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Antibody Synthesis Induced by Endogenous Internal Images

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ABSTRACT

In this study, immunization with a vaccine consisting of multiple $F(ab')_2$ fragments of affinity-purified antitetanus toxoid antibodies covalently bound to a carrier protein successfully induced antitetanus toxoid antibodies. Further studies showed that this vaccine preparation contained no biologically detectable tetanus antigen. The induced antitetanus antibody (Ab1′) titer was higher than the titer of antibodies binding control antigens. The immunizing $F(ab')_2$ preparation did not elicit a secondary antitetanus response from mice primed with tetanus toxoid and, hence, appeared free of tetanus epitopes. The specificity of Ab1′ was established by absorption and inhibition with antigen. Immunization with antitetanus $F(ab')_2$ (Ab1′) fragments appears to have elicited naturally occurring autologous antitetanus toxoid antibody (Ab1′) through an idiotypic pathway. As predicted by network theory, anti-idiotype (Ab2) and antitetanus (Ab1′) cycled reciprocally. Clonotypic characterization of Ab1′ using isoelectric focusing and affinity immunoblotting showed increases in Ab1′ titer to be the result of increased synthesis by limited subsets of antitetanus toxoid B-cell clones and not increased synthesis by multiple clones, as is characteristic of antigen-driven Ab1 responses. Many Ab1 and Ab1′ clonotypes had identical pIs, suggesting that they either share V region genes or are the product of the same B-cell clones. These findings indicate that immunization with polyclonal multivalent Ab1 preparations can trigger active synthesis of antibodies with the same specificity. The results provide further evidence for naturally occurring idiotypic cascades that could be exploited for studies of catalytic antibodies.

Index Entries: Auto-anti-idiotype; idiotype network; internal image; tetanus.

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INTRODUCTION

A novel approach to antibody induction involves using anti-idiotype antibodies of the Ab2β type as immunogens. This approach is based on the idiotype network (*1*) prediction of an ordered cascade of antibody epitopes and paratopes (*2*) first documented by Urbain et al. (*3*) and Cazanave (*4*). This cascade (recently reviewed in refs. *5–7*) consists of antibodies produced in response to the antigen stimulus (Ab1) and anti-idiotypic antibodies (Ab2) that are specific for idiotopes either outside (Ab2α) or within (Ab2β) the antigen-binding site of Ab1. Ab2 has the capacity, under proper circumstances, to induce anti-anti-idiotypic antibodies (Ab3). The Ab2β subset is thought to bear an internal image of antigen and to be capable of inducing an Ab3 population that binds the antigen that was used to elicit Ab1. This subset is known as Ab1′, because it is Ab1-like in its specificity for antigen. Ab2β molecules were first suggested to have vaccine potential by Nisonoff and Lamoyi (*8*) and Roitt et al. (*9*).

Most idiotype-based vaccine studies have focused on the anti-idiotypic Ab2 antibodies (*5–7,10*), particularly the Ab2β subset. However, there is compelling evidence supporting the role of non-Ab2β idiotypic pathways in the induction of immunity (*11*). Other studies have documented an image-like function by non-Ab2β antibodies (*12–14*). These data have raised questions about the true nature of idiotypic images (*15*). Few studies have specifically emphasized the role of non-Ab2 pathways and the idiotype cascade in the induction of specific immunity.

This article shows that immunization with a multivalent form of polyclonal Ab1 F(ab′)$_2$ fragments prepared from purified antitetanus toxoid antibodies induces antibodies specific for tetanus toxoid in the absence of antigen stimulation. The specificity of the resulting Ab1′ was established by absorption and inhibition with antigen. As network theory predicts, Ab2 and Ab1′ titers cycled alternatively throughout the study. A sensitive biological assay was used to show that Ab1′ induction was not the result of residual tetanus toxoid epitopes in the immunizing preparation. Isoelectric focusing analysis showed that only limited numbers of clonal products contributed to the Ab1′ population.

MATERIALS AND METHODS

Generation and Purification of Ab1 F(ab′)$_2$ Fragments

Antitetanus toxoid (TT) antibody from a rabbit that was hyperimmunized with the C-fragment of TT was affinity purified on a TT-Sepharose column. Antibodies were eluted with 0.1*M* glycine-HCl (pH 2.5) and dialyzed against pH 7.2 phosphate-buffered saline (PBS). F(ab′)$_2$ fragments of affinity-purified Ab1 were collected by G-150 Sephadex gel-fil-

tration chromatography (Pharmacia, Piscataway, NJ) following pepsin digestion (*16*).

Tetanus toxoid (Connaught Laboratories, Swiftwater, PA), bovine serum albumin (BSA), or hen egg ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) were bound to CNBr-activated Sepharose 4B according to the manufacturer's recommendations (Pharmacia).

Immunization to Elicit Ab2 and Autologous Ab3

Ab1-F(ab')$_2$ fragments were coupled to keyhole limpet hemocyanin (KLH) by copolymerization with glutaraldehyde (*17*). Recipient rabbits (85-1 and 85-2) were matched for Ig allotype with the donor of the Ab1-F(ab')$_2$ and injected with 8.5 mg of glutaraldehyde-polymerized Ab1-F(ab')$_2$-KLH conjugate in complete Freund's adjuvant (CFA) subcutaneously and were boosted 3 wk later with 6.93 mg of Ab1-F(ab')$_2$-KLH in incomplete adjuvant. Weekly blood samples were collected.

Idiotype and Antigen-Binding ELISAS

Wells of flat-bottom polyvinyl chloride assay plates (Falcon #3912) were coated overnight at 4°C with antigen or F(ab')$_2$ fragments (50 μL/well at 5–10 μg/ml) in borate saline buffer (BSB), pH 8.0. The plates were then treated with BSB containing 1% BSA (BSB-BSA) for 2 h at T_r to inhibit nonspecific binding. Following three washes with PBS containing 0.05% Tween 20 (PBS-Tween), 50 μL of sample or control sera diluted optimally (based on preliminary titrations) in PBS-Tween containing 0.25% BSA were added to each well. The plates were covered and incubated for 1 h at T_r. The plates were washed three times with PBS-Tween, and 50 μL of a 1:1000 dilution of horseradish peroxidase-conjugated goat antirabbit IgG (Fc-specific) antibody (Organon Tecknika, Durham, NC) in PBS-Tween were added to each well. After 1 h at T_r, the plates were washed with PBS-Tween, and 50 μL of a 400 μg/mL *o*-phenylenediamine (Sigma Chemical Co.) substrate solution were added. Plates were developed in the dark at T_r for 10 min, and the reaction was stopped by adding 50 μL of 2*N* H_2SO_4/well. The OD at 492 nm was read on a Titerec Multiscan using water as a blank.

Inhibition and Absorption Studies

Serum samples were absorbed on TT or OVA affinity columns. In inhibition experiments, antiserum was mixed with free TT toxoid in concentrations ranging from 0–24 μg/mL and incubated for 1 h at 37°C. After incubation, the inhibited sample was assayed using a TT or control antigen-binding ELISA. Binding activity of inhibited or absorbed samples was then compared with uninhibited or unabsorbed samples containing the same concentration of antibodies to determine percent inhibition.

Isoelectric Focusing–Affinity Immunoblotting of Ab1′

Isoelectric focusing was done as described previously (*18*) with minor modifications. Test sera were applied in 18-μL aliquots to acrylamide gels (6.6%T and 3%C) containing 1% Tween 20 and 2% carrier ampholytes synthesized as described earlier (*19*). Electrophoretic separation was done for 2 h at a final constant power of 30 W. Antibodies were then transferred by diffusion to antigen-coated nitrocellulose for analysis of Ab1′ clonotype distribution by affinity immunoblotting (*20*). Nitrocellulose sheets (0.45 μm, Schleicher & Schuell, Keene, NH) were hydrated in 0.5*M* sodium bicarbonate containing 10 μg/mL tetanus toxoid and incubated overnight at T_r on a rocking platform. The antigen-coated sheets were then treated by washing with PBS containing 10% Tween 20 to block further protein uptake. The TT-coated sheet was laid on the gel for 15 min following isoelectric focusing before gel fixation and staining. The sheet was rinsed by rocking in PBS-Tween for 1 h, immersed for 1 h in affinity-purified goat antirabbit $F(ab')_2$ antibody diluted 1:10,000 in PBS-Tween, washed in PBS-Tween, and then immersed for 1 h in a 1:5000 dilution of rabbit anti-goat IgG serum that had been absorbed over a TT column. After thorough washing, antibody bands were developed with horseradish peroxidase-conjugated goat antirabbit IgG (Fc-specific) antibody (Organon Tecknika) diluted 1:10,000 in PBS-Tween. Following a 1-h rinse in PBS-Tween and several PBS rinses, the sheet was immersed in a solution of 600 μg/mL 3,3-diaminobenzidine (Sigma Chemical Co.) and 0.03% H_2O_2 for 5 min to visualize antibody bands. The sheets were then rinsed thoroughly with water and air-dried.

Passenger Epitope Assay

Three groups of BALB/c mice (3/group) were primed with antigen or antigen mixtures to attempt to detect the presence of those antigens in the Ab1-$F(ab')_2$ fragments. These groups were:

1. Primed with 200 μg of glutaraldehyde polymerized TT;
2. Primed with normal rabbit $F(ab')_2$ fragments; and
3. Primed with a mixture of the two preparations.

Once the antibody responses returned to preinoculation levels (74 d), mice were challenged with glutaraldehyde-polymerized TT, normal rabbit $F(ab')_2$ fragments, or Ab1-$F(ab')_2$. The presence of anti-TT and anti-$F(ab')_2$ antibody was assayed by ELISA using a 1:5000 dilution of horseradish peroxidase-conjugated antimouse immunoglobulin (Organon Tecknika).

RESULTS

Immunization of a group of six recipient rabbits with allotype-matched $F(ab')_2$ fragments of affinity-purified rabbit antitetanus toxoid Ab1 in the

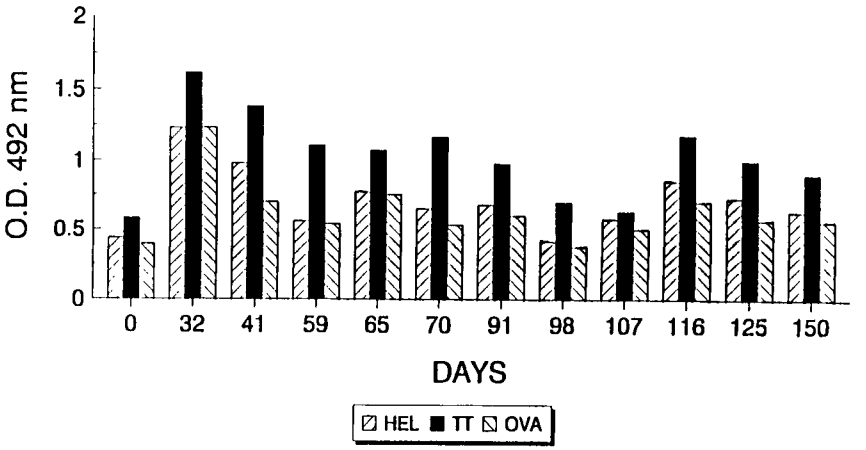

Fig. 1. The antigen-binding activities of rabbit 85-1 sera collected from 0–150 d postimmunization are shown. Data are presented as the OD 492 nm obtained from the binding of a 1:625 dilution of serum. Injections were on days 0 and 21. HEL (hen egg lysozyme) and OVA (hen ovalbumin) were used as control antigens (from Seferian, P. G. and Rodkey, L. S. [1992] *Vaccine Res.* **1,** 373–382, with permission).

soluble form (two rabbits), glutaraldehyde-polymerized $F(ab')_2$ fragments (two rabbits), or $F(ab')_2$ fragments conjugated to KLH (two rabbits) induced detectable antitetanus toxoid antibody responses in both rabbits immunized with the KLH-$F(ab')_2$ conjugates. Sera were analyzed by ELISA for specific binding of the nominal antigen (TT) and two unrelated control antigens (hen egg lysozyme and hen ovalbumin). Binding of the carrier KLH was also assayed as a positive control. As expected, the response to KLH was very strong (data not shown). Following immunization with Ab1-KLH conjugate, the TT antibody titer was substantially greater than background binding of the control antigens that were tested, suggesting that injection of Ab1 had preferentially induced TT-specific Ab1'. ELISA binding data for one rabbit, 85-1, is shown in Fig. 1.

The ability of TT to inhibit the reaction of Ab1' with coated plates and absorb Ab1' was evaluated in order to verify the specificity of the putative Ab1'. Serum samples, excluding the preinoculation sample, were pooled and preincubated with soluble TT before application to a TT ELISA assay. Preincubation with soluble TT inhibited the Ab1' activity of pooled serum by 59%. In contrast, preincubation with OVA had little effect (4% inhibition) on Ab1' activity. A one-step absorption on a small Sepharose-TT column removed 63% of the Ab1' activity.

Immunization of the recipient rabbit with Ab1-$F(ab')_2$ fragments also induced the production of Ab2 as would be expected if Ab1' activity were induced through the idiotype network. Sera contained anti-idiotypic antibodies that preferentially bound to the Ab1-$F(ab')_2$ fragments used for injection (Fig. 2). The sera did not contain antibodies that bound significantly to allotype-matched $F(ab')_2$ fragments on non-TT antibodies. The

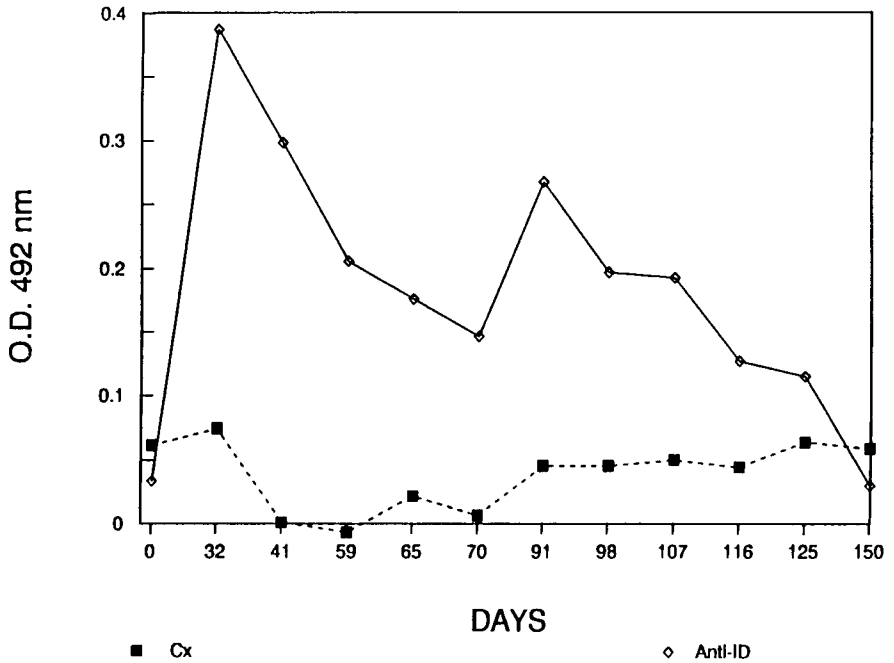

Fig. 2. Anti-idiotype antibody of sera from rabbit 85-1 using a 1:125 dilution of sera drawn on days 0–150 postimmunization. The solid line depicts the binding of Ab1-F(ab')$_2$ fragments (Anti-ID). The broken line depicts the binding of normal allotype-matched control rabbit F(ab')$_2$ (Cx). Injections were on days 0 and 21 (from Seferian, P. G. and Rodkey, L. S. [1992] *Vaccine Res.* **1**, 373–382, with permission).

weak binding of unrelated F(ab')$_2$ fragments can likely be attributed to activity directed against epitopes exposed by pepsin digestion, such as homoreactant (*21*) or parareactant (*22*).

The role of idiotypic interactions in the generation of anti-TT antibody was evaluated by assaying both Ab2 and Ab1′ in each serum sample. The results of these assays are expressed as titer relative to preinoculation titer in order to present the results of these two assays on the same scale (Fig. 3). The relative titer of Ab2 (solid line) was substantially greater than the titer of autologously induced Ab1′ (broken line). The Ab2 and Ab1′ cycled-reciprocally.

Although affinity-purified antitetanus Ab1 antibodies underwent extensive dialysis, pepsin digestion, gel-filtration chromatography, and further exhaustive dialysis after purification, the remote possibility existed that some TT or hydrolytic fragments bearing sequential epitopes might have leached from the affinity column matrix during low-pH elution and were carried along either free or bound in the paratope of the Ab1-F(ab')$_2$ molecules through the purification and pepsin-digestion procedures. To check for the presence of contaminating antigen fragments in the immunizing Ab1-KLH preparation, a sensitive in vivo biological assay was

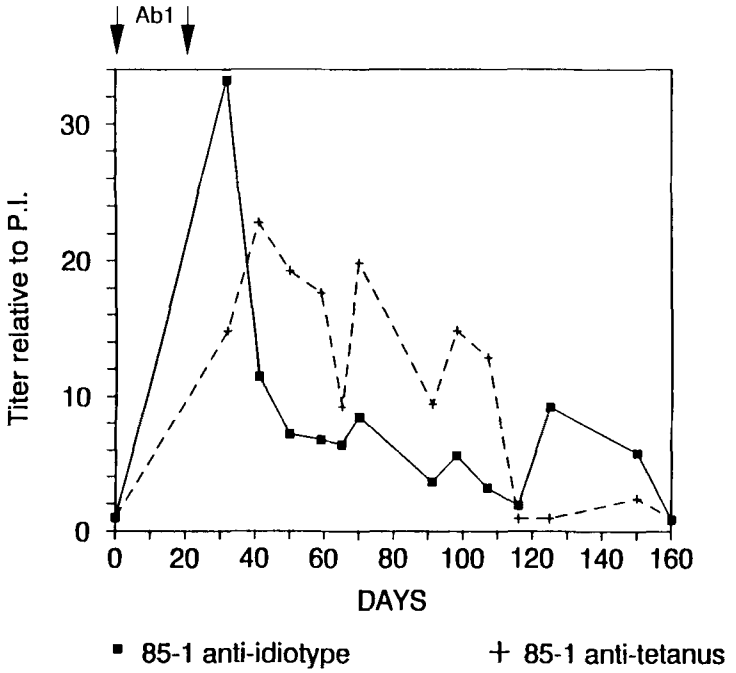

Fig. 3. The reciprocal expression of anti-idiotype (Ab2) and antitetanus (Ab1') antibody in rabbit 85-1. The solid line depicts anti-idiotype antibody levels, and the broken line depicts antitetanus antibody levels. Ab1 injections are indicated by arrows at the top (from Seferian, P. G. and Rodkey, L. S. [1992] *Vaccine Res.* **1**, 373–382, with permission).

done. Three groups of BALB/c mice were given priming injections. One group was primed with TT, a second group was primed with normal rabbit $F(ab')_2$ fragments, and a third group was primed with a mixture of the two. The priming using a mixture of rabbit $F(ab')_2$ fragments and TT was designed to elicit and expand a pool of $F(ab')_2$-specific T-helper cells as well as TT-specific B-cells. Following primary immunization of these three groups of mice, the antibody titers of anti-TT and anti-$F(ab')_2$ were monitored by ELISA. The mice were then left untreated until their titers dropped to preinoculation levels. At this time the mice were reimmunized with the Ab1-$F(ab')_2$ fragments, TT, or normal $F(ab')_2$ fragments. The presence of even trace amounts of contaminating TT molecules or fragments in the Ab1-$F(ab')_2$ preparation would be expected to stimulate an anamnestic response to TT in mice initially primed with either TT or the combination of TT and unrelated rabbit $F(ab')_2$ fragments.

The ELISA results for the binding activity of these mouse sera to TT are presented in Fig. 4A, and the control data for binding to normal rabbit $F(ab')_2$ fragments in Fig. 4B. Priming of mice with TT alone followed by booster injections of normal rabbit $F(ab')_2$ fragments elicited negligible antibody responses to $F(ab')_2$ fragments or TT (open boxes). Priming of

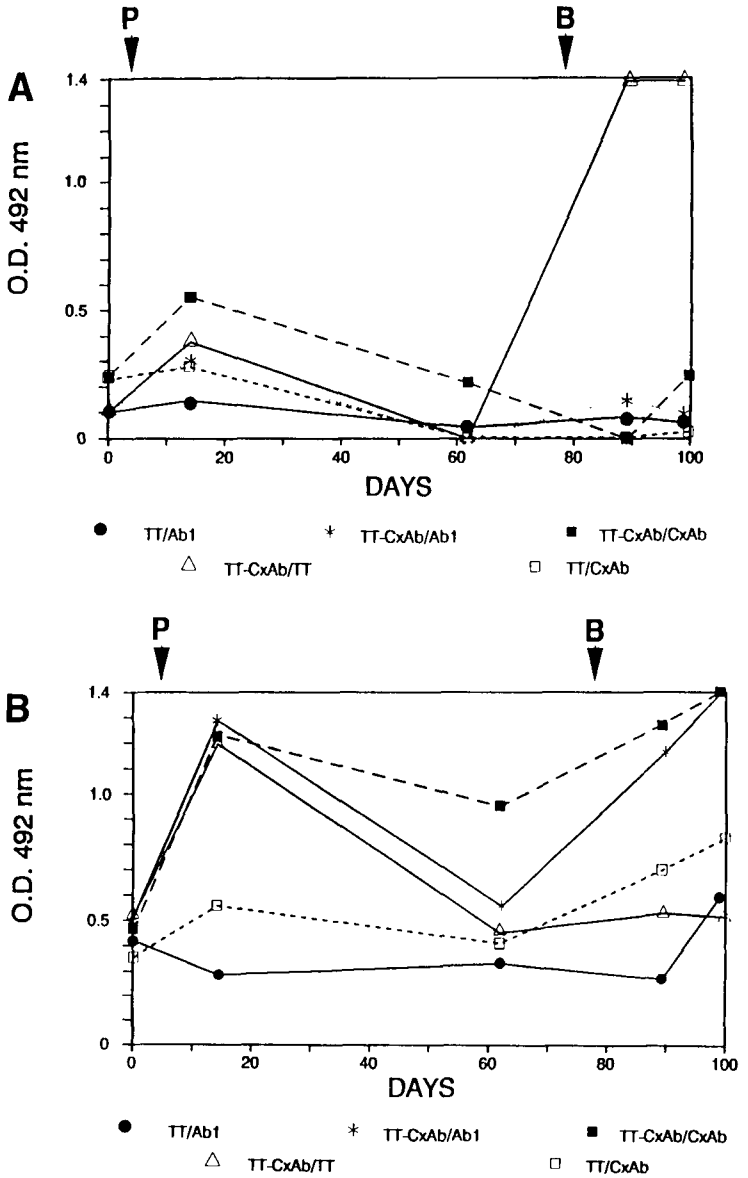

Fig. 4. (**A**) The antitetanus response of mice immunized to determine if the Ab1-F(ab')$_2$ fragment preparation was contaminated with antigen fragments. BALB/c mice were primed (P) with 200 μg of glutaraldehyde polymerized TT (TT), 200 μg of normal unrelated rabbit F(ab')$_2$ fragments (CxAb), or a combination of TT and normal F(ab')$_2$ fragments (TT-CxAb) (100 μg of each). The mice were then boosted (B) with 100 μg of glutaraldehyde polymerized TT alone (TT), 100 μg of normal unrelated rabbit F(ab')$_2$ fragments (CxAb), or 100 μg of Ab1-F(ab')$_2$ fragments (Ab1). Closed circles represent TT primed, Ab1-F(ab')$_2$ fragment boosted (TT/Ab1); open triangles represent TT + F(ab')$_2$ fragment

mice with TT followed by boosting with Ab1-F(ab')$_2$ fragments also elicited negligible levels of antibody-binding F(ab')$_2$ fragments or TT (closed circles). Both treatments gave results consistent with eliciting only a primary response to TT and to F(ab')$_2$ fragments in these mice, and suggested that no TT fragments could be detected in the Ab1-F(ab')$_2$ by this biological assay. On the other hand, priming of mice with a mixture of normal F(ab')$_2$ fragments and TT and challenging with TT gave the expected boost in TT antibody titer consistent with a secondary response (open triangles). This shows that the mixture of normal F(ab')$_2$ fragments and TT had effectively primed the mice against TT. Mice primed with the mixture of normal F(ab')$_2$ fragments and TT and later challenged with normal F(ab')$_2$ fragments also showed the expected secondary response to the F(ab')$_2$ fragments, but only a primary response to TT (closed boxes). The last group of mice were primed with a mixture of normal F(ab')$_2$ fragments and TT, and were later challenged with the Ab1-F(ab')$_2$ preparation (asterisks). This challenge induced a strong secondary response specific for the F(ab')$_2$ fragments as expected, but showed only a primary response to TT. The slight rise in TT titer is similar in intensity to the effect of immunization on the antibody titer to control antigens (data not shown). The presence of a secondary response to F(ab')$_2$ and only a primary response to TT in mice initially primed with both rabbit F(ab')$_2$ fragments and TT show that no biologically detectable TT fragments contaminated the Ab1-F(ab')$_2$ fragment preparation that had elicited the Ab1' response.

Clonal analysis of the Ab1' IgG antibody was done using isoelectric focusing and affinity immunoblotting. Affinity immunoblots of sera from rabbit 85-1 and from rabbit 85-2, respectively, are shown in Fig. 5A and B. These results revealed that increases in Ab1' titer were the result of increased synthesis by only limited subsets of B-cell clones synthesizing anti-TT. This is in contrast to antigen-driven increases in Ab1 titer that characteristically result from increased synthesis by numerous B-cell clones (+AB1 lane in 5A and AB1 lane in 5B). A low, but detectable level of anti-TT antibody in preinoculation serum (lane 2) was found in 85-1, but not in 85-2. The expression of some clonotypes was upregulated and downregulated at different times (*see* arrows in Fig. 5A).

Fig. 4. (*cont'd*). primed, TT boosted (TT-CxAb/TT); asterisks represent TT + unrelated rabbit F(ab')$_2$ fragment primed, Ab1-F(ab')$_2$ fragment boosted (TT-CxAb/Ab1); open boxes represent TT primed, unrelated rabbit F(ab')$_2$ fragment boosted (TT/CxAb); closed boxes represent TT + unrelated rabbit F(ab')$_2$ fragment primed, unrelated F(ab')$_2$ fragment boosted (TT-CxAb/CxAb). (**B**) The anti-F(ab')$_2$ response of mice immunized to determine if the Ab1-F(ab')$_2$ fragment preparation was contaminated with antigen fragments. Immunization protocol and symbols are as described in the legend of A (from Seferian, P. G. and Rodkey, L. S. [1992] *Vaccine Res.* **1**, 373–382, with permission).

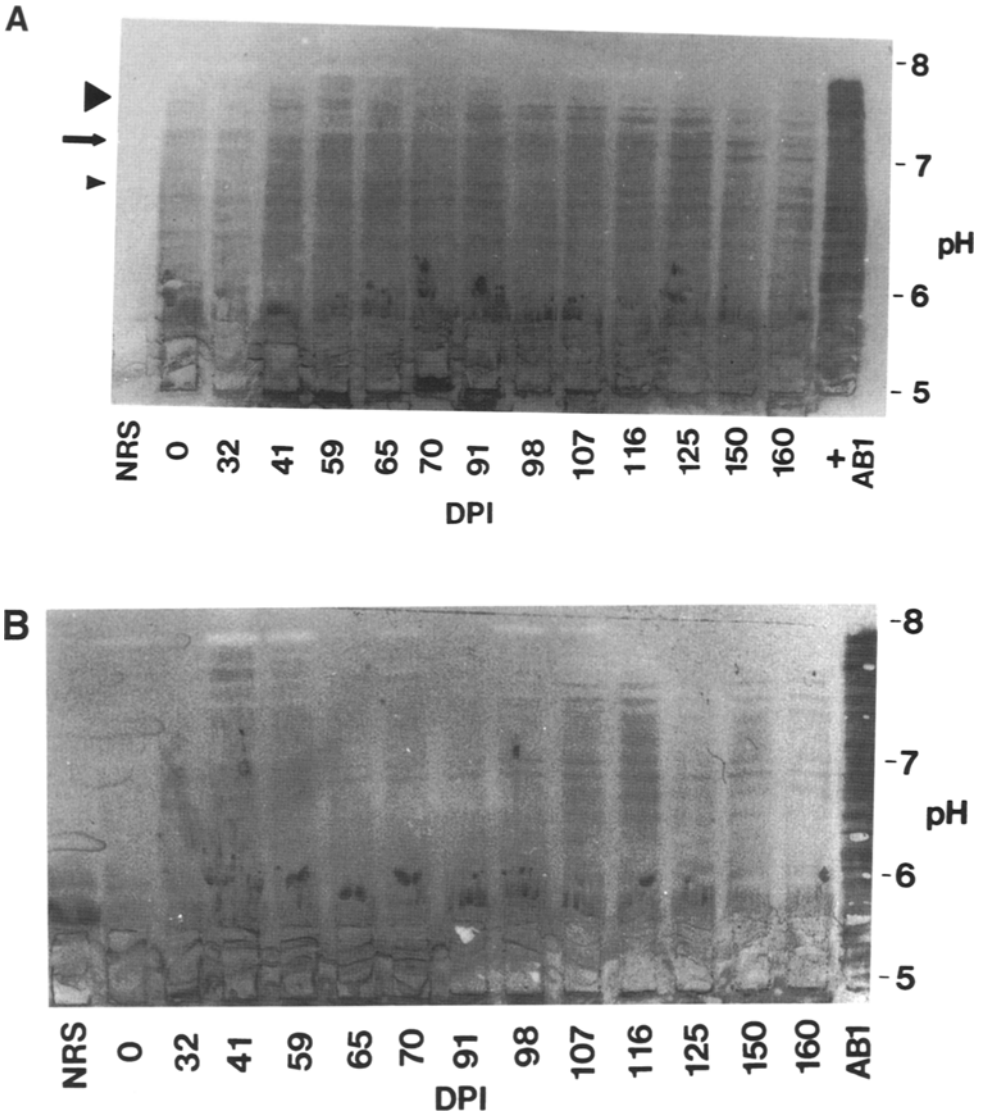

Fig. 5. (**A**) Affinity immunoblot of rabbit 85-1 antitetanus Ab1′ antibodies in sera obtained from 0–160 d postimmunization. Arrowheads indicate antibody clonotypes that fluctuate independently in their level of expression. NRS is normal rabbit serum included as a negative control. + AB1 represents a known antitetanus Ab1 serum as a positive control. (**B**) Affinity immunoblot of rabbit 85-2 antitetanus Ab1′ antibodies in sera obtained from 1–160 d postimmunization. NRS is normal rabbit serum included as a negative control. AB1 is a known antitetanus Ab1 serum included as a positive control (from Seferian, P. G. and Rodkey, L. S. [1992] *Vaccine Res.* **1,** 373–382, with permission).

DISCUSSION

This article documents an example of antibody production elicited in the absence of detectable antigen. A multivalent preparation of Ab1 was used to induce antibodies of the same specificity actively. The injection of $F(ab')_2$ fragments of affinity-purified antitetanus toxoid IgG antibodies coupled to a carrier protein induced the production of antitetanus toxoid Ab1' in the absence of nominal antigen stimulation.

The use of a polyclonal Ab1 preparation to induce the production of Ab1' was reported previously by Forni et al. (23). Injection of IgM anti-SRBC or antidextran induced plaque-forming cells (PFC) of the same specificity as the antibody used for immunization. The IgM antibodies induced responses to both T-dependent and T-independent antigens. IgG antibody was found in their studies to be ineffective. Our studies utilized $F(ab')_2$ fragments of polyclonal IgG antibody molecules copolymerized on KLH. Polymerization to KLH creates a functionally multivalent complex consisting of both idiotopes and paratopes. These data, coupled with Forni et al.'s (23) reported success using IgM and failure using IgG to induce Ab1' synthesis, suggest that multivalency could be the critical property required for effective induction of Ab1' using Ab1.

The hypothesis that idiotype network interactions are involved in the induction of Ab1' by the injection of Ab1 is supported by the reciprocal cycling of Ab2 and Ab1', which was documented here. This pattern suggests that one antibody is driving the synthesis of another and is characteristic for naturally occurring auto-anti-idiotypic responses. Reciprocal cycling of idiotypic anti-*Streptococcus pneumoniae* PFC and naturally occurring anto-anti-T15 PFC following antigen stimulation was described by Kelsoe and Cerny (24). Thomas et al. (25) showed cyclic expression of antibodies with different idiotypes in a diabetic individual receiving insulin therapy, and Brown and Rodkey (26) and Rodkey and Adler (27) reported natural cycling of idiotype and anti-idiotype during immune responses.

A sensitive in vivo assay was used to study the possibility that Ab1' activity was inadvertently induced by the presence of whole antigen or antigen fragments in the Ab1 preparation that leached from the affinity column used to isolate the Ab1. The results of this assay showed that biologically detectable antigen did not contaminate the Ab1-$F(ab')_2$ fragment preparation. Forni et al. (23) addressed the question of contaminating antigen by showing that treatment with dextranase abolished the immunogenicity of dextran, but had no effect on the ability of anti-dextran IgM to induce PFC.

It is unlikely that the anti-TT-binding activity, which was detected in the recipient's serum in this study, was the result of the presence of free Ab1-$F(ab')_2$ fragments released from the inoculum. First, the Ab1-$F(ab')_2$ fragments used for immunization were covalently coupled to KLH.

Second, the anti-TT response was shown to wax and wane in a cycling pattern. Ab1-F(ab')$_2$ fragments freed from the conjugate would not be expected to cycle. Third, the rate of titer increase of anti-TT antibody is characteristic of active synthesis. Last, the most convincing argument against antigen release is that the enzyme conjugate used in the ELISA assay to detect anti-TT binding activity was specific for the Fc portion of IgG, and the immunizing preparation contained only F(ab')$_2$ fragments of Ab1.

Clonal analysis of the Ab1' response showed that the heterogeneity of IgG Ab1' clonotypes were more restricted than that of Ab1 elicited using whole antigen. Fluctuations in the expression of certain individual Ab1' clonotypes suggest that these clones are being independently regulated. This type of clone-specific regulation is similar to previous reports of idiotype regulation (*26*). The observation of the enhanced expression of some Ab1' clones at a time when the overall titer is declining is reminiscent of findings in the human anti-insulin response where the expression of a particular idiotype had no detectable effect on the total antibody response (*25*). The cofocusing of Ab1' and Ab1 antibodies from an allotype-matched rabbit suggests that both sets of antibodies may use the same or very similar V-gene conversion strategies to generate these molecules. It is also possible that Ab1 and Ab1' are merely different designations for antibodies produced by the same B-cells. It is difficult, perhaps impossible, to differentiate Ab1 and Ab1' serologically, because they have the same binding patterns. Data showing that Ab1 and Ab1' bind the same epitope (*28*) support the idea that V-gene conversions are similar or identical for both Ab1 and Ab1'.

Members of the idiotypic cascade (Ab1, Ab2, and Ab3) were originally described following serial immunization of individual rabbits in experiments by Cazanave (*4*) and Urbain et al. (*3*). Ab3 generated in this fashion did not show Ab1' activity without additional stimulation with antigen. In the present study, the induction of antitetanus toxoid Ab1' did not require further antigen stimulation. The existence and activity of idiotype cascades were also shown following the injection of monoclonal antibodies (*29,30*) and while generating hybridomas (*28,31*). Naturally occurring members of an idiotypic cascade were recently identified in a study of maternal–neonatal network interactions (*32*). The importance of naturally occurring idiotype cascades is emphasized by recent data suggesting involvement in the pathogenesis of AIDS (*33,34*).

This article documents the natural induction of antitetanus toxoid antibody following immunization of a recipient rabbit with purified polyclonal antitetanus toxoid antibody and the clonotypic characterization of the induced Ab1'. These findings support network theory and provide evidence of the potential for the development of Ab1 vaccine preparations. The isolation of extremely pure polyclonal or monoclonal Ab1 is relatively simple when compared to purification of internal image-bearing

Ab2β, the inactivation of infectious agents, isolation of pathogen subunits, or synthesis and purification of genetically engineered antigens or functional antigen fragments. The restricted heterogeneity of the Ab1' response observed may be an asset, if for example, the missing clonotypes exhibit autoreactivity or are nonneutralizing. Additionally, idiotypic pathways have been shown to activate B-cell clones that are refractory to stimulation with antigen (*35,36*).

It is likely that the property of idiotypic connectivity as shown in this and other articles could be utilized for efficient production of abzyme molecules. Injection of an enzyme should elicit production of subsets of antibodies with substrate-like structural properties. The ensuing Ab2 response may contain subsets of antibodies with enzyme activities in their paratope pockets. This approach is likely to be a more efficient approach to abzyme production than simple injection of substrate. This idiotypic connectivity, if properly utilized, could provide sources of reagents with enzymatic activities that might be useful in some therapeutic situations. Experiments to test this hypothesis are in progress.

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DISCUSSION

L. S. Rodkey

Gabibov: The idiotype-anti-idiotype system seems to be operative in animals and humans, in autoimmune disease and in HIV infections. Normal individuals who have been immunized with recombinant gp120 will also produce anti-idiotypic antibodies that also bind to the receptor for the virus.

Hansen: At what point after immunization are the concentrations of the anti-idiotype internal image antibodies maximized and the concentration of the primary antibodies minimized?

Rodkey: That is totally unpredictable. We have been looking at various type of responses over 10 years, and it seems to be highly individual-specific. You have to do the assays in every case to find the peak concentrations. Typically, the Ab1 disappears very rapidly on Ab2 production because Ab1–Ab2 immune complexes are produced. These idiotype–antiidiotype complexes can be shown very nicely even in normal immunized animals at small concentrations. Once you trigger the system, there is massive clearance of the complexes. Then, of course, once the Ab1 is cleared out, you can start detecting the presence of Ab2. The Ab2 really arrived earlier than its detection, but it was tied up in the complexes. The timing is related to old axioms about antigen dose, interval of immunization and kind of antigen, whether it is a "good antigen" like KLH or a "poor antigen," like carbohydrates. Those variables really eliminate any predictability in the system, unfortunately.

Paul: Is there a regulatory significance to the network?

Rodkey: Absolutely. It is very clear that the cycling has been seen in humans and mice. It has been seen in tissue culture. It has been seen in the intact animal. It has been seen in animals that were not even immunized by the investigator, but once the anti-Id response is functional, the original idiotype disappears. This is not a clonal deletion type of disappearance. It is a clonal freezing, and later those clones can reappear. You are not killing the cells by the anti-Id response in the auto-anti-idiotype system. You are turning the system off temporarily.

Paul: If indeed that is the mechanism, would you not predict that the anti-Id, Ab2, would predominate over Ab1? Presumably, the concentration of Ab2 is greater than Ab1 in order to exercise the regulatory function. Where does the network end? Ab3, Ab4?

Rodkey: That is an earth-shaking question and a common question about the idiotypes. For all practical purposes, the idiotype works one step after the other, Ab1, Ab2, Ab3, and so forth. There is clearly some other mechanism for central kinds of interactions. Cyclic regulation of the Ab1 by Ab2 followed by Ab1 followed by another Ab2 seems to occur.

Paul: Either the concentrations or affinities of the secondary antibodies would need to be greater relative to the primary antibody.

Rodkey: Not necessarily. You should also consider anti-idiotypic T-cells. You may need far less of the secondary antibody to get regulation of the true primary antibody.