterization of monoclonal a-Id is not always an easy task. Immunochemical characterization of monoclonal a-Ids usually implies testing for the three following parameters: 1/ its property to detect a conformational epitope in the Fab region of an immunoglobulin molecule; 2/ its property to detect idiotype presence in a mixture of polyclonal idiotypes or different monoclonal idiotypes; 3/ and for immunochemical characterization of a-Id it is necessary to detect the paratope specificity of the idiotype bearing immunoglobulin. Discovering the paratope specificity of the idiotype is essential for final immunochemical characterization of a-Id. According to the third criterium, monoclonal anti-paratopic antibodies are classified as Ab2$\beta$ or Ab2$\gamma$ a-Id.

During the characterization of Y7 MoAb we followed these three criteria.

Enzymatic treatment of IgMs results in the production of asymmetric fragments, like Fab$\mu$ $(C\mu 2)_2$, fragments which have been already reported for pepsin (19), pronase and proteinase K digestion (20). Y7 MoAb bound to IgM $\lambda$ DJ fragments from 180 to 56 kDa only under nonreducing conditions. These results confirmed that the Y7 conformational specific epitope is present in the Fab of IgM $\lambda$ DJ immunogen and fulfilled the first criterium of an a-Id which is defined as conformational determinant (21).

The detection of cross-reactive idiotypes (CRI) in the mixture of polyclonal idiotypes by a-Id was very low and assay specific. Using of a hemaglutination assay, 8% of the cold agglutinin cross-reactive idiotypes (CRI) were detectable among normal immunoglobulins (22).

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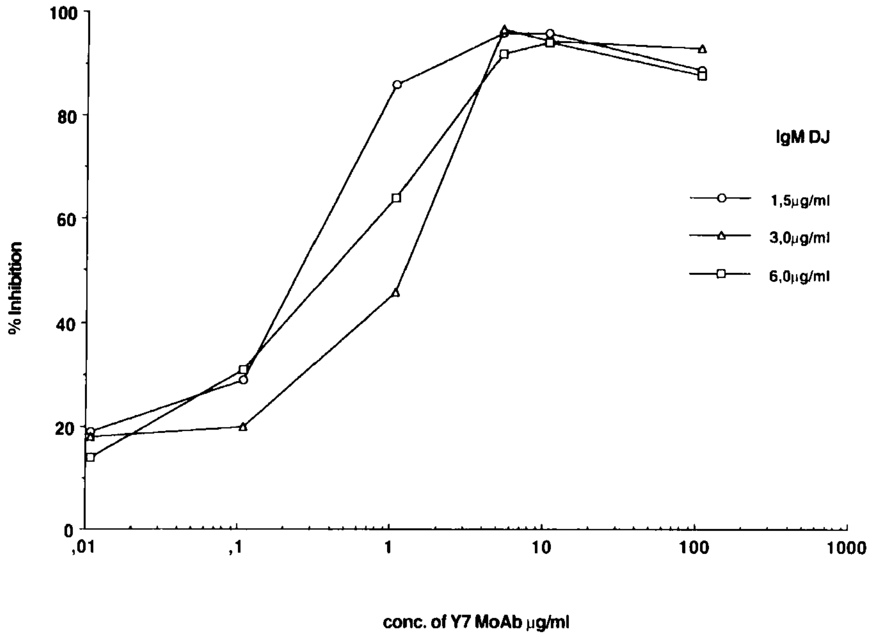


Figure 5. Dose-dependent inhibition of IgM λ DJ binding to albumin/PC in the presence of different concentration of Y7 MoAb. Different concentrations of phosphorylcholine specific IgM DJ in competitive ELISA are presented: 1.5 μg (o-o) 3 μg (□-□) and 6 μg (Δ-Δ).

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Isotype or allotype epitope specificity was excluded by testing the isolated Y7 MoAb against standard Ig preparations. According to some investigators (23) testing of monoclonal a-Id should be done on several monoclonal Ig antigens. In our experiment we tested only monoclonal IgM λ BR as an individually different Ig antigen.

The high reactivity with polyclonal human IgM detected in the direct ELISA can be explained by the presence of cross-reactive idiotypes. Besides these, the existence of IgM rheumatoid factors (RF) in the mixture of polyclonal human IgM is also possible. Although, the polyclonal human IgM was negative in the Latex RF

test, this does not exclude the possibility of the existence of low concentrations of naturally occuring IgM RF in this preparation. Meanwhile presence of Y7 CRI in the mixture of polyclonal human IgM was studied in an independent experiment and reported to be less than 5% (24). Therefore, the nonspecific binding registered in our ELISA was more than 25%. A posibility of nonspecific hydrophobic interactions between Ig molecules may also be an explanation for low specificity in direct ELISA.

The third part of a-Id detection was the classification of Y7 MoAb as an anti-paratopic antibody. The inhibition of IgM λ DJ binding to phosphorylcholine in the presence of Y7 MoAb classified it immunochemically as Ab2β a-Id. According to Reisen et al. (25) among 904 human sera with myeloma (IgA, IgG and IgM) tested, only one monoclonal IgM κ had the phosphorylcholine paratope. Since agar gel precipitation with pneumococcus C polysaccharide (PnC) was used as the screening procedure in this experiment, the low frequency of this paratope may be not valid. The nonhydrophobic nature of PC excludes the possibility of nonspecific interaction due to hydrophobicity. Besides that, this interaction exhibits dose-dependence. The interaction through the phosphate ion, as Kabat et al. (26) suggested for two monoclonal human IgMs, cannot be excluded even in our case and may be compared to phosphorylcholine specific mouse myeloma protein (McPC603).

Further biological evaluation of such an anti-Id will maybe explain whether it has any role in network or clonal regulation of the immune system.

**ACKNOWLEDGMENT**

This work was supported by the Scientific Fund of Serbia.

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