

AUTOREGULATION OF AN ANTIBODY RESPONSE VIA NETWORK-INDUCED AUTO-ANTI-IDIOTYPE*

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Numerous reports document the frequent occurrence of rheumatoid antibodies (RA)¹ during hyperimmunization with many antigens. Bacterial carbohydrate antigens are frequently used to elicit antibody responses in which RA are detected. Antibody responses to certain streptococcal vaccines (1–5) have been shown to contain substantial amounts of RA reactive with the Fc region of autologous IgG. Recently, RA antibodies were detected in the sera of rabbits hyperimmunized with vaccine prepared from *Micrococcus lysodeikticus* (6).

Idiotypic determinants of immunoglobulins, first described by Oudin and Michel (7) and Kunkel et al. (8) are located in the Fab portion of the IgG molecule. Idiotypic determinants elicit specific anti-idiotypic antibodies in heterologous species (9, 10), in isologous species (11, 12), and even within the same individual that synthesized the idio type (13). Niels Jerne (14) first conceptualized the “lymphocyte network” or “idiotypic network”, in which antibodies (idiotypes) may be regulated in their quantitative expression by the production of anti-idiotypic antibodies within the same individual.

There are currently two examples of apparent natural regulation of antibody synthesis by autologous anti-idiotypic responses, both in inbred animal systems. In the first (15), Kluskens and Kohler immunized BALB/c mice with pneumococcal R36A vaccine to induce antibody specific for phosphorylcholine (PC) bearing the TEPC-15 idio type and showed suppression of a plaque-forming cell (PFC) response to the vaccine with antiserum that could be removed by absorption with TEPC-15 molecules. In the same system, Cosenza (16) detected anti-idiotypic plaques using sheep erythrocytes (SRBC) coated with TEPC-15. These plaques were detectable only after the anti-PC response had peaked and began to decline. In the second system, McKearn et al. (17) reported the production of autologous anti-idiotypic antibodies in inbred rats after repeated immunizations with alloantigens from a different inbred strain. The autologous anti-idiotypic antibody was specific only for antibody directed toward the alloantigen difference in the two inbred strains of rats.

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; DMF, dimethylformamide; HA, hemagglutination; PBS, phosphate-buffered saline; PC, phosphorylcholine; RA, rheumatoid antibodies; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

The present investigation documents studies of an individual outbred rabbit in which the initial antibody response to *M. lysodeikticus* was later modified by the natural production of autologous anti-idiotypic. The reaction was detected because of the simultaneous occurrence of RA.

Materials and Methods

Immunogens. Whole *M. lysodeikticus* was obtained from Worthington Biochemical Corp., Freehold, N. J.

Immunization Protocol. Random-bred New Zealand White rabbits were immunized with vaccine essentially according to Osterland et al. (18) and Strosberg et al. (19).

Electrophoretic Procedures

ZONE ELECTROPHORESIS. Serum samples were electrophoresed on Gelman cellulose acetate membranes in Gelman high resolution buffer, pH 8.8 (Gelman Instrument Co., Philadelphia, Pa.)

POLYACRYLAMIDE GELS. Alkaline-urea polyacrylamide gel electrophoresis was performed according to Reisfeld and Small (20). Samples were completely reduced and carboxymethylated according to Chapuis and Koshland (21).

Autoradiographic Isoelectric Focusing. Isoelectric focusing of antibodies in polyacrylamide gels followed by autoradiographic band visualization was done following the method of Braun et al. (22), in the LKB Multiphor apparatus (LKB Instruments, Inc., Rockville, Md.).

Ultracentrifuge Studies. Sedimentation analyses of preinoculation and immune serum was performed on a Beckman Spinco model E analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) equipped with schlieren optics.

Micrococcal Cell Wall Vaccine. Lyophilized whole micrococcus was suspended in normal saline and washed by centrifugation. Cell walls were made by treatment of whole cells in a Braun homogenizer (B. Braun Instruments, San Francisco, Calif.) followed by fractional centrifugation. Cell walls were resuspended in saline, washed in distilled water, and then lyophilized.

Cell Wall Solubilization. Cell walls were digested with ribonuclease A (20 $\mu\text{g}/\text{ml}$) for 1 h at 37°C. The cells were washed, suspended in pH 2.0 saline, and digested with 500 $\mu\text{g}/\text{ml}$ pepsin for 2 h at 37°C. After washing, 2.5-g cells were suspended in 100 ml of a 0.05 M ammonium acetate buffer, pH 6.8, containing 3 mg of lysozyme. The suspension was incubated for 24 h at 37°C and insoluble material was sedimented by centrifugation. The supernate was removed, dialyzed against distilled water, and lyophilized.

Immunoabsorbents

MICROCOCCAL CELL WALL CARBOHYDRATE. Before attachment of soluble cell wall antigen, a hexamethylenediamine spacer arm was attached to Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). Approximately 20 g of washed Sepharose 4B was suspended in distilled water and the pH was adjusted to 11.5. 3 g of CNBr, dissolved in dimethylformamide DMF, was added for 10 min while maintaining the pH between 11 and 12. The CNBr-activated suspension was washed with ice-cold 0.1 M NaHCO_3 , pH 10.0. After washing, the suspension was transferred to 100 ml of ice-cold 0.1 M NaHCO_3 buffer, pH 10.0, containing 3 g of hexamethylenediamine, and swirled for 24 h at 4°C.

Approximately 250 mg of cell wall material was dissolved in 100 ml of distilled water and the pH was adjusted to 11.5. 2 g of CNBr dissolved in DMF was added dropwise to the solution. After the pH had stabilized, HCl was added to adjust the pH to 8.5, and the solution was mixed with 20 g hexamethylenediamine-modified Sepharose 4B resin suspended in 60 ml 0.5 M NaHCO_3 buffer, pH 8.5. After reaction at 4°C for 24 h, the resulting conjugate was washed with distilled water and PBS.

INSOLUBILIZED RABBIT IGG. Two insolubilized rabbit IgG preparations were used. The first consisted of rabbit IgG, purified by salt precipitation and ion-exchange chromatography from pooled normal rabbit serum coupled to hexamethylenediamine-modified Sepharose 4B resin in the presence of glutaraldehyde. The second was prepared from rabbit IgG, purified as above, and insolubilized according to the method of Avrameas and Ternynck (23).

STAPHYLOCOCCAL IMMUNOABSORBENT. The Cowan I strain of *Staphylococcus aureus* was used to prepare an IgG absorbent according to Kessler (24).

Purification of Anti-Micrococcal Antibody. Isolation of specific antibody was essentially according to Wikler (25). Micrococcal antiserum was added to the Sepharose 4B micrococcus-CHO immunoabsorbent and unbound protein was rinsed through with 0.02 M phosphate buffer, pH 7.5. Bound Ig was eluted stepwise in three peaks as follows: peak B eluted in the presence of PBS made 5% (wt/vol) in glucose; peak C was obtained by elution in the presence of 0.02 M phosphate, 0.5 M NaCl, 5% (wt/vol) glucose buffer, pH 7.5; and peak D by elution in the presence of 0.2 M acetic acid.

Preparation of Immunoglobulin Fragments. Both normal IgG and anti-micrococcus CHO F(ab')₂ fragments were isolated from peptic digests (26).

Iodination of F(ab')₂. F(ab')₂ fragments were iodinated by either the ICl technique of McFarlane (27), or by a modification (28) of the chloramine-T method.

Dilution-Induced Precipitation Assay. Serum samples were routinely examined for direct precipitation by diluting 50 or 100 μ l antiserum to 0.5 or 1.0 ml, respectively, in phosphate-buffered saline (PBS) and incubating the solution at room temperature for 1-3 h. After reaction, samples were centrifuged and the pellet was washed three times with PBS. The amount of precipitate was determined by dissolving the precipitate in NaOH and measuring the absorbance of the resulting solution at 278 nm.

Inhibition of Dilution-Induced Precipitation. The effects of serum, saccharide compounds, fetuin glycopeptide, and other components on dilution-induced precipitation were examined using a standard 1:10 dilution of 102 serum. Whole normal, anti-micrococcal, or anti-streptococcal sera were added in increasing amounts to centrifuge tubes followed by addition of phosphate-buffered saline (PBS), where necessary, to 1.0 ml. 100 μ l of 102 serum was added to these solutions, and the precipitation reaction was allowed to proceed for 1 h at room temperature. After incubation, the solutions were centrifuged and the amount of precipitate was determined as described. When saccharide compounds were used as inhibitor, 0.28 M solutions were prepared in PBS. All inhibitors used, with the exception of specific antisera, were from commercial sources. Fetuin glycopeptide material was prepared according to Sela et al. (29) from a pronase digest of fetuin followed by ion-exchange chromatography and gel filtration.

Rheumatoid Antibody Assay. Detection of anti-IgG activity in 102 serum was performed using hemagglutination (HA) and coprecipitation assays. HA experiments were performed using IgG-sensitized rabbit erythrocytes. A 2% suspension of washed rabbit blood group F erythrocytes in PBS was incubated with an equal volume of diluted anti-rabbit blood group F antiserum. Sensitized erythrocytes were washed and adjusted to 1% (vol/vol).

Coprecipitation assays were performed using rabbit anti-bovine serum albumin (anti-BSA) and BSA at equivalent ratios. To 100 μ l of 102 serum, 100 μ l rabbit anti-BSA serum and 17 μ l BSA at 10 mg/ml were added. After incubation, the amount of protein in the precipitate was determined by absorbance measurements at 278 nm of NaOH-solubilized material. Control experiments included 102 serum in the presence of BSA and normal rabbit serum, in addition to normal rabbit serum in the presence of BSA and anti-BSA. The amount of anti-IgG in 102 serum was quantitated by summation of the absorbance values obtained for the two controls and subtraction of this value from the absorbance when 102 serum was incubated in the presence of the heterologous precipitating system.

Indirect Radioimmunoassay. The technique used to detect autogenous anti-idiotypic was a modification of a previously described method (13). Briefly, 102 serum was diluted in PBS and 1- to 5-ng aliquots of ¹²⁵I-F(ab')₂ were mixed and incubated with the diluted 102 preparation. Goat anti-rabbit Fc γ serum was added to precipitate complexes. Some experiments utilized antibody preparations eluted from a micrococcal-specific F(ab')₂-Sepharose 4B conjugate. Antibody eluting as peaks B and C from a micrococcal cell-wall material Sepharose 4B conjugate were pooled, and F(ab')₂ fragments were prepared. F(ab')₂ fragments were coupled to a hexamethylenediamine Sepharose 4B matrix in the presence of glutaraldehyde. Serum from rabbit 102 was added to the absorbent and washed through with PBS. Antibody was eluted with 0.2 M glycine-HCl buffer, pH 2.5. After dialysis, eluted material was concentrated. Aliquots of ¹²⁵I-F(ab')₂ prepared from 102 antibody that was eluting as peaks B or D, were added, mixed, and goat anti-rabbit Fc γ was added to precipitate complexes. Supernates and precipitates were assayed for radioactivity. Percentages of radioactivity precipitated were calculated after correcting for: (a) background radioactivity, (b) trichloroacetic acid (TCA)

precipitability of the iodinated antigen, and (c) precipitate trapping of radioactivity (normal serum control).

The effect of ligand on $^{125}\text{I-F(ab')}_2$ facilitated precipitation in 102 serum was assayed by incubating 1–5 ng $^{125}\text{I-F(ab')}_2$ isolated as peak B, C, or D, with a 5% (wt/vol) glucose, 0.3 M NaCl in PBS solution before the addition of 102 serum diluted 1:100 in 5% (wt/vol) glucose, 0.3 M NaCl in PBS. After precipitation, the percent inhibition of binding was calculated by comparing the counts precipitated under these conditions, with the counts precipitated in nonligand-treated samples. The effect of nonradiolabeled homologous and isologous (with regard to specificity) F(ab')_2 , isolated as peak B or D, on peak B or D $^{125}\text{I-F(ab')}_2$ facilitated precipitation in diluted 102 serum was performed by incubating increasing amounts of nonlabeled fragments with diluted 102 serum before addition of labeled F(ab')_2 . Inhibition was calculated relative to counts precipitated in uninhibited control solutions.

Results.

Dilution-Induced Precipitation. When serum isolated from rabbit 102 during the second round of hyperimmunization with *M. lysodeikticus* vaccine was diluted at room temperature in neutral buffer, a reaction characteristic of insoluble immune complex formation in precipitin assays. Depicted in Fig. 1 are the results obtained when serum from the second round of immunization was diluted 1:15 in the presence of PBS. Maximum precipitation occurred with serum collected $\cong 7$ wk after the second immunization was initiated, and declined in a cyclical fashion for serum isolated in later weeks. The amount of precipitate formed in any single serum sample was dependent upon the pH of the diluent (Fig. 2). Formation of precipitate occurred over a relatively narrow pH range, i.e., pH 6.5–9.0 with a pH range of 7–8 resulting in maximum precipitation. Also shown in Fig. 2 are the results of experiments which examined the relationship between precipitate formation and the extent of dilution. As depicted, maximum precipitation was observed between 1:20–1:60 diluted samples.

Because precipitate formation in diluted serum was characteristic of immune complex reactions, precipitates were examined for immunoglobulin. As shown in Fig. 3, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed proteins that migrated with rabbit γ - and light chains. Also shown in Fig. 3, is an analysis of Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) fractionated 102 serum. Material eluting as excluded fractions in the presence of 0.1 M sodium acetate buffer, pH 4.5, was pooled and concentrated to the original serum volume. This solution was neutralized with NaOH, and the precipitate was isolated by centrifugation. When this material was examined by SDS-PAGE, only γ - and light-chain size proteins were observed. In addition, dissolved precipitate reacted with goat anti-Fcy in immunodiffusion assays.

To examine the association of micrococcal-specific antibody in the precipitation phenomenon, the ability of radiolabeled *M. lysodeikticus* solubilized cell wall antigen to be incorporated in the precipitate was tested. For these experiments, the nondialyzable fraction of lysozyme-digested *M. lysodeikticus* cell walls was tyraminated (30) and the tyraminated product radiolabeled in the presence of Na^{125}I using the chloramine T method (28). Addition of 10 ng ^{125}I -tyramine-carbohydrate to 102 serum was followed by 1:10 dilution in PBS. 80% of the radioactivity was incorporated in the insoluble complex.

Immune Complexes and Rheumatoid Antibodies. The unique behavior of 102 serum upon dilution suggested the presence of pre-existing immune complexes. Although a shoulder of more rapidly sedimenting molecular species was obvious in immune serum

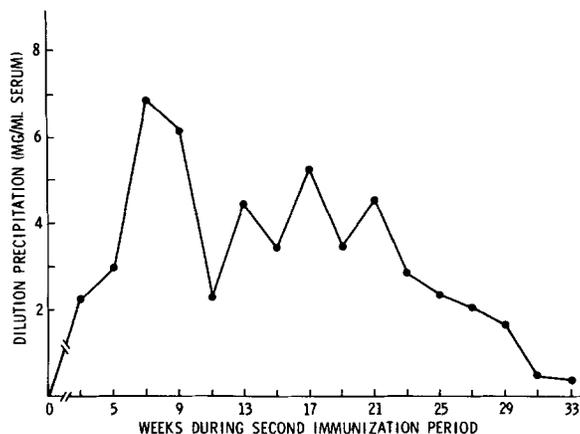


FIG. 1. The amount of precipitate formed (●) upon dilution of serum 102 isolated during the second immunization period. Values were obtained from absorbance measurements of NaOH-dissolved material.

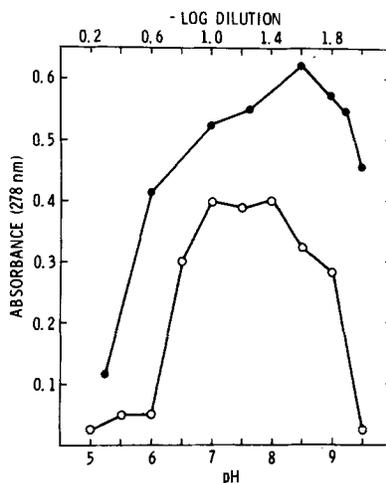


FIG. 2. The effect of pH (○), or extent of dilution (●), upon precipitate formation.

and absent in preinoculation serum, as evidence by schlieren patterns of ultracentrifuged samples, a high concentration of discrete higher molecular weight complexes was not apparent (Fig. 4). In addition, cryoglobulin formation was not observed in 102 serum. These data suggested that formation of complexes upon dilution was specific and not a result of nonspecific aggregation. Indeed, dilution of 102 serum with pooled normal rabbit serum resulted in precipitate formation equal to the amount obtained using buffer.

Preinoculation and first and second immunization period serum samples were tested for their ability to effect HA of rabbit erythrocytes coated with rabbit blood group substance-specific antibody. Whereas no hemagglutinating activity was detected in preinoculation serum, immune serum from both the first and second immunization periods revealed extensive agglutinating activity. The maximum titer

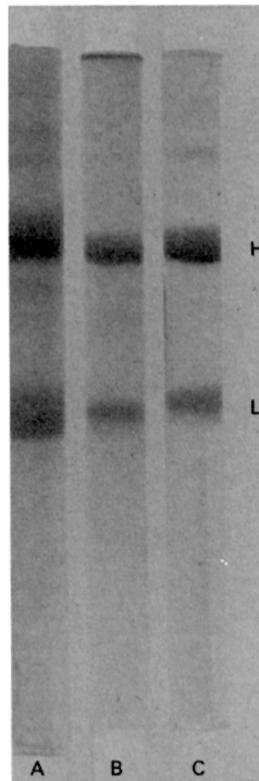


FIG. 3. SDS-PAGE completely reduced and carboxymethylated normal rabbit IgG (A); serum 102 dilution-induced precipitate, reduced and carboxymethylated before (B), or after (C), Sephadex G-100 chromatography of whole serum.

observed in first-round serum exceeded 1:320, whereas for second-round serum, titers as high as 1:160 were detected. Noncoated control erythrocytes were not agglutinated. Because the effect of dilution-induced precipitation on hemagglutination titers was unpredictable, the presence of RA in sera collected during the second immunization period was substantiated by additional methods. First, the ability of RA to interact with immune complexes in the process of formation was used as a specific assay for anti-IgG. Heterologous immune complexes were allowed to form in 102 serum by addition of equivalent amounts of BSA and rabbit anti-BSA. Under these conditions, ≈ 2.8 mg immunoglobulin/ml 102 serum was coprecipitated by the heterologous complex. In addition to these assays, anti-IgG antibody was detected by specific immunoabsorption. Approximately 3 mg IgG was eluted from a rabbit IgG-Sepharose 4B immunoabsorbent column after passage of 1.0 ml 102 serum. That RA was involved in dilution-induced precipitation was suggested by the inhibitory effect then observed, when this anti-IgG-depleted 102 serum was subsequently tested for precipitating activity.

Serum collected as the fall-through peak from the rabbit IgG-Sepharose 4B conjugate was concentrated to the serum volume applied to the column and diluted in PBS. No precipitation was observed at any dilution tested under these conditions. Further, addition of glutaraldehyde-insolubilized rabbit IgG to 102 serum, followed

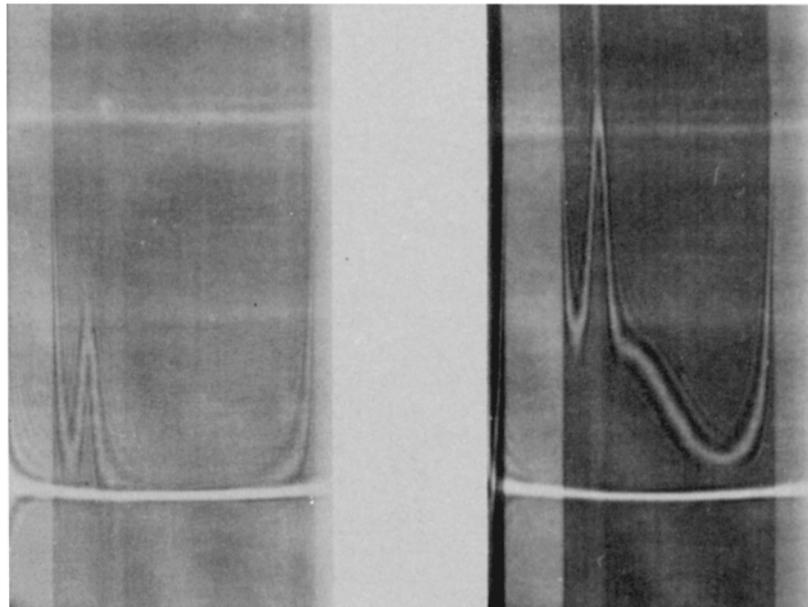


FIG. 4. Schlieren patterns of preinoculation serum (left frame), and immune serum isolated during the second immunization period (right frame) from rabbit 102. Sedimentation is from left to right; Temperature, 20°C; Speed, 56,000 rpm.

by incubation, centrifugation, and dilution of the supernate in PBS resulted in 31% inhibition of precipitate formation relative to a control.

Dilution-Induced Precipitation Inhibition Studies. Although RA appeared to have a significant role in the precipitation phenomenon, other experiments had indicated participation of specific antibody. It was important therefore to examine whether the dilution-induced precipitation reaction was modifiable by the presence of soluble ligands representative of immunodominant determinants associated with the micrococcal cell wall. The effect of various saccharide or saccharide-containing compounds as inhibitors of precipitation was tested. The results of these experiments are listed in Table I. The most effective inhibitors were glucose and mannuronic acid, with a combination of the two yielding maximum inhibition of precipitate formation. When 102 serum was diluted 1:10 in PBS-5% (wt/vol) mannuronic acid or glucose, 37% and 28% inhibition, respectively, could be effected. When the two saccharide compounds were present simultaneously, 47% inhibition of precipitation occurred. When similar concentrations of glucose were examined for inhibition of RA activity in both HA and coprecipitation assays in the BSA-anti-BSA system, no differences were detected in agglutination of IgG-sensitized erythrocytes or in the amount of Ig coprecipitated. In addition, absorption of 102 serum over the micrococcal immunoabsorbent resulted in 28% inhibition of subsequent precipitation when the fall-through serum was concentrated to the original volume and diluted 1:10 in PBS. These results clearly indicated an active and specific involvement of micrococcal antigen-reactive antibodies in the precipitation process, and in particular, those reactive with saccharide ligands homologous to the immunodominant carbohydrate determinants of the micrococcal cell wall, i.e., glucose and mannuronic acid (25).

TABLE I
Effect of Various Inhibitors on Dilution-Induced Precipitation of Serum 102

Inhibitor*	Percent inhibition
Phosphate buffer	0
0.3 M NaCl	0
0.25 M glycine	0
<i>N</i> -acetylgalactosamine	5
<i>D</i> -galactose	9
<i>N</i> -acetylmuramic acid	10
<i>D</i> -mannose	14
<i>N</i> -acetylmannosamine	14
<i>N</i> -acetylglucosamine	14
<i>D</i> -glucose	18
<i>D</i> -mannuronic acid	37
<i>D</i> -glucose + 0.3 M NaCl	20-28
<i>N</i> -acetylglucosamine + 0.3 M NaCl	22
<i>D</i> -glucose + <i>N</i> -acetylglucosamine + 0.3 M NaCl	26-31
<i>D</i> -glucose + <i>D</i> -mannuronic acid	47

* All inhibitor substances were prepared in 0.02 M phosphate buffer, pH 7.2. Carbohydrate solutions were prepared at 0.28 M concentration.

Micrococcal Antigen In 102 Serum. Experiments designed to detect the presence of micrococcal antigen in 102 serum were performed. Initial experiments examined the effect of lysozyme treatment on the ability of 102 serum to form precipitate upon dilution. After incubation of 102 serum in the presence of the enzyme, subsequent dilution yielded an amount of precipitate identical to that of nonenzyme-treated control samples. Further, no immune complex formation was observed in Ouchterlony double-diffusion experiments between opposing wells of 102 serum and other anti-micrococcal serum samples, although all samples formed precipitin lines against purified, lysozyme-digested cell wall material. In addition, small volumes of other hyperimmune anti-micrococcal antisera were added in sequential fashion to 102 serum in attempts to detect precipitate formation. Precipitin reactions were never observed. Although by direct precipitin methods there was no evidence for the presence of micrococcal antigens in 102 serum, it was possible that these methods were not sufficiently sensitive to detect small quantities of these antigens. It was necessary therefore to design an alternate assay system.

Advantage was taken of the dilution-precipitation phenomenon. If micrococcal antigens were present in 102 serum, other anti-micrococcal $F(ab')_2$ should coprecipitate. Specifically purified anti-micrococcal antibodies from two individual hyperimmune rabbit sera were isolated by affinity chromatography, and their respective $F(ab')_2$ were prepared from peptic digests. The fragments were radiolabeled and incubated in the presence of 102 serum. After addition of 2-5 ng ^{125}I - $F(ab')_2$ and incubation of the mixture, the solutions were diluted with PBS and the precipitated radioactivity was determined. As shown in Table II, no significant precipitation of counts occurred. Although specific ^{125}I - $F(ab')_2$ were not associated with precipitated complexes, it was possible that competition of endogenous specific antibody had inhibited fragment reactivity with antigen. In an attempt to isolate complexed antigen from competing soluble antibodies, additional experiments utilized the $Fc\gamma$ reactivity

TABLE II
*Binding of Isologous Anti-Micrococcal $^{125}\text{I-F(ab')}_2$ to Precipitate Formed during Dilution of Serum 102 or to *S. aureus* Exposed to Whole 102 Serum*

Antibody fragment source (rabbit number)	Radioactivity bound			
	Direct precipitation		<i>S. aureus</i>	
	Serum 102	NRS*	Serum 102	NRS*
			%	
92-6	2	2	13	17
92-6	7	5	15	13
93-6	1	1	2	2
93-6	2	1	2	2

* Fragments were added to normal rabbit serum (NRS) or to *S. aureus* incubated in the presence of NRS, and the amount of radioactivity remaining in tubes or associated with *S. aureus* was determined.

of *Staphylococcus aureus* protein A as an immunoabsorbent. Whole bacteria were added to 102 serum and incubated. After incubation, the suspensions were pelleted and washed with PBS made 1% with BSA. The pellets were resuspended in PBS-BSA and to the suspensions were added specific $^{125}\text{I-F(ab')}_2$. After incubation, resuspension, and washing in PBS-BSA, pellets were examined for bound radioactivity. The results of these assays are also shown in Table II. As depicted, no significant binding occurred. These data indicated that complex formation upon dilution did not involve participation of micrococcal cell wall antigen.

Autogenous Anti-Idiotypic In 102 Serum. Results obtained by ligand inhibition of dilution-induced precipitation, the absence of RA-activity inhibition by the same ligands, and the inability to detect soluble or immune complex-associated micrococcal antigens in 102 serum suggested that precipitation involved the simultaneous interaction of micrococcal-specific antibody, rheumatoid factor, and autogenous anti-idiotypic antibodies. Addition of excess idiotypic, as well as the addition of excess normal rabbit IgG to 102 serum, would therefore be expected to inhibit, in the former instance, the anti-idiotypic-endogenous idiotypic interaction and, in the latter, the RA-Fcγ interaction. To test these possibilities, 102 serum was diluted with PBS and the supernate was concentrated to the original serum volume. Precipitate-depleted serum was then used as a diluent for whole 102 serum. As depicted in Fig. 5, 102 precipitate-depleted serum was capable of 100% inhibition of whole 102 dilution-induced precipitation. Also shown in Fig. 5 are the results obtained when whole 102 serum was diluted in the presence of pooled normal rabbit serum or hyperimmune isologous anti-micrococcal (41% of serum protein as IgG) or anti-streptococcal (30% of serum protein as IgG) serum. Both the anti-micrococcal and anti-streptococcal hyperimmune sera were capable of inhibiting precipitation when used as diluent for 102 serum; however, comparison of the 50% inhibition endpoints obtained from the dose-response curves revealed that substantially more anti-streptococcal or isologous anti-micrococcal serum was required to effect inhibition compared to autologous, precipitate-depleted 102 serum. Further, neither pooled normal rabbit nor preinoculation sera were inhibitory when used as diluent. These results suggested that excess idiotypic(s)

IDIOTYPE NETWORK AUTOREGULATION

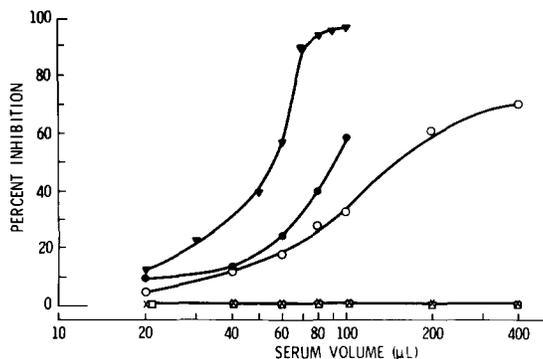


FIG. 5. Inhibition of dilution-induced precipitation in the presence of precipitate-depleted serum 102 (▲); micrococcal antiserum 99 (●); streptococcal antiserum H3 (○); preinoculation serum 102 (×); or normal rabbit serum (□). The amount of inhibition was calculated relative to an uninhibited serum 102 control diluted in the presence of 0.02 M phosphate, 0.15 M NaCl buffer, pH 7.2.

and/or Fc γ in the supernate of precipitate-depleted serum was capable of blocking subsequent complex formation in other 102 serum samples. To further examine these possibilities, purified normal rabbit IgG and IgG purified from 102 serum as affinity chromatographed peaks B, C, or D were used as inhibitors of dilution-induced precipitation. As shown in Table III, the presence of $\cong 2$ mg of 102 peak B, C, or D anti-micrococcal antibody inhibited precipitation by $>90\%$. A similar degree of inhibition was effected only after 11 mg of normal rabbit IgG was added. In addition, the amount of precipitate formed upon dilution in the presence of 4 mg of normal rabbit IgG F(ab') $_2$ was identical to the amount formed in the control containing no fragments. These data indicated that specific anti-micrococcal antibodies obtained from rabbit 102 were capable of far more efficient interaction and suggested that the specificities of the interacting molecules were associated with combining sites as well as with Fc regions.

It was necessary, therefore, to demonstrate that 102 micrococcal-specific F(ab') $_2$ could be specifically bound by antibody present in 102 serum. F(ab') $_2$ was prepared from purified, pooled normal rabbit IgG and from antibody eluted as either peak B, C, or D after affinity chromatography of 102 serum. These radiolabeled fragments were then incubated individually with diluted 102 serum, and immune complexes were precipitated by a goat anti-rabbit Fc γ antiserum. The results of these experiments are presented in Table IV. Although ^{125}I -F(ab') $_2$, prepared from normal rabbit IgG were not reactive, precipitation of radioactivity ranged from 10 to 49% when specific 102 fragments were used. When fragment binding in preinoculation serum was examined, a substantial percentage of peak D ^{125}I -F(ab') $_2$, 21% of TCA-precipitable counts, was precipitated upon facilitation. When 1 ml of preinoculation serum was passed over the micrococcal affinity column, 1.4 mg of peak D antibody was eluted.

Specific fragment binding activity was not unique to the serum samples depicted in Table IV; similar reactivity was detectable during the second immunization period.

Because RA was clearly present in 102 serum, binding of specific ^{125}I -F(ab') $_2$ could have resulted from contamination of purified antibody with anti-IgG in micrococcal affinity column isolates. It was important to determine that the radioactivity precipitated was not a result of RA ^{125}I -F(ab') $_2$ binding to rabbit Fc γ . To examine this

TABLE III
Inhibition of Dilution-Induced Precipitation in the Presence of Normal Rabbit IgG or Serum 102-Specific Antibodies Isolated by Affinity Chromatography as Peak B or Peak D

Inhibitor		Precipitate formed*	Inhibition
Source	Amount		
	mg	^A 278 ^{nm}	%
No inhibitor	0	0.259	0
Normal IgG F(ab') ₂	4	0.258	0
Normal IgG	2	0.105	59
Normal IgG	11	0.022	91
Peak B	2	0.023	91
Peak D	2	0.023	91

* Precipitate was solubilized in 0.05 N NaOH.

TABLE IV
Indirect Radioimmunoassay for Detection of Autogenous Anti-Idiotype in Serum 102

Serum sample	Percent counts precipitated ¹²⁵ I-F(ab') ₂ source			
	Normal rabbit IgG	Peak B	Peak C	Peak D
Preinoculation*	3	12	10	21
Week 38 first‡	3	36	22	49
Week 10 second	2	34	23	45
Week 30 second	2	29	20	49

* Application of 1 ml of preinoculation serum 102 to solubilized micrococcal cell-wall-Sepharose 4B conjugate yielded, upon gradient elution, 1.4-mg antibodies eluting as peak D.

‡ Immunization period, first or second.

possibility, heterologous immune complexes were formed in the presence of peak B, C, or D or normal rabbit IgG ¹²⁵I-F(ab')₂. As seen in Table V, a small percentage of radioactivity was associated with the complexes when micrococcal-specific fragments were examined. Thus, RA activity was present in affinity column isolates; however, the percentage of binding observed was significantly less than that associated with the 102 serum assays.

Inhibition of Autogenous Anti-Idiotype-Idiotype Binding. Experiments were done to examine the effect of unlabeled, specific F(ab')₂ on binding of specific ¹²⁵I-F(ab')₂ in 102 serum. As shown in Fig. 6, preincubation of the unlabeled homologous peak F(ab')₂ with 102 serum significantly inhibited the subsequent interaction of radiolabeled fragments. Inhibition of binding was dose dependent, and approached 100%. Further, the ability of unlabeled, specific fragments to inhibit was not restricted to homologous fragments as evidenced by the significant cross-inhibition observed when unlabeled peak D fragments were tested in the presence of radiolabeled peak C fragments, and vice versa. These results were in contrast to those obtained when either normal rabbit IgG F(ab')₂ or affinity-purified peak B or D F(ab')₂ isolated from other

TABLE V
Rheumatoid Factor Activity in F(ab')₂ Prepared from Micrococcal-Specific Affinity-Purified Serum 102 Antibodies

Heterologous immune complexes* (source)	Radioactivity precipitated‡			
	NF(ab') ₂	Peak B	Peak C	Peak D
	%			
NR IgG + anti-IgG Fc	0.6	3.8	1.2	11.5
NRS + anti-IgG Fc	3.4	8.9	5.3	11.9
BSA + anti-BSA	1.8	7.4	5.6	13.2
Anti-BSA + anti-IgG Fc	2.0	4.4	1.7	6.2

* Immune complexes were formed at equivalence using normal rabbit (NR) IgG and goat antiserum; normal rabbit serum (NRS) and goat antiserum, BSA and rabbit anti-BSA, or rabbit anti-BSA and goat antiserum.

‡ Radiolabeled F(ab')₂ were prepared for normal rabbit IgG (NF(ab')₂) or from antibodies specifically purified from serum 102 as gradient elution peak B, C, or D.

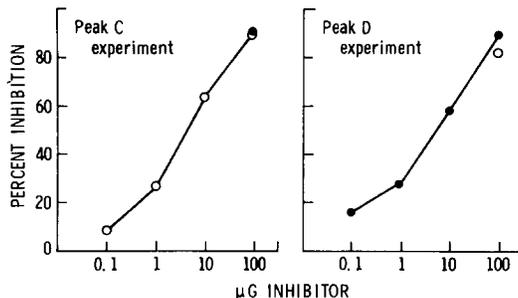


FIG. 6. Inhibition by unlabeled F(ab')₂, isolated from peak C (○), or peak D (●), of binding in indirect radioimmunoassay of peak C or peak D ¹²⁵I-F(ab')₂ in dilute serum 102.

hyperimmune anti-micrococcal sera was used as inhibitor of radiolabeled 102 fragment binding. No inhibition was observed. The lack of inhibition observed under these conditions correlated with the data from previous experiments designed to detect soluble micrococcal antigen in 102 serum, i.e., insignificant precipitation of radioactive F(ab')₂ occurred when 102 serum was diluted after the addition of anti-micrococcal fragments isolated from two other rabbits.

Additional inhibition experiments were performed which utilized the well documented evidence for the ligand modifiability of private idiotypic specificities. As described in Table VI, glucose significantly inhibited the ability of radiolabeled F(ab')₂ to be bound in the indirect radioimmunoassay. Although binding of peak B or C fragments was inhibited by 36–37%, respectively, essentially no effect upon peak D reactivity was observed. This result is not surprising because Wikler (25) has shown that the peak D antibody has a predominant specificity for micrococcal peptidoglycan, not for the CHO antigen of micrococcus. These results provide further evidence for a ligand-modifiable idio-anti-idio interaction, and corroborate that the ligand modification of dilution-induced precipitation is associated with autogenous anti-idiotypic.

Purified Autogenous Anti-Idiotypic. One final approach to substantiate the presence of autogenous anti-idiotypic in 102 serum was an experiment designed to examine ¹²⁵I-

TABLE VI
Ligand Inhibition in Indirect Radioimmunoassay of Serum 102 Autogenous Anti-Idiotype-Idiotype Interaction

F(ab') ₂ source	Radioactivity precipitated		Inhibition
	(+)*	(-)	
	%		%
Normal rabbit IgG	1	1	0
Peak B	18	28	36
Peak C	11	17	37
Peak D	41	42	1

* Glucose present at 0.2-M concentration.

TABLE VII
Reactivity of Serum 102 Anti-(Peak B, C, F(ab')₂) Antibodies with Serum 102 Radiolabeled Peak B or Peak D F(ab')₂ in Indirect Radioimmunoassay

F(ab') ₂ source	Radioactivity precipitated
	%
Normal rabbit F(ab') ₂	2
Peak B	25
Peak D	41

F(ab')₂ binding in the absence of 102 serum. Therefore, serum 102 peak B and C antibodies, were used to prepare F(ab')₂ for conjugation to Sepharose 4B. The peak B and C F(ab')₂-Sepharose 4B conjugate was then used as a specific immunoabsorbent for idiotype-reactive antibodies in 102 serum. Antibodies eluted from this conjugate after passage of 102 serum were subsequently examined in indirect radioimmunoassays for their ability to react with homologous peak ¹²⁵I-F(ab')₂. As shown in Table VII, normal rabbit ¹²⁵I-F(ab')₂ was not significantly precipitated whereas ≈25 and 40% of radiolabeled peak B and D fragments, respectively, were complexed.

Clonotype Redistribution during Auto-anti-Idiotype Response. Sera from the first-, second-, and third-round immunizations of rabbit 102 were analyzed for clonotype distribution using the method of analytical isoelectric focusing followed by radioactive antigen localization. Samples of two different first-, second-, and third-round sera were electrofocused, fixed in the gel, exposed to ¹²⁵I-micrococcal antigen, washed, dried, and exposed to x-ray film. The autoradiograph (Fig. 7) shows substantial clonotype differences between first-, second-, and third-round sera. Samples 3 and 4 (first round) contain anti-micrococcal antibody clonotypes migrating predominantly in the pH range of 7–8. In the second-round response, in which the auto-anti-idiotypic antibodies were detected (samples 5 and 6), there is a dramatic decrease or absence of the antibodies in the pH 7–8 range and a concomitant increase or first emergence of several clonotypes in the pH range 6.5–7.4. After a 51-wk rest period, the third-round immunization schedule was begun and sera from the peak of this third-round response (where an auto-anti-idiotype response was not detectable) showed (samples 7 and 8) the reappearance of the clonotypes in the pH range of 7–8 in addition to the clonotypes that were predominant in the second-round response. This suggests that

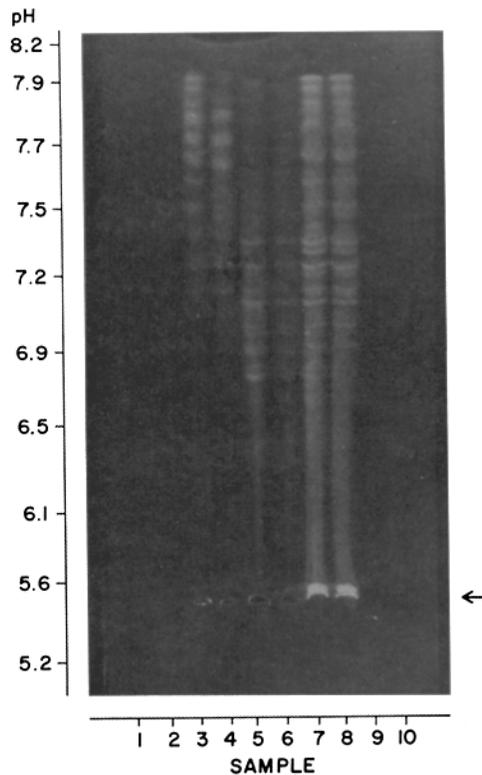


FIG. 7. Autoradiogram of isoelectric focusing analysis of rabbit 102 anti-micrococcal antisera. Samples 1 and 10 are pooled normal rabbit serum; samples 2 and 9 are rabbit 102, preinoculation; sample 3 is day 190 of first-round response; sample 4 is day 230 of first-round response; sample 5 is day 127 of second-round response; sample 6 is day 161 of second-round response; sample 7 is day 34 of third-round response; sample 8 is day 41 of third-round response. 20 μ l of each sample was applied to the plate. The pH gradient is shown at the left. The sample application point is indicated by an arrow.

the clonotypes that were absent in the second-round response were re-expressed in the third-round response in the absence of detectable auto-anti-idiotypic.

Discussion

The mechanisms responsible for the precipitation reaction in diluted 102 serum are not well understood; however, results of the present study suggest that a triad of interacting components involving micrococcal cell-wall specific antibodies, rheumatoid factor, and autogenous anti-idiotypic was involved. The reaction is not a common occurrence, but simple dilution of serum to assay for precipitate formation is not a commonly used analytical technique. We have now analyzed over 600 high-titer, anti-streptococcal, and anti-micrococcal antisera, many of which contained RA, and have seen this reaction only in second-round sera from rabbit 102.

Binding experiments clearly demonstrated that autogenous anti-idiotypic was produced in the serum of rabbit 102 during a second-round immune response to *M. lysodeikticus* vaccine. That binding was specifically associated with 102 antibody

interaction with 102 anti-idiotypic was substantiated by several experimental approaches. Micrococcal cell-wall antigen could not be detected in 102 serum by either precipitin or direct binding assays which utilized intact antibodies or $F(ab')_2$ fragments isolated from other anti-micrococcal sera. In addition, when similarly prepared $F(ab')_2$ fragments were tested for their ability to be precipitated in the presence of 102 serum by indirect radioimmunoassay, only fragments prepared from 102 serum antibodies were significantly reactive. Further, binding was not associated with pepsin agglutinator (31) activity, because non-102 anti-micrococcal $F(ab')_2$ or normal rabbit IgG $F(ab')_2$ were not significantly reactive in 102 serum. The absence of reactivity in 102 serum toward pooled normal rabbit IgG $F(ab')_2$ suggested further that the binding reactions observed did not involve, to a significant extent at least, latent or natural anti-allotype specificities. Finally, purified autogenous anti-idiotypic reacted only with anti-micrococcal $F(ab')_2$ isolated from 102 serum.

The idiotypic nature of the reactions observed in 102 serum was suggested by both fragment and ligand inhibition studies. Only specific 102 $F(ab')_2$ fragments were capable of inhibition in radioimmunoassay. The extensive cross-inhibition observed between heterologous-peak 102 $F(ab')_2$ suggested that a particular idiotypic(s) was not confined to specific gradient-eluted fractions, but was represented to some extent in all fractions.

One can detect certain shared idiotypic specificities in different mice of the same strain. The determinants which reflect these specificities are apparently associated primarily with the framework residues or relatively invariant portion of the V_H region. Such observations have led to the proposal that the corresponding structural genes encoding combining site sequences are in the germ line (32). In most instances, such shared idiotypic specificities reflect nonligand-modifiable idiotypic-anti-idiotypic interactions. The TEPC-15 system is an example of this type. In contrast to the TEPC-15 anti-idiotypic reaction, the anti-idiotypic reactions in the present study were clearly ligand-modifiable. The immunodominant residues present on the micrococcal, cell wall carbohydrate structure have been shown to consist of glucose and *N*-acetylaminomannuronic acid (25). Clearly, the most effective inhibitors of dilution-induced precipitation were the same (glucose) or similar (mannuronic acid) saccharide compounds. Because the rheumatoid factor-associated reaction was not inhibited by these compounds, and because micrococcal antigen was not detectable in 102 serum, inhibition of dilution-induced precipitation must have been a result of ligand modification. In addition, radiolabeled $F(ab')_2$ binding in 102 serum was specifically inhibited in the presence of glucose, with the exception of peak D fragment binding (Table VI).

The lack of inhibition observed for peak D fragment binding suggests multiple anti-idiotypic specificities related either to idiotypic determinants associated with peak D antibodies specific for micrococcal antigenic determinants other than glucose (i.e., peptidoglycan), or to nonligand-modifiable combining site residues. The inhibition of dilution-induced precipitation by mannuronic acid suggests that different micrococcal antigenic determinant specificities were involved. This reasoning does not, however, explain the ability of peak D $F(ab')_2$ fragments to completely inhibit binding of ^{125}I - $F(ab')_2$ isolated from peak C or vice versa in radioimmunoassay. It is possible that significant cross-idiotypic specificity was present and the requirements for ligand-modifiability of the reaction were more stringent.

Results obtained in the present study suggested that, in addition to autogenous anti-idiotypic antibodies reactive with homologous rabbit IgG Fc were present in 102 serum. Indeed, these antibodies were apparently directly involved in the dilution-induced precipitation reaction. Significant inhibition of precipitation was observed after absorption of 102 serum with glutaraldehyde-insolubilized rabbit IgG, or after passage of whole 102 serum over an IgG-Sepharose 4B conjugate. Further, albeit at relatively high concentration, the presence of normal rabbit IgG or anti-streptococcal hyperimmune serum effectively inhibited precipitation. That RA was involved was substantiated by results of HA experiments which clearly demonstrated the presence of IgG-reactive antibody. The degree of HA observed, in addition to the data obtained from SDS-PAGE analysis suggested that the RA in 102 serum was of the IgG class.

The association in the present study of RA with dilution-induced precipitation is reminiscent of results obtained over 20 yr ago in a study of low temperature-induced precipitation in human rheumatoid sera. Christian (33) observed that prior dilution of these sera in normal saline significantly increased the amount of precipitate formed upon subsequent storage of the samples at 4°C. Although immunoglobulin was shown to be present in these precipitates, the mechanism(s) responsible for their enhanced precipitability under dilute conditions was not fully elucidated.

The presence of auto-anti-idiotypic in second-round sera was established in this study by several approaches, including specific purification of these antibodies. Further experiments showed a direct observable suppressive effect of these antibodies on the production of specific subpopulations of anti-micrococcal antibodies. During the period in which autologous anti-idiotypic antibodies were detected, antibodies that made up a significant part of the first-round response with a pI of 7-8 were absent or substantially reduced in quantity. This effect was not permanent. These same antibodies reappeared in substantial quantities in a third-round response after a 51-wk rest period. It therefore appears that the natural auto-anti-idiotypic suppression observed in this animal was abrogated during the rest period and further boosting of the animal with antigen. These data are in complete agreement with the earlier results of Kohler et al. (34), who showed that treatments of adults with auto-anti-idiotypic caused a reversible suppression of responsiveness but the suppression was permanent in neonates.

Several investigators (35-37) have clearly shown that T-cell receptors share idiotypes with immunoglobulins. It is tempting to speculate that auto-anti-idiotypic responses such as this one could significantly alter T-cell reactivity. The opposite effect (auto-anti-idiotypic T cells affecting B-cell responses) is clearly also a possibility, although not approached in this study.

The precise mechanism of auto-anti-idiotypic-mediated immunosuppression is unclear. In the present study, RA was found to be present during a period of auto-anti-idiotypic-mediated suppression and to participate in the dilution-precipitation of idiotypic and auto-anti-idiotypic. Kohler et al. (38) have shown that the Fc portion of their anti-idiotypic is necessary for suppression of responses in the TEPC-15 and HOPC-8 systems. These authors suggested that suppression might be mediated by the interaction of anti-idiotypic with the antigen receptor and the Fc receptor thereby cross-linking the two receptors on the lymphocyte. The present study suggests an alternative mechanism in which suppression may be mediated by Fc-specific rheumatoid factor. The present study also supports the regulatory capacity of anti-idiotypic

described by Cazenave (39), Urbain et al. (40), Hart et al. (41), Binz and Wigzell (42), and Bona and Paul (43).

Examples of natural auto-anti-idiotypic responses have so far been confined to inbred lines of animals (15-17). The present investigation has focused on a normal outbred animal as an example of auto-anti-idiotypic regulation of an immune response to a foreign antigen occurring as component part of the immune response. Clearly, such examples lend strong support to the lymphocyte or idiochrome network theory of Jerne (14).

Summary

The antibody response of a single outbred rabbit was studied throughout three rounds of injections with *Micrococcus lysodeikticus* vaccine over a 31-mo period. The first-round response was characterized by a vigorous anti-micrococcus response and a strong anti-IgG rheumatoid factor response. The second-round response consisted of a triad of interacting molecules: anti-micrococcal antibodies, autoanti-idiotypic antibodies specific for distinct clonotypes of the first-round anti-micrococcal antibodies, and Fc-specific anti-IgG rheumatoid factor. The interacting triple complex was detected because of the formation of an immune complex that became insoluble upon dilution of the serum. Complex formation was inhibited in the presence of saccharide compounds known to be major immunodominant determinants of the micrococcal cell-wall carbohydrate polymer. The same saccharides did not affect the reaction of rheumatoid factor with IgG. Direct-binding radioimmunoassays ruled out mediation of the dilution-precipitation reaction by soluble micrococcal antigens. Specific absorption of rheumatoid factor inhibited the dilution-precipitation reaction. Auto-anti-idiotypic antibodies were specifically purified from second-round sera, directly confirming the presence of these antibodies. Suppressible effects of auto-anti-idiotypic antibodies on distinct antibody clonotypes were shown by gel isoelectric focusing of first-, second-, and third-round sera. Clonotypes expressed in the first round of immunizations were reduced in quantity or absent when auto-anti-idiotypic antibodies were detectable. Greatly enhanced levels of initial synthesis of new clonotypes of anti-micrococcal antibodies were detected during the period of auto-anti-idiotypic synthesis. The third-round sera, devoid of detectable auto-anti-idiotypic, contained clonotypes characteristic of both first- and second-round antisera. Thus, auto-anti-idiotypic-mediated suppression appeared to be reversible. The data are interpreted as lending strong support for concepts of autoregulation of immune processes in normal outbred animals via an idiotypic network.

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References

1. Bokisch, V. A., D. Bernstein, and R. M. Krause. 1972. Occurrence of 19S and 7S anti-IgG's during hyperimmunization of rabbits with streptococci. *J. Exp. Med.* **136**:799.
2. Bokisch, V. A., J. W. Chiao, D. Bernstein, and R. M. Krause. 1973. Homogeneous rabbit 7S anti-IgG with antibody specificity for peptidoglycan. *J. Exp. Med.* **138**:1184.

3. Herd, Z. L. 1973. Antiglobulins and cryoglobulins in rabbits producing homogeneous streptococcal antibodies. *Immunology*. **25**:923.
4. Herd, Z. L. 1973. Experimental cryoglobulinemia. Production and properties of streptococcus-induced rabbit cryoglobulins. *Immunology*. **25**:931.
5. Onica, D., F. Mihalcu, R. Lenkei, M. Gherman, and M. Tudor. 1977. Autoantibodies detected in rabbits hyperimmunized with Group A, C, and G, streptococcal vaccines. *Infect. Immun.* **18**:624.
6. Hamers, R., C. Hamers-Casterman, W. Van DerLoo, A. D. Strosberg, and P. De Baetselier. 1975. Rheumatoid factor appearance in *Micrococcus lysodeikticus* immunization and its interference with allotype specific reactions. *Z. Immunitaetsforsch.* **149**:187.
7. Oudin, M. J., and J. Michel. 1963. A new form of allotypy of rabbit γ -globulins apparently correlated with antibody function and specificity. *C. R. Acad. Sci. Paris*. **257**:805.
8. Kunkel, H. G., M. Mannik, and R. G. Williams. 1963. Individual antigenic specificities of isolated antibodies. *Science (Wash. D. C.)*. **140**:1218.
9. Braun, D. G., and R. M. Krause. 1968. The individual antigenic specificity of antibodies to streptococcal carbohydrates. *J. Exp. Med.* **128**:969.
10. Kuettner, M. G., A. L. Wang, and A. Nisonoff. 1972. Quantitative investigations of idiotypic antibodies. VI. Idiotypic specificity as a potential genetic marker for the variable regions of mouse immunoglobulin polypeptide chains. *J. Exp. Med.* **135**:579.
11. Sakato, N., S. H. Hall, and H. N. Eisen. 1977. Restricted heterogeneity of antibodies to idiotypes of isologous immunoglobulins. *Immunochemistry*. **14**:621.
12. Urbain, J., N. Tasiaux, R. Leuwenkroon, A. Van Acker, and B. Mariame. 1975. Sharing of idiotypic specificities between different antibody populations from an individual animal. *Eur. J. Immunol.* **5**:570.
13. Rodkey, L. S. 1974. Studies of idiotypic antibodies. Production and characterization of autoantiidiotypic antisera. *J. Exp. Med.* **139**:712.
14. Jerne, N. K. 1974. Towards a network theory of the immune system. *Ann. Immunol. (Paris)*. **125c**:373.
15. Kluskens, L., and H. Kohler. 1974. Regulation of immune response by autogenous antibody against receptor. *Proc. Natl. Acad. Sci. U.S.A.* **71**:5083.
16. Cosenza, H. 1976. Detection of anti-idiotypic reactive cells in the response to phosphorylcholine. *Eur. J. Immunol.* **6**:114.
17. McKearn, T. J., F. P. Stuart, and F. W. Fitch. 1974. Anti-idiotypic antibody in rat transplantation immunity. I. Production of anti-idiotypic antibody in animals repeatedly immunized with alloantigens. *J. Immunol.* **113**:1876.
18. Osterland, C. K., E. J. Miller, W. W. Karakawa, and R. M. Krause. 1966. Characterization of streptococcal group-specific antibody isolated from hyperimmune rabbits. *J. Exp. Med.* **123**:599.
19. Strosberg, A. D., C. Hamers-Casterman, W. Van Der Loo, and R. Hamers. 1974. A rabbit of allotypic phenotype $a_1a_2a_3b_4b_5b_6$. *J. Immunol.* **113**:1313.
20. Reisfeld, R. A., and P. A. Small. 1966. Electrophoretic heterogeneity of polypeptide chains of specific antibodies. *Science (Wash. D. C.)*. **152**:1253.
21. Chapuis, R. M., and M. E. Koshland. 1974. Mechanism of IgM polymerization. *Proc. Natl. Acad. Sci. U.S.A.* **71**:657.
22. Braun, D. G., K. Hild, and A. Ziegler. 1979. Resolution of immunoglobulin patterns by analytical isoelectric focusing. In *Research Methods in Immunology*. I. Lefkovits and B. Pernis, editors. Academic Press Inc., New York. In press.
23. Avrameas, S., and T. Ternynck. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoabsorbents. *Immunochemistry*. **6**:53.
24. Kessler, S. W. 1975. Rapid isolation of antigen from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617.

25. Wikler, M. 1975. Isolation and characterization of homogeneous rabbit antibodies to *Micrococcus lysodeikticus* with specificity to the peptidoglycan and to the glucose-N-acetylamino-mannuronic acid polymer. *Z. Immunitaetsforsch.* **149**:193.
26. Nisonoff, A., F. C. Wissler, L. N. Lipman, and D. L. Woernley. 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. *Arch. Biochem.* **89**:230.
27. McFarlane, A. S. 1958. Efficient trace labeling of proteins with iodine. *Nature (Lond.)*. **182**:53.
28. Hunter, R. 1970. Standardization of the chloramine-T method of protein iodination. *Proc. Soc. Exp. Biol. Med.* **133**:989.
29. Sela, B-A., J. L. Wang, and G. M. Edelman. 1975. Antibodies reactive with cell surface carbohydrates. *Proc. Natl. Acad. Sci. U.S.A.* **72**:1127.
30. Braun, D. G., E. Kjemis, and R. Cramer. 1973. A rabbit family of restricted high responders to the streptococcal Group A-variant polysaccharide. Selective breeding narrows the isoelectric focusing spectra of dominant clones. *J. Exp. Med.* **138**:645.
31. Mandy, W. J. 1967. A new serum factor in normal rabbits. III. Specificity for antigenic determinants uncovered by papain or pepsin digestion. *J. Immunol.* **99**:815.
32. Barstad, P., S. Rudikoff, M. Potter, W. Konigsberg, M. Cohn, and L. Hood. 1974. Immunoglobulin structure: Amino terminal sequences of mouse myeloma proteins that bind phosphorylcholine. *Science (Wash. D. C.)*. **183**:962.
33. Christian, C. L. 1959. A study of rheumatoid arthritis sera: comparison of spontaneous precipitates and gamma globulin-induced precipitates. *Arthritis Rheum.* **2**:289.
34. Kohler, H., D. R. Kaplan, and D. S. Strayer. 1974. Clonal depletion in neonatal tolerance. *Science (Wash. D. C.)*. **186**:643.
35. Binz, H., and H. Wigzell. 1975. Shared idiotypic determinants on B and T lymphocytes reactive against the same antigenic determinants. IV. Isolation of two groups of naturally occurring idiotypic molecules with specific antigen binding activity in the serum and urine of normal rats. *Scand. J. Immunol.* **4**:591.
36. Krawinkel, U., M. Cramer, T. Imanishi-Kari, R. S. Jack, K. Rajewsky, and O. Makela. 1977. Isolated hapten-binding receptors of sensitized lymphocytes. I. Receptors from nylon wool-enriched mouse T lymphocytes lack serological markers of immunoglobulin constant domains but express heavy chain variable portions. *Eur. J. Immunol.* **8**:566.
37. Cazenave, P.-A., J. M. Cavaillon, and C. Bona. 1977. Idiotypic determinants on rabbit B- and T-derived lymphocytes. *Immunological Rev.* **34**:34.
38. Kohler, H., B. C. Richardson, D. A. Rowley, and S. Smyk. 1977. Immune response to phosphorylcholine. III. Requirement of the Fc portion and equal effectiveness of IgG subclasses in anti-receptor antibody-induced suppression. *J. Immunol.* **119**:1979.
39. Cazenave, P.-A. 1977. Idiotypic-anti-idiotypic regulation of antibody synthesis in rabbits. *Proc. Natl. Acad. Sci. U.S.A.* **74**:5122.
40. Urbain, J., M. Wikler, J. D. Franssen, and C. Collignon. 1977. Idiotypic regulation of the immune system by the induction of antibodies against anti-idiotypic antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **74**:5126.
41. Hart, D. A., A. L. Wang, L. L. Pawlak, and A. Nisonoff. 1972. Suppression of idiotypic specificities in adult mice by administration of antiidiotypic antibody. *J. Exp. Med.* **135**:1293.
42. Binz, H., and H. Wigzell. 1976. Specific transplantation tolerance induced by autoimmunization against the individual's own, naturally occurring idiotypic, antigen-binding receptors. *J. Exp. Med.* **144**:1438.
43. Bona, C., and W. E. Paul. 1979. Cellular basis of regulation of expression of idiotype. I. T-suppressor cells specific for MOPC 460 idiotype regulate the expression of cells secreting anti-TNP antibodies bearing 460 idiotype. *J. Exp. Med.* **149**:592.