

GENERATION OF ANTI-IDIOTYPIC AND ANTI-ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES IN THE SAME FUSION

Support of Jerne's Network Theory¹

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Upon immunization of mice with a mAb (290A-167) directed against an epitope of Lol p I (the major allergenic determinant of *Lolium perenne*), both anti-idiotypic (aId) mAb (Ab2) and anti-aId mAb (Ab3) were produced. The Ab2 displayed the following internal image properties of Lol p I: it can be affinity-purified on an immobilized Id column; its binding to the anti-Lol p I mAb (290A-167) is inhibited by Lol p I; it inhibits in a dose-response fashion the binding of the specific Id to Ag. It is recognized by anti-Lol p I antisera from different species such as mouse, human, and goat. The Ab3 which binds to Lol p I was also produced from the same fusion. This binding was inhibited significantly by aId mAb (Ab2), anti-Lol p I mAb (290A-167) and Lol p I. These data indicate that the two mAb with specificity for Lol p I (290A-167 and Ab3) share similar reactivity to the Ag and that aId mAb is the internal image of the epitope recognized by the Id. We showed also that the capacity of rabbit aId Ab directed against the 290A-167 Id to inhibit the binding of Ab1 and Ab3 to Ag was almost abolished by passage over a Ab3-coated Sepharose column. This would suggest that not only are the two mAb with reactivity to Lol p I (Ab1 and Ab3) directed against identical epitopes, but that they in fact shared identical idiotopes as well. The production of identical mAb upon immunization with either the Ag or the aId mAb supports that the conceptual framework proposed by Jerne finds its biologic application in the course of an immune response.

aId³ Ab were originally described by Oudin and Michel (1) and by Kunkel et al. (2) as markers that distinguished the V regions of specific Ab molecules. Jerne (3) postulated later that they play an essential regulatory role in the immune network system. Indeed since there is coexistence, within each individual's repertoire of complementary V region partners, the Id could serve as regula-

tory targets for communication between Ag-reactive cells in the absence of extrinsic Ag. Experimental animals are clearly capable of producing aId Ab to their own Ig and these could, in turn, modulate the first Ab response (4-9). In humans (5-9) aId Ab occur in the course of normal immune response, such as that to tetanus toxoid (10, 11); they are also involved in the physiopathology of some disorders such as autoimmune diseases (12-14) or immunodeficiencies (15), and in the IgE Ab response related to allergens (16-18), where the levels are influenced by seasonal exposure and immunotherapy (17, 18). Four categories of Ab2 have been identified and the best known are Ab2 α , which recognizes the Ab1 idiotopes, and the Ab2 β , which is complementary to the Ab1 paratope and represents the internal image of the Ag. This work aims to produce and characterize aId mAb (Ab2) upon immunization of syngeneic mice with murine mAb (290A-167) (19, 20) directed against one epitope of Lol p I, the major allergenic determinant of *Lolium perenne*, one of the grass pollens. Since the concept of internal image stems from the possibility of raising anti-aId Ab (Ab3) that will recognize the original Ag, this work aims also to produce, from the same fusion, and subsequently characterize, the Ab3 that should occur in the course of this immune response.

MATERIALS AND METHODS

Ag and Antisera

Lol p I was purified by immunosorbent techniques from crude extracts of *L. perenne* (Omega Laboratories, Montreal) as described previously (19). Anti-Lol p I mAb 290A-167 (γ 2b, κ), 348A-6 (γ 1, κ) and 539A-6 (γ 2a, κ) were produced in our laboratory and previously characterized (19, 20). These mAb were purified from murine ascitic fluids by passing the fluids through a protein A-Sepharose CL4B column. AgE, the major allergen of ragweed, was obtained from the National Institutes of Health (NIH, Bethesda, MD). Human Lol p I specific IgG Ab fraction and goat anti-Lol p I antiserum were produced and purified as previously described (21).

Immunization of Mice and Production of Hybridomas

Syngeneic mice (BALB/c) were first immunized with i.p. injection of 50 μ g of either one of the two purified anti-Lol p I mAb (290A-167, 539A-6) emulsified in CFA. Then the mice were injected i.p. with 50 μ g of the same mAb emulsified in IFA every 3 wk for 6 mo. Retroorbital bleedings were performed and serum samples were tested for the presence of the different activities as described thereafter. Seven and five days before fusion, 50 μ g of mAb were given i.v. The mice were then sacrificed and the spleen cells fused with SP2/O-Ag14 myeloma cells as previously described (19).

Detection of Ab2 and Ab3 Activities

The anti-anti-Lol p I mAb (aId mAb, Ab2) activity was measured on the basis of the ability of murine serum or hybridoma supernatant

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³ Abbreviations used in this paper: aId, anti-idiotypic; Ab, antibody; Lol p I, Ag Lol p I of *Lolium perenne*; AgE, Ag E of ragweed pollen.

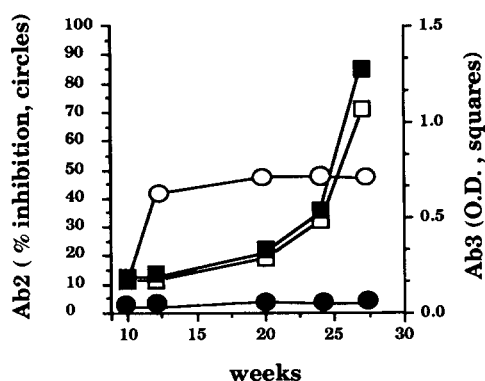


Figure 1. Kinetic of the detection in mouse serum (1/100) of anti-290A-167 (binding-inhibition of 290A-167 to Ag, expressed in percent inhibition on the left axis, circles) and anti-Lol p I (direct binding on Lol p I expressed in optical densities (OD) on the right axis, squares) responses upon immunization with anti-Lol p I mAb (290A-167, open symbols, and 539A-6, closed symbols) on day 0.

to inhibit the binding of anti-Lol p I mAb (290A-167) to solid phase-fixed Lol p I (21–23). Briefly, cyanogen-activated cellulose disks (Whatman, England) were coated with Lol p I (100 ng per disk in 100 mM borate buffer, pH 8.0), followed by 0.9 M ethanolamine to saturate additional protein-binding sites. Then, ^{125}I -labeled anti-Lol p I mAb (290A-167), at concentration corresponding to 50% of maximum binding, was incubated with 100 μl of serum (1/100) or hybridoma supernatant to be tested for 2 h at room temperature; the mixture was then incubated with the coated disks for 4 h at 4°C and the radioactivity was counted in a LKB gamma counter after extensive washes. The percentage of inhibition was calculated as follows:

$$\left[1 - \left(\frac{\text{cpm bound in presence of inhibitor}}{\text{cpm bound in absence of inhibitor}} \right) \right] \times 100$$

The anti-Lol p I (Ab3) activity in serum or hybridoma supernatant was screened in a direct Ag-binding assay by using Lol p I-coated disks. Briefly, serum or hybridoma supernatant were incubated with Lol p I-coated disks for 18 h at 4°C. After washes, ^{125}I -goat anti-mouse Ig antiserum (Amersham, UK) was added and incubation continued for 6 h at 4°C. The radioactivity was then counted.

Purification of mAb

Hybrid cells producing the appropriate mAb were then propagated in the peritoneal cavity of female BALB/c mice after prior injection with pristane (0.5 ml per mouse). The ascitic fluid was harvested 10 to 14 days later, and ald mAb (Ab2) and anti-ald mAb (Ab3) were purified by passing the fluid through a protein A-Sepharose CL4B column. They were then eluted and dialyzed against 10 mM PBS. Ab2 was also purified by affinity chromatography by using Id-Sepharose CL4B column. Then the bound material was eluted with glycine-HCl (pH 2.8) and dialyzed against PBS (pH 7.4). The isotype was determined by Ouchterlony analysis. An interactive computer program was used for the determination of the affinity constant of the mAb by nonlinear regression analysis of inhibition assay data (24). A fixed amount of labeled Ag and different amounts of unlabeled Ag were then incubated with solid-phase fixed mAb as described later.

Radiolabeling of Proteins

Purified mAb (30 μg) or Lol p I Ag were iodinated by using Iodogen (Pierce Chemical Co., Rockford, IL), and free ^{125}I was separated from ^{125}I -labeled mAb or Ag by passage through an Econo-Pac 10DG column (Bio-Rad, Richmond, CA). Specific activity was estimated in accordance with the instructions of the manufacturer.

Id Specificity of Ab2 and Ab3

Binding inhibition of anti-Lol p I mAb (Ab1 or Ab3) to Lol p I. The saturation point of ^{125}I -labeled anti-Lol p I mAb (Ab1 or Ab3) was determined by the addition of various concentrations of the labeled mAb to the Ag-coated disks for 4 h at 4°C and the bound radioactivity was counted after extensive washes. In a competitive assay (21), ^{125}I -labeled anti-Lol p I mAb at concentration corresponding to 50% of maximum binding was incubated with various concentrations (78 to 5000 ng/ml) of inhibitors for 12 h at room temperature; the mixture was then incubated with the Lol p I-coated disks for 4 h at 4°C and the radioactivity was counted. The percentage of inhibition was calculated as previously described (20). The inhibitors used were as follows: 1) ald mAb (Ab2) purified by affinity chromatography either using an immobilized Id column or a protein A column 2) the rabbit ald Ab directed against the Id 290A-167 (23), either before or after passage over an Ab3-Sepharose CL4B column as indicated; 3) the anti-Lol p I mAb used for immunization (290A-167) and the anti-ald mAb (Ab3) obtained from the same fusion. Used as controls were two anti-Lol p I mAb directed against a different epitope of Lol p I (348A-6, 539A-6), and the Lol p I Ag or the AgE.

Binding inhibition of anti-Lol p I mAb (Ab1 or Ab3) to ald mAb (Ab2). These experiments were carried out as described above except that ald mAb (Ab2)-coated disks were used with a constant quantity of ^{125}I -anti-Lol p I mAb (Ab1 or Ab3) and various concentrations of inhibitors (as indicated).

RESULTS

Production of ald and anti-ald upon immunization with anti-Lol p I mAb. A panel of three mAb directed against three distinct non-overlapping and nonrepetitive epitopes (19) of Lol p I were recently produced and characterized in our laboratory (20–23). Two of them (290A-167 and 539A-6) were then used to immunize syngeneic mice. Mouse sera were tested for the presence of two different activities: 1) Ab inhibiting the binding of labeled 290A-167 mAb to Lol p I (Ab2) and 2) Ab binding directly to Lol p I Ag (Ab3). As shown in Figure 1, an anti-290A-167 Ab response was detected after 10 wk only in mice immunized with the 290A-167 Id (no response could be detected before 8 wk, not shown) and the peak response seen at 12 wk. An anti-Lol p I activity was detectable at 26 wk upon immunization with the two anti-Lol p I mAb (290A-167 and 539A-6). Immunization with unrelated mAb did not yield any specific Ab response (not shown). The mouse spleen cells were then fused for production of Ab-secreting hybridoma as previously described (20–23). From this fusion, one hybridoma, termed A7H2,

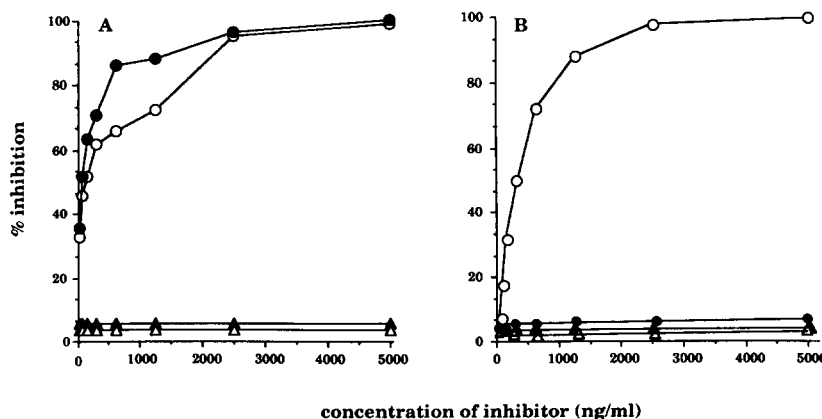


Figure 2. Binding inhibition of 290A-167 (A) and 348A-6 (B) anti-Lol p I mAb to Lol p I by Lol p I (○—○), A7H2 (Ab2) (●—●), unrelated Ag (AgE) (△—△), or anti-Lol p I mAb 539A-6 (▲—▲). The bindings of 290A-167 and 348A-6 to Lol p I are $54,246 \pm 6,253$ and $40,238 \pm 7,856$ cpm $\pm$ SEM, respectively. Each point represents the mean value of three experiments.

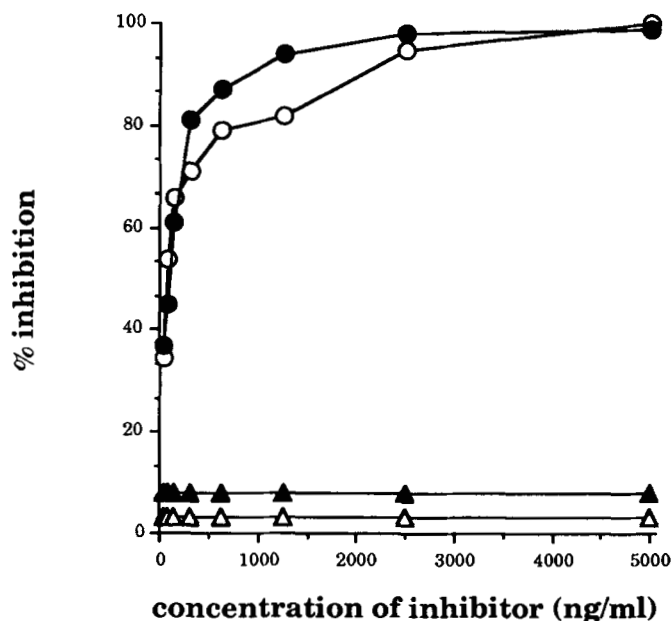


Figure 3. Binding inhibition of anti-Lol p I mAb (290A-167) to ald mAb (A7H2) by Lol p I (○—○), A7H2 (Ab2) (●—●), unrelated Ag (AgE) (△—△), or anti-Lol p I mAb 539A-6 (▲—▲). The binding of 290A-167 to A7H2 is $24,568 \pm 4,152$ cpm $\pm$ SEM. Each point represents the mean value of three experiments.

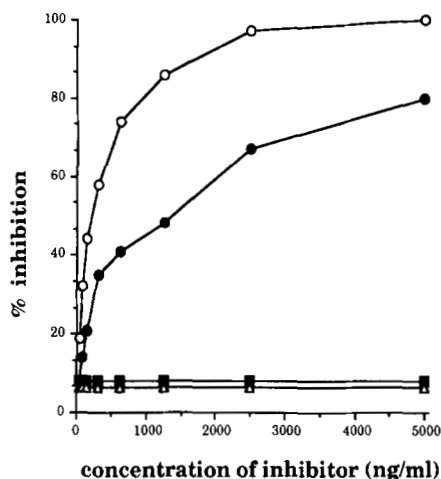


Figure 4. Binding inhibition of anti-ald mAb (1H4) to Lol p I by Lol p I (○—○), A7H2 (Ab2) (●—●), 348A-6 (■—■) or unrelated Ag (ragweed) (△—△). The direct binding of Ab3 to Lol p I is $44,854 \pm 6,784$ cpm $\pm$ SEM. Each point represents the mean value of three experiments.

produced Ab that effectively inhibited the binding of 290A-167 to Lol p I without showing specific binding to Lol p I. This mAb was referred to as ald mAb or Ab2. From the same fusion, another hybridoma (1H4) producing Ab able to recognize Lol p I Ag (anti-ald mAb or Ab3) was selected. These two mAb, shown by Ouchterlony analysis to be IgG1 κ , were then purified over protein A-Sepharose column from ascitic fluids. The ald mAb were also purified by affinity chromatography with the use of an Id-Sepharose column. All the results reported herein were obtained by using protein A-Sepharose-purified mAb but were comparable to those obtained by affinity chromatography with mAb (not shown).

Idiotypic specificity of Ab2. Figure 2A shows that binding of labeled 290A-167 to Lol p I is inhibited in a dose-response fashion by A7H2 mAb (Ab2) and by Lol p I Ag, but not by unrelated Ag nor by an anti-Lol p I mAb

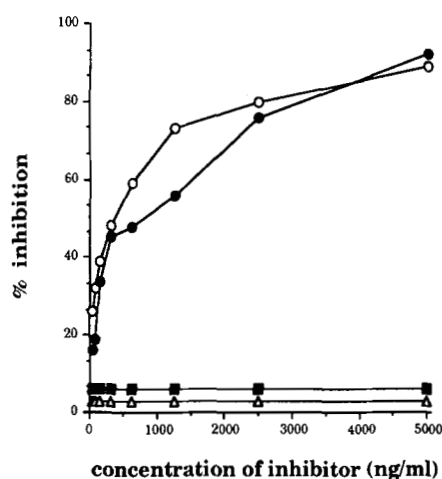


Figure 5. Binding inhibition of 1H4 (Ab3) to A7H2 (Ab2) by Lol p I (○—○), A7H2 (Ab2) (●—●), 348A-6 (■—■) or unrelated Ag (ragweed) (△—△). The binding of Ab3 to Ab2 is $18,687 \pm 1,112$ cpm $\pm$ SEM. Each point represents the mean value of three experiments.

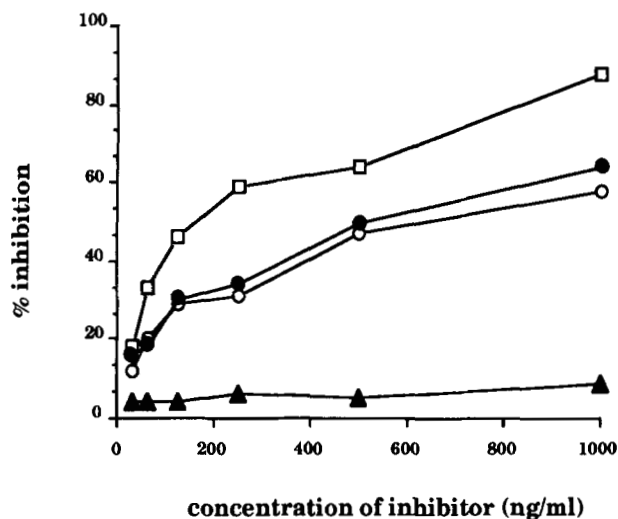


Figure 6. Binding inhibition of 290A-167 to Ab2 (A7H2) by 290A-167 (□—□), purified human anti-Lol p I Ab (○—○), goat anti-Lol p I anti-serum (●—●) and serum from nonimmunized goat (▲—▲). Each point represents the mean value of three experiments.

of different specificity. Binding of the 348A-6 Id is effectively inhibited by Lol p I but not by Ab2, by unrelated Ag nor by an anti-Lol p I mAb of different specificity (Fig. 2B), which supports the fine specificity of ald mAb. In order to show that the idiotype determinant recognized by the ald Ab (A7H2) is associated with the Ag-binding site, we reported in Figure 3 the ability of Lol p I but not unrelated Ag to inhibit the binding of Ab1 to Ab2. Addition of Ab2 as opposed to mAb of different specificity abolished also completely this response. Altogether, these data confirm that Ab2 is highly specific and directed against an epitope located inside the binding site of the Id 290A-167.

Ab2 is the internal image of Ag. The binding inhibition of Ab1 to fixed Lol p I or Ab2 by the Ab2 and Lol p I is absolutely necessary but not sufficient to conclude that Ab2 bears the internal image of the epitope recognized by anti-Lol p I mAb (290A-167). Indeed, an ald Ab (Ab2 α), although not bearing the internal image of the Ag, may recognize an idiotope expressed on the paratope of the Id and therefore interfere with this binding. The concept of internal image stems from the possibility of raising Ab

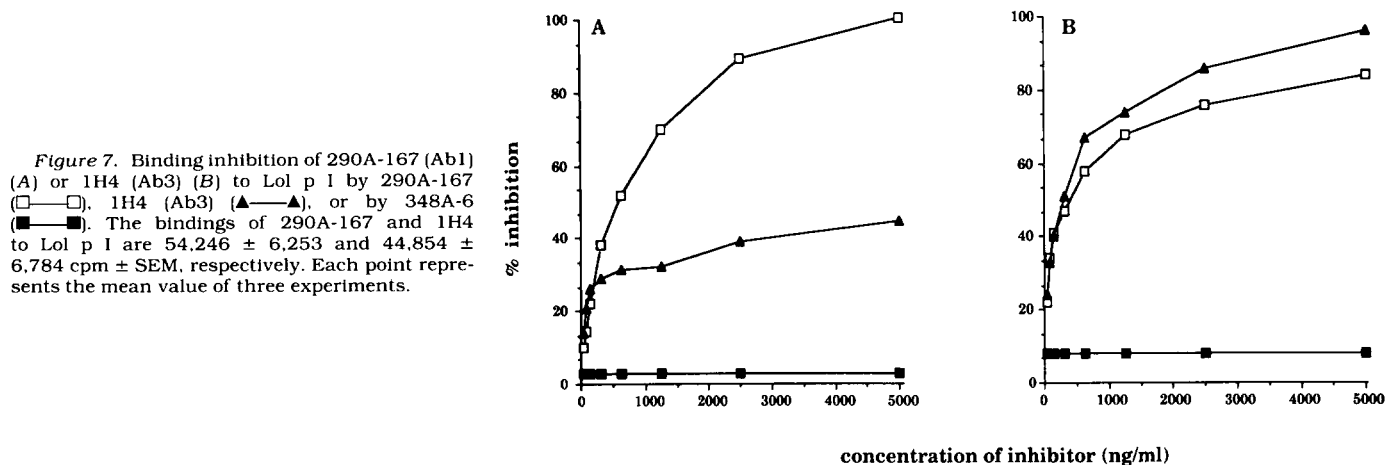


Figure 7. Binding inhibition of 290A-167 (Ab1) (A) or 1H4 (Ab3) (B) to Lol p I by 290A-167 (□), 1H4 (Ab3) (▲), or by 348A-6 (■). The bindings of 290A-167 and 1H4 to Lol p I are $54,246 \pm 6,253$ and $44,854 \pm 6,784$ cpm $\pm$ SEM, respectively. Each point represents the mean value of three experiments.

TABLE I
Affinity constants for Lol p I and Ab2 (A7H2)

mAb with Anti-Lol p I Activity	Affinity Constant (mol/liter)	
	Lol p I	A7H2
290A-167 (Ab1)	4.3×10^{-9}	1.18×10^{-9}
1H4 (Ab3)	2.9×10^{-8}	2.48×10^{-9}

that are directed against Ab2 but recognize the original Ag; anti-ald Ab or Ab3. Taking advantage of the Id network concept, we looked for the presence of Ab3 from the same fusion. In fact, one mAb (1H4) was shown to bind specifically to Lol p I Ag. This binding was inhibited by Ab2 (A7H2) and by Lol p I Ag (Fig. 4). This suggests that Ab3 possesses specificity similar to that of Ab1 in terms of reactivity with Lol p I. Furthermore, Ab3 (1H4) binds to Ab2 (A7H2), and this binding was inhibited in a dose-dependant manner by Ab2 and Lol p I Ag (Fig. 5). Finally, it could be seen on Figure 6 that both goat anti-Lol p I antiserum and purified human anti-Lol p I Ab inhibited significantly the binding of radiolabeled 290A-167 Id to solid phase-fixed Ab2. However we previously reported that the goat antiserum could not inhibit the binding of Id to rabbit polyclonal anti-Id Ab (23) but this discrepancy could be explained by the use of ald mAb herein. Altogether, these data support the concept that Ab2 (A7H2) is the internal image of the epitope recognized by the Id 290A-167.

Comparison of idiotypic specificity of Ab1 and Ab3 mAb. We tested the ability of Ab1 or Ab3 to inhibit the

binding of Ab1 to Lol p I and to Ab2. Figure 7A shows that Ab1 binding to Lol p I is significantly inhibited by itself and, to a lesser degree, by Ab3 mAb. The difference of binding inhibition by Ab1 and Ab3 could have been explained by the production of Ab3 by an Ab2 which is not the internal image of Ag (Ab2 α). But the significant inhibition of Ab3-binding to Ag by both mAb strengthens the fact that the Ab2 carry the internal image of Ag (Fig. 7B). The difference in the profile of inhibition of Ab1 to Lol p I by Ab1 and Ab3 could be explained by a higher affinity of Ab1 than Ab3 for the Ag. Study of the affinity constants of the two mAb shows that this is indeed the case (Table I). Up to now, the data suggest that Ab1 and Ab3 are directed against an identical epitope of Lol p I. Furthermore, the binding of Ab1 or Ab3 (Fig. 8, A and B, respectively) to Ab2 is inhibited by Ab1 or Ab3 in a comparable fashion. This observation suggests that Ab2 has comparable affinity for both Ab1 and Ab3 (Table I) and that the two anti-Lol p I mAb (Ab1 and Ab3) share the same paratopic region. These data strongly support that Ab3 has not been elicited by an innocent bystander and Ab2 is in fact the internal image of the epitope recognized by Ab1. We next examined whether Ab3 expresses an Id similar to that of Ab1. For this purpose, we used polyclonal rabbit ald Ab directed against the paratopic and nonparatopic region of the Id 290A-167, as previously reported (21). This anti-290A-167 Ab inhibited significantly the binding of Ab1 and Ab3 to Lol p I (Fig. 9, A and B, respectively). After passage over an Ab3-

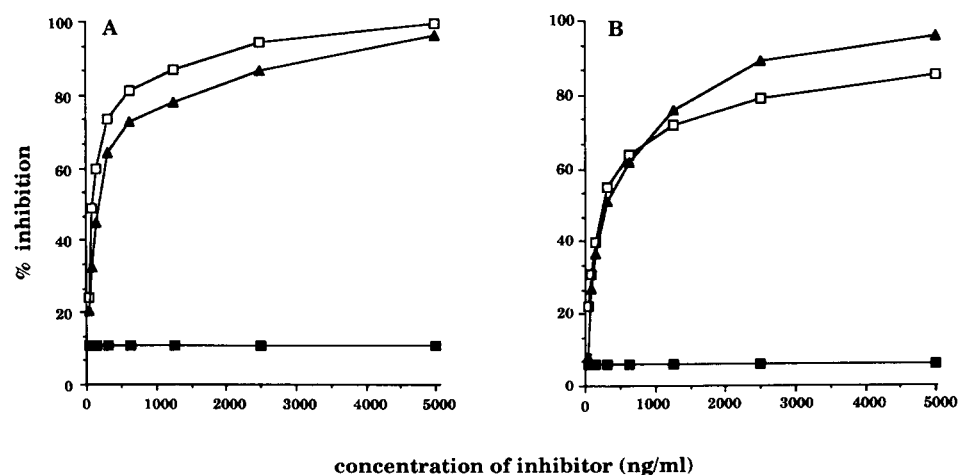
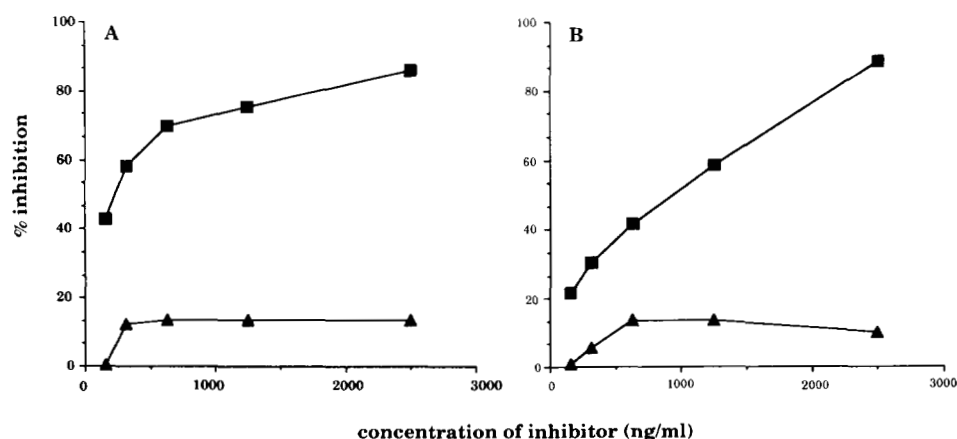


Figure 8. Binding inhibition of 290A-167 (Ab1) (A) or 1H4 (Ab3) (B) to A7H2 (Ab2) by 290A-167 (□), 1H4 (Ab3) (▲), and 348A-6 (■). The bindings of 290A-167 and 1H4 to A7H2 are $24,568 \pm 4,152$ and $18,687 \pm 1,112$ cpm $\pm$ SEM, respectively. Each point represents the mean value of three experiments.

Figure 9. Binding inhibition of Ab1 (A) or Ab3 (B) to Lol p I by rabbit ald Ab (anti-290A-167 mAb) before (■—■) and after (▲—▲) adsorption over an Ab3-coupled Sepharose column. Each point represents the mean value of three experiments.



coated column, the resulting material failed to inhibit the binding of Ab1 or Ab3 to Ag. This observation suggests that these two mAb with specificity for Lol p I share the same Id.

DISCUSSION

The idiotypic network theory, put forward by Jerne (3, 25) implies a means of regulating available repertoires of interacting Ab (Ab1, Ab2, Ab3...) generated during the course of any immune response. It is predictable from this hypothesis that all the molecules involved in this network should be found after immunization with Ag. In the present study, we are reporting, in the same fusion, the production of ald mAb (Ab2) directed against an anti-Lol p I mAb (290A-167) previously produced and described in our laboratory (19, 20) and an anti-ald mAb (Ab3). The ald mAb (Ab2) displayed internal image properties of Lol p I and could be considered as an Ab2 β according to the following criteria: 1) this Ab can be affinity-purified on an immobilized Id column; 2) it binds to the anti-Lol p I mAb (Ab1) and not to Lol p I and this binding is inhibited by itself, by Ab1 as well as by Lol p I, but not by unrelated Ag or anti-Lol p I mAb of different specificity. The Ab2 does not affect the binding of an anti-Lol p I mAb of different specificity to Ag, which supports its fine specificity; 3) it inhibits in a dose-response fashion the binding of Ab1 to Ag and this inhibition is comparable to that of Lol p I, whereas irrelevant Ag had no effect; and 4) it is recognized by anti-Lol p I antisera of different species such as mouse, human, and goat.

This observation alone would not lead us to conclude that the Ab2 bears the internal image of the Ag, because reactivity of Ab2 with idiotope outside the combining site of Ab1 cannot be ruled out. The essential notion of an internal image of the Ag borne by anti-Id Ab is the basis for using these as potential surrogate Ag which could mimic the stereospecificity and functionality of the original Ag (26) as elegantly shown by Reilly and Roots (27) with glucagon and insulin receptors as models. In fact, the capacity of ald Ab to induce the production of protective Ab and cellular immunity, probably through the generation of naturally occurring anti-ald Ab (Ab3) (28) as proposed by the network theory, was clearly shown in reovirus, *Streptococcus* infections, and experimental parasitic infections such as schistosomiasis (29, 30). Since the essential notion of an internal image of the Ag borne by ald Ab is the basis for using these as potential

surrogate Ag, we looked also, in this work, upon immunization with anti-Lol p I mAb, for the generation of Ab3 in the same fusion. Such a finding would confirm Jerne's theory and prove that Ab2 is really the internal image of Ag. In fact, Bona et al. (31) have reported that immunization of syngeneic mice with Ab1 lead to the production of Ab2 and Ab3 responses where however, the Ab3 failed to recognize Ag, possibly because of the difficulty of detecting Ag-binding activity of Ab3 in their system. But there are number of instances of Ab3 displaying Ab1-like Ag-binding activity (32, 33). We actually found one mAb that binds to Ab2. This binding of Ab3 to Ab2 can be inhibited by both Lol p I and Ab2. Furthermore, we found that this mAb binds also to Lol p I, which binding is inhibited completely by Ab2 or Ag. These data suggest that the two anti-Lol p I mAb (Ab1 and Ab3) share similar reactivity to the Ag and support that Ab2 is the internal image of the epitope recognized by Ab1.

We then compared the idiotypic specificities of Ab3 with that of Ab1. We showed that the binding of Ab1 to Ag is inhibited by itself and to a lesser degree by Ab3, whereas the two anti-Lol p I mAb (Ab1 and Ab3) inhibit the binding of Ab3 to Lol p I. This discrepancy could be explained by a difference of affinity between the two mAb, confirmed by measuring their affinity constants for Lol p I. The above observations thus suggest that the two anti-Lol p I mAb are directed against identical epitope on the molecule. The earlier detection in murine serum of Ab2 at 10 wk after immunization as compared to 26 wk for the Ab3 support that the Ab3 could not have been elicited by an innocent bystander. The possibility nonetheless remains that Ab3 expresses an idiotope outside the combining site that is different from that of Ab1. In order to address this question, we used a rabbit ald Ab directed against the 290A-167 Id and succeeded in inhibiting the binding of the two anti-Lol p I mAb to the Ag. If these two mAb share the same Id, it should be possible to adsorb out the anti-Ab3 and anti-Ab1 activities of rabbit ald Ab by passage over an Ab3-coated column. Our results support this hypothesis, confirming that Ab1 and Ab3 share the same Id. The importance of this observation resides in the fact that mAb sharing common Id could be produced upon immunization with either the Ag or the ald mAb. Our results further confirm that the conceptual framework proposed by Jerne is possible and may play a role in the maintenance of immunologic homeostasis by forming a network with multiple connections.

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