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Autoanti-Idiotypes Exhibit Mimicry of Myocyte Antigens in Virus-Induced Myocarditis

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Mice infected with coxsackievirus B develop immunologically mediated inflammatory myocarditis in heart tissue that results in the development of autoantibodies with multiple idiotypes. The specificity and temporal development of autoantibodies produced during coxsackievirus B3 infection were assessed. Antiviral idiotypes and anti-idiotypic antibodies against coxsackievirus B3 idiotypes were detected and quantitated over 21- and 42-day periods, respectively. Both polyclonal and monoclonal anti-idiotypes exhibited greater but nonspecific binding to heart, liver, kidney, and spleen cells from virus-exposed animals and normal tissue. Binding of anti-idiotypes was also demonstrated to myosin and to solubilized heart-associated antigens but not to virus. Western immunoblot analysis revealed that monoclonal and polyclonal anti-idiotypes selectively bound to hypertonic, salt-extracted, solubilized proteins of myocyte extracts of virus-exposed animals.

Earlier reports from this laboratory have focused on the role of anti-idiotypic antibodies (anti-Ids) in the abrogation and regulation of virus-induced, autoimmune murine myocarditis (19, 20–22). Both polyclonal and monoclonal anti-Ids have been shown to greatly modulate or inhibit the extent of myocarditis, either by 4-h pretreatment of mice with polyclonal or monoclonal anti-Ids (20) or by active immunization of susceptible animals with anti-Ids (20, 22). Other studies have demonstrated the modulatory effects of anti-Ids on the chemotaxis of macrophages participating in the inflammatory response associated with myocarditis (21).

The mechanism(s) whereby these anti-Ids exert their effects on the immune system during induction of autoimmune myocarditic disease is not clear, yet their effects on macrophages and the murine immune apparatus are obvious. Thus, the effects of anti-Ids on the immune apparatus in this system may represent multiple avenues of network activation leading to diverse mechanisms of pathogenesis.

Experimental evidence (1, 2, 7, 8, 13, 25) in other systems suggests that multiple infectious organisms have antigenic determinants associated with their outer membranes that exhibit antigenic mimicry of specific tissue determinants of certain mammalian cells (2, 19, 25, 26). Moreover, various investigators (7, 9, 13, 14, 17, 18) have suggested that populations of anti-Ids, acting as internal images, might function as antigenic epitopes in molecular mimicry of determinants associated with a particular organism or immunogen. Similarly, in the myocarditis model, if molecular mimicry by anti-Ids plays a role in infectious autoimmune myocarditis, anti-Ids might serve as a second source of offending epitopes in temporal development of the disease.

This investigation attempted to assess (i) whether autoanti-Ids are developed during coxsackievirus B3 (CVB3) infection and (ii) whether anti-Ids generated during exposure to a myocarditic dose of CVB3 exhibit affinity for normal or virus-exposed myocytes or solubilized proteins associated with myocytes.

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We now report a temporal profile of autoanti-Ids against anti-CVB3 idiotypes developed during the course of viral infection concomitant with development of specific idiotypic antibodies during CVB3-induced myocarditis. We also demonstrate the affinity of monoclonal and polyclonal anti-Ids to bind intact myocytes as well as solubilized myocyte proteins of virus-exposed animals and induction of an autoantibody, ab-3, capable of binding monoclonal or polyclonal idiotypes developed after CVB3 infection.

MATERIALS AND METHODS

Mice and immunization. BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and bred in University of Texas Health Science Center facilities. Adolescent 4- to 6-week-old males were used. BALB/c donors of anti-CVB3 antibodies were immunized intraperitoneally (i.p.) with 10^8 PFU of UV-irradiated CVB3 in Freund complete adjuvant, followed by weekly injections for at least 6 to 8 weeks of 10^7 PFU of virus i.p. (3–5, 19). Syngeneic BALB/c donors of mouse anti-Ids were immunized subcutaneously with 50 μg of protein A-purified immunoglobulin G (IgG) emulsified with Freund complete adjuvant. Groups of five to seven mice were boosted weekly with i.p. injections of 50 μg of IgG for 10 to 14 weeks and bled weekly after the sixth injection via the retro-orbital sinus. Mice were bled after the fifth injection, and antibody titers were routinely assessed by the enzyme-linked immunosorbent assay (ELISA). Serum was separated in Microtainer capillary blood separator tubes (Becton Dickinson, Rutherford, N.J.).

Virus production and viral antigen preparation. CVB3 was prepared by procedures reported previously (19–22). Confluent HeLa cell cultures were inoculated with 50 to 75 PFU of CVB3 per cell. After lysis of HeLa cells 12 to 14 h later, cells were centrifuged at 10,000 × *g* for 30 min in a Beckman J-21 B centrifuge with a type JA20 rotor. The virus lysate was adjusted to a molarity of 0.5 M NaCl, and 10% (wt/vol) polyethylene glycol (6,000 to 8,000 Da; Sigma Chemical Co., St. Louis, Mo.) was added. The solution was stirred for 12 min and held at 4°C for 24 h. The virus precipitate was

centrifuged for 20 min at 15,000 × *g*, layered on a linear CsCl gradient (1.27 to 1.37 g/ml) in tryptose-phosphate buffer (0.2% tryptose-phosphate broth, 0.1 M NaCl), in an SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 4°C for 12 h (19). After centrifugation, the density of each fraction of the gradient was assessed with a refractometer, and the localized viral protein band was collected in 0.2-ml fractions for measured densities ranging from 1.335 to 1.345. The virus band was collected and dialyzed for 5 h at 4°C against phosphate-buffered saline (PBS).

Tissue sectioning and staining. Portions of the apical section of each mouse heart were fixed in neutral 10% Formalin, sectioned, and stained with hematoxylin and eosin. Six to eight sections from each tissue sample were examined for myocarditis, which was defined as discrete foci of degenerated myocytes associated with focal mononuclear cell infiltrate (5, 19, 21). Each tissue section was read blindly, and foci of myocarditic lesions were blindly counted and recorded.

Preparation of DNP-ovalbumin. The dinitrophenol (DNP)-ovalbumin immunogen was prepared by conjugation of 0.2 ml of dinitrofluorobenzene to 500 mg of ovalbumin (Sigma Chemical Co., St. Louis, Mo.). The ovalbumin was dissolved in 100 ml of 0.85 M saline with dinitrofluorobenzene by stirring at 25°C for 30 min. Sodium bicarbonate was slowly added to obtain a pH of 8.0, and the solution was permitted to stand for 2.0 h at 25°C. The preparation was refrigerated at 4°C overnight and dialyzed the next day in a 20:1 volume of 10 changes of 0.85 M saline. Preparations were dispensed in 0.5 ml increments and stored at −20°C until used.

Preparation of cell suspensions. Heart, liver, kidney, and spleen were surgically extirpated from each experimental animal. Tissue was finely minced with a sterile, sharp scissors and expressed with a small syringe plunger through a 40-mesh stainless steel screen, using RPMI 1640 medium. The expressed cells were gently aspirated with a Pasteur pipette, collected in a 15-ml centrifuge tube, and washed three times in medium at 500 × *g*. The cells were then suspended in 3 to 5 ml of medium and counted, and 50 μl of cell suspension was dispensed on 96-well microtiter plates at 10^6 cells per ml.

Solid-phase ELISA. The detection of anti-Ids was assessed by using a solid-phase micro-ELISA. Briefly, mouse $F(ab')_2$ anti-CVB3 idiotype-bearing fragments were deposited in 96-well microtiter plates (20–22). After washing, the wells were coated with $F(ab')_2$ and a filler consisting of 10% goat serum and 1% bovine serum albumin. Next, the antigen-coated plates were washed six times, and serum unknowns and/or controls were layered on the $F(ab')_2$ fragment-covered plates. After overnight incubation at 4°C, the plates were washed six times in distilled water. The plates were then incubated for 3 h with a horseradish peroxidase-conjugated goat anti-mouse Fc. After incubation with anti-immunoglobulin serum, the plates were washed and the appropriate concentration of substrate was added to detect the presence of the enzyme and binding of the presumable anti-Id to $F(ab')_2$. Radial immunodiffusion plates (Boehringer Mannheim, Indianapolis, Ind.) were used to assess levels of murine IgM and IgG in test animal sera.

We routinely quantitate the amount and intensity of color reading by scanning the 96-well test plate in a recording spectrophotometer (MR580 Micro Elisa Autoreader, Dynatech, Alexandria, Va.). The wells with color changes of 0.05 or above are considered positive. This figure for positive reactivity is based on a statistical analysis of 100 negative wells in the assay containing all reagents in the test except specific antibody and ordinarily ranges from 0.000 to 0.050.

Protein A separation of IgG fractions. All murine serum antibodies were purified by protein A-Sepharose (Sigma) separation. One gram of Sepharose was swollen in PBS to a volume of 3.5 to 4.0 ml. The Sepharose gel was packed in a Bio-Rad Econo (Bio-Rad Laboratories, Richmond, Calif.) column measuring 7 mm by 30 cm and washed several times with PBS for 30 to 45 min. The samples were diluted 1:5 in PBS, adjusted to a pH of 7.5, and layered on top of the gel surface. Samples were permitted to run into the column, and approximately 2.5 to 5 ml of eluant was collected. After the column was washed with PBS, IgG was eluted with 1 M acetic acid (pH 4.5) and immediately neutralized. Fractions were analyzed at 280 nm with a Beckman model DB-G spectrophotometer; the peak of absorbance was collected and dialyzed in PBS overnight. The amount of protein was calculated from the optical density reading at 280 nm.

Antibody digestion and Fab preparation. $F(ab')_2$ fragments of the anti-Ids (ab-2) were prepared as described earlier (20). Briefly, serum immunoglobulin or ascites fluids containing antibodies or anti-Ids (3.0 mg) were dissolved in 0.14 M sodium acetate-sodium chloride buffer with 60 mg of pepsin at pH 4.0. The mixture of antibody and enzyme was incubated for 18 h at 37°C and adjusted to a pH of 8.0 with 0.1 M NaOH. This preparation was then dialyzed against 0.15 M saline-borate buffer (pH 7.5) with a molecular weight cutoff of 60,000, yielding 53% of $F(ab')_2$ fragments.

Affinity chromatography. Cyanogen bromide-Sepharose 4B (Pharmacia, Inc., Piscataway, N.J.) was used for coupling anti-CVB3 IgG (CVB3 absorbed, acid eluted) for purification and characterization of the anti-Ids. One gram of gel was swollen in 0.003 M HCl and washed for 15 min on a glass filter. Next, serum (500 μg/ml) was dissolved in 5 ml of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl) and mixed with the swollen gel. The mixture was rocked slowly at 25°C for 60 min, and excess ligand was washed away with buffer. Remaining active groups were blocked with 0.1 M Tris hydrochloride buffer (pH 8.0). The resulting gel was washed three times with alternating cycles of 0.1 M acetate buffer (pH 4.0) and 0.1 M Tris buffer (pH 8.0). Coupled Sepharose (5 ml) was resuspended by gently agitating the column in 2 volumes of PBS and allowed to incubate for 30 min at 37°C in a small column. The sample containing anti-Id was applied to the column and permitted to flow for 30 min. The eluant was washed over the column two additional times, and the column was washed with 200 ml of PBS. To elute the anti-Ids, the Sepharose was carefully resuspended by gentle rocking of the column in 3 M $MgCl_2$. This procedure was performed two additional times; the eluate was collected and dialyzed overnight against two changes of distilled water to remove excess salt and then in PBS.

Western immunoblotting. A Bio-Rad apparatus for performing nitrocellulose electroblotting was used to transfer bands to nitrocellulose for further development with specific peroxidase-conjugated antibodies. Electrophoresed gels were sandwiched with nitrocellulose paper, using blotting paper, domestic scouring pads, and plastic racks with holders. Blotting buffer was made up with a 20% volume of methanol in regular electrophoresis buffer. Air bubbles were carefully pressed out of the gel sandwich, and the gel was immersed in the tank of electroblot buffer. Transfer of antibody proteins was conducted at 30 V and 125 mA for 16 to 18 h. After transfer, the gel was removed and stained with Coomassie blue as a control to detect any residual peptides;

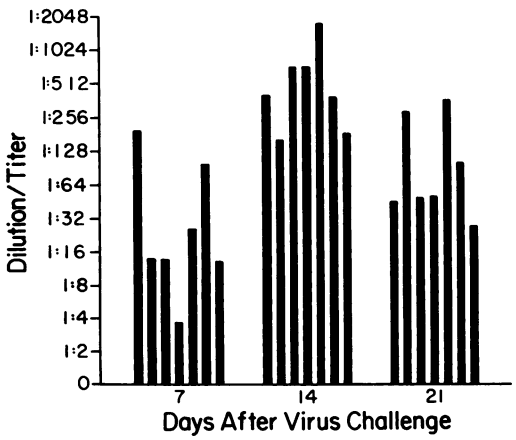

FIG. 1. Titers of autoanti-CVB3 (ab-1) idiotypic antibody in syngeneic BALB/c mice challenged with 10^{-7} PFU of CVB3. Each bar represents the mean serum titer from two animals. Each titer represents the last well exhibiting a positive absorbance reading above 0.050. Preimmune serum titers of 14 mice ranged from <0 to 1:2 prior to challenge with virus.

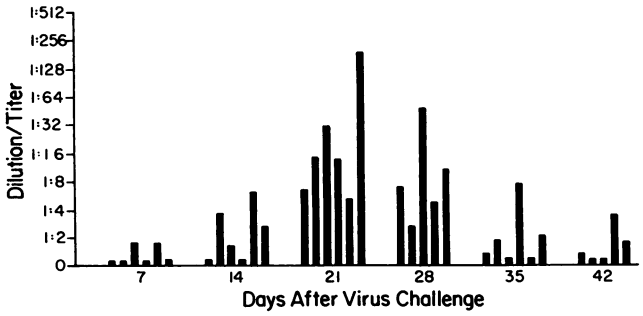

FIG. 2. Autoanti-Id titers (ab-2) detected by ELISA in syngeneic BALB/c mice challenged with 1,000 PFU of CVB3 virus during a myocarditic infection. $F(ab')_2$ fragments of individual animals were prepared on each of the days indicated. Each bar represents the mean individual ab-2 titers from two animals. After day 42, all animals were sacrificed and assessed histopathologically for the presence of myocarditis.

the nitrocellulose paper was washed thoroughly in a 4.0% concentration of dry milk as a blocking agent to bind nonspecific binding sites on the nitrocellulose. The nitrocellulose was then incubated with the appropriate probe antibody for at least 2.5 h or overnight in a wet, humid environment. After incubation, the nitrocellulose was carefully washed and sites of antibody binding were detected by using an indirect technique, by flooding the nitrocellulose with a Western blot peroxidase-conjugated goat anti-murine IgG (Bio-Rad) diluted 1:3,000 at room temperature.

RESULTS

Assessing levels of anti-CVB3 antibodies developed during the induction of myocarditis. To establish base-line levels of anti-CVB3 idiotypes, syngeneic BALB/c mice were inoculated with UV-inactivated CVB3 and assessed for levels of anti-CVB3 idiotypes binding or neutralizing virus antigen at various times after exposure to virus. Antibodies of the IgM and IgG subclasses were assessed for the presence of anti-CVB3 idiotypes by their ability to bind CVB3 antigen(s) in a solid-phase ELISA (20, 22). BALB/c mice were inoculated i.p. with 10^7 PFU of UV-inactivated CVB3 and bled on days 7, 14, and 21 days after virus challenge. Each heat-inactivated mouse serum was serially diluted and tested against CsCl-purified CVB3 antigen-coated 96-well microtiter plates. Ab-1 titers are shown in Fig. 1.

At day 7, IgM antibodies ranged from a low serum titer of 1:4 to a high titer of 1:256, with most antiviral antibody titers in the 1:16 range (Fig. 1). After 14 days, antiviral antibody titers were raised, ranging from 1:128 to 1:2,048 (Fig. 1). After 21 days, IgG antibodies bearing anti-CVB3 idiotypes declined to levels that ranged from 1:64 to 1:512. Random testing of mice after day 21 resulted in antibody titers ranging from 1:16 to 1:256 in individual mice. Negative controls in each ELISA included normal, heat-inactivated mouse serum, a positive anti-CVB3 reference control having an antibody titer greater than 1:2,048, and a virus specificity control utilizing mengovirus as antigen.

Assessing individual mice for autoanti-Ids (ab-2) against antiviral idiotypes. The next series of experiments was aimed at assessing the levels of autoanti-Ids (ab-2) occurring subsequent to a regular CVB3 challenge. Syngeneic BALB/c mice were injected i.p. with 10^{-7} PFU of virus. Mice were bled individually on days 7, 14, 21, 28, 35, and 42. Individual mouse sera were subjected to a pepsin digestion to obtain serum $F(ab')_2$ fragments, presumably containing autoanti-Ids (ab-2), from animals at each of the above-designated times. Next, the dialyzed, purified, $F(ab')_2$ fragments from individual animals were attached to a polystyrene microtiter plate and titrated with a CVB3-absorbed, protein A affinity-purified anti-CVB3 IgG antibody (ab-1) in doubling dilutions. Marginal levels of anti-Id were detected on day 7 or 14; ab-2 titers ranged from less than 1:2 to 1:8 on day 14 (Fig. 2). On the other hand, some but not all anti-Id titers rose considerably by day 21, ranging from 1:8 to 1:256 in different animals. Routine controls testing the F(ab)s with normal serum failed to detect binding to the $F(ab')_2$ fragments. Titers of anti-Id persisted but declined after day 21, ranging from less than 1:2 to 1:4 by day 42 (Fig. 2). Sections of heart tissue from each animal were assessed for myocarditis; myocarditis indices ranged from 2+ to 4+ for all animals challenged with CVB3.

In vitro assessment of autoantibodies (ab-1 and ab-2) binding to intact normal or CVB3 virus-exposed murine tissue cells. Earlier experiments in our laboratory suggested preferential but nonspecific binding of polyclonal anti-Ids to CVB3-exposed myocyte populations. Because of these results (20), we wondered whether selective binding of anti-Ids to myocytes, possibly acting as internal images of viral epitopes, might exhibit mimicry for myocyte viral receptors, thus competing for virus-myocyte attachment. The next series of experiments sought to assess the binding characteristics of a monoclonal anti-Id (anti-Id88) (22) and polyclonal ab-1 on normal or CVB3-exposed viable myocyte cell populations. Normal animals or animals exposed to a myocarditic dose (1,000 PFU) of CVB3 were sacrificed 7 days after virus exposure. Heart, liver, kidney, and spleen cell populations were removed, set up as single-cell suspensions, and fixed to an ELISA plate. Monoclonal anti-Id88 and polyclonal ab-1 were diluted and assessed for the ability to bind each of the normal or CVB3-exposed cell populations. Antibody binding was assessed with an enzyme-conjugated goat anti-mouse Fc antibody. Each bar in Fig. 3 represents mean titers of four separate experiments. Binding of monoclonal or polyclonal anti-Id to heart, liver, kidney, and spleen cells was observed when cells were obtained from

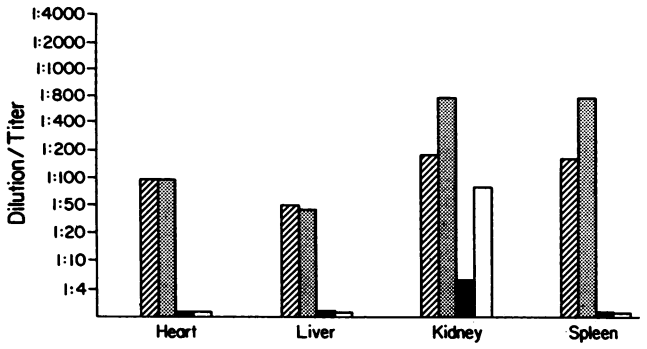

FIG. 3. Quantitative comparison of autoanti-Id and idiotypic antibody binding to normal or CVB3-exposed BALB/c cell populations in vitro. Cell populations were incubated with monoclonal anti-Id88 or a polyclonal ab-1. Antibody-cell binding was assessed with a peroxidase-conjugated goat anti-mouse IgG antibody. Bars represent anti-Id88 on CVB3-exposed animals (▨), anti-Id88 on normal cells (▩), polyclonal ab-1 on CVB3-exposed animals (■), and ab-1 on normal cells (□). Each bar represents the mean titers of four separate experiments run on different days with different pools of animals.

normal animals or animals exposed to CVB3; on the other hand, when these same murine cell populations were incubated with the control polyclonal anti-CVB3 ab-1, marginal to negative binding was observed in heart, liver, and spleen cells (Fig. 3). There was some binding of antibody to kidney cells observed with ab-1 as well as with the anti-Id88 (Fig. 3).

Testing of autoantibodies against noncellular, solubilized heart-associated antigens, virus, and antiviral idiotypes. The range of antigen binding of polyclonal or monoclonal anti-Ids, along with other autoantibodies present during the disease process, was assessed by using heart-associated antigens, myosin, antiviral idiotypes, and CVB3. Hypertonic salt extracts, myosin, solubilized virus antigen, and $F(ab')_2$ fragments of ab-1 were deposited on an ELISA plate, and dilutions of each of the autoantibodies were tested against these various antigens in four separate experiments. Polyclonal anti-Ids were able to bind solubilized salt extracts of myocyte tissue from CVB3-exposed animals as well as extracts of normal myocyte tissue used as controls (Table 1). However, these anti-Ids did not bind purified virus antigen (Table 1). A similar pattern of binding of the monoclonal anti-Id88 was observed except that the monoclonal anti-Id failed to bind solubilized proteins extracted from normal myocytes (Table 1). On the other hand, ab-3, which should be similar to ab-1 in terms of specificity, failed to bind ab-1 $F(ab')_2$ fragments; however, ab-3 did predictably bind virus antigen in addition to myosin and the solubilized salt extracts (Table 1). Ab-1 was able to bind virus antigen in titers that ranged from 1:1,024 to 1:4,096 but did not bind myosin or normal KCl extracts to any significant degree (Table 1).

Western blot analysis of monoclonal and polyclonal anti-Id binding to myocyte antigens. Because we had demonstrated binding of both polyclonal and monoclonal anti-Ids to myocytes in vitro, Western blot analysis was done to visualize the binding of anti-Ids to myocyte proteins and to assess the numbers and relative molecular weights of myocyte proteins to which syngeneic, monoclonal, and polyclonal anti-Ids were binding. Hypertonic KCl extracts were prepared from normal or CVB3-exposed BALB/c myocytes as reported earlier (24). After extraction, graded amounts of the solubilized proteins were electrophoresed on a slab gel and then transfer blotted to nitrocellulose. The blotted proteins were then probed with monoclonal anti-Id88, and a syngeneic, affinity-purified, polyclonal ab-2 was developed against anti-CVB3 idiotypes. Binding of the anti-Ids to the solubilized myocyte proteins was assessed with a peroxidase-conjugated goat anti-mouse IgG specially prepared for Western immunoblot assay (Bio-Rad).

Binding of the monoclonal anti-Id88 occurred in multiple bands of proteins from CVB3-exposed animals ranging from approximately 75 to 150 kDa. Similar binding patterns of proteins were evident when probed with the syngeneic, affinity-purified polyclonal anti-Ids (Fig. 4). On the other hand, probing the KCl extracts from normal animal myocytes failed to show either the intensity or numbers of bands observed with the experimental anti-Ids (Fig. 4). Testing of polyclonal anti-Ids developed against idiotypes specific for an unrelated antigen, DNP-ovalbumin, failed to demonstrate binding to CVB3-exposed or normal myocyte salt-extracted proteins.

Analysis of monoclonal anti-Id88 and polyclonal anti-Ids, using syngeneic, polyclonal ab-3s against anti-Id preparations. The specificities of the monoclonal anti-Id88 and the polyclonal anti-Ids used in these experiments were assessed by the development of anti-anti-Ids (ab-3s) against each of the anti-Ids. Ab-3s generated against anti-Ids will presumably

TABLE 1. Dilution titers of autoanti-Ids tested against myosin, CVB3 antigen, or murine heart-associated antigens

Antibody tested	Titer with given antigen[a]				
	ab-1 $F(ab)_2$	Myosin[b]	KCl extract		CVB3 antigen[c]
			CVB3	normal	
Polyclonal anti-Id (CVB3)	128, 256	200, 40	200	252	<1:10
	1,048, 512	400, 200	256	400	
				200	
Monoclonal anti-Id88	256, 512	2,048, 400	200	<1:4	<1:2
	512	100, 40	200		
	1,048				
Anti-antiidiotypic (ab-3)	<1:2	200, 800	200	256	512, 1,064
	<1:4	1,600, 2,048	256	200	128, 256
Anti-CVB3 (ab-1)	<1:8	80, 50		<1:2	2,048, 2,048
	<1:2	4, 40			1,024, 4,096

[a] Last dilution with a positive absorbance reading above 0.050.
[b] 200 μg of rabbit myosin per test well.
[c] 2 μg of solubilized virus antigen per test well.

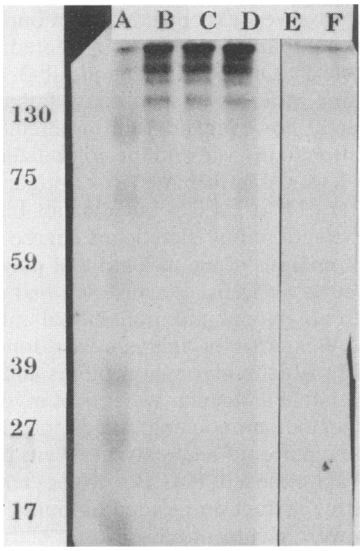

FIG. 4. Immunoblot analysis of hypertonic KCl extracts of myocyte tissue from CVB3-exposed or normal myocyte tissue from BALB/c mice. KCl extracts were electrophoresed, blotted to nitrocellulose, and probed with monoclonal anti-Id88, polyclonal anti-Id, or normal IgG. Lanes: A, molecular weight markers ranging from 17 to 130 kDa; B to D, hypertonic salt extracts from animals infected with CVB3 (lanes B and C, polyclonal anti-Id; lane D, monoclonal anti-Id88); E and F, extracts of myocytes from normal, uninfected syngeneic animals.

possess idiotypes able to bind the original immunogen, in this case CVB3 (20). Previous studies indicate that our ab-3s are able to bind specifically CVB3 antigen(s) but are unable to neutralize viable virus in vitro (20, 22). Several groups of syngeneic BALB/c mice were immunized with protein A affinity-purified anti-Id88 or affinity-purified polyclonal anti-Ids. Separate preparations of protein A-separated serum fractions containing the ab-3s were tested against $F(ab')_2$ fragments of anti-Id88 and polyclonal anti-Ids. Ab-3s made against either the monoclonal anti-Id88 or the polyclonal anti-Id preparations bound solubilized CVB3 antigen in three experiments (Table 2). Moreover, each of the Ab-3s bound $F(ab')_2$ fragments of each anti-Id used to generate the ab-3 as well as homologous anti-Id $F(ab')_2$ fragments (Table 2). On the other hand, none of the ab-3 preparations bound F(ab') fragments prepared from nonimmune, syngeneic $F(ab')_2$ (Table 2). Earlier assays testing the specificity of these ab-3 preparations on $F(ab')_2$ fragments of anti-Ids against idiotypes binding DNP-ovalbumin were negative; absorbance readings ranged from 0.013 to 0.048 at dilutions of less than 1:2.

DISCUSSION

In the studies reported here, autoanti-Ids developed during induction of myocarditis by CVB3 were detected and quantitated. Polyclonal anti-Ids as well as a monoclonal anti-Id (anti-Id88) bound to heart, liver, kidney, and spleen cells from CVB3-exposed as well as normal animals. Results indicated high titers of antibody against virus epitopes 21 days after exposure to the virus and the concurrent development of autologous anti-Ids specific for antiviral idiotypes during a 42-day period. Both monoclonal and polyclonal anti-Ids bound solubilized, hypertonic salt extracts of syngeneic heart tissue from virus-exposed animals and, to a lesser extent, solubilized determinants from uninfected murine heart tissue. Anti-Ids also bound myosin and solubilized heart-associated antigens but not virus antigens.

The abrogation or inhibition of myocarditis by anti-Ids on the one hand (20) and possible exacerbation or mediation of myocarditis after the administration of viable virus, with the subsequent development of auto anti-Ids, create a complexity and paradox in dissecting the mechanism(s) involved in cardiomyopathy (6, 10, 11, 29). This complexity suggests that multiple network activation(s) by anti-Ids may be operative during the development of myocarditis (6, 11, 29), and network activation(s) may depend on subtle but discrete quantitative relationships between idiotype and anti-idiotype. For example, our earlier studies demonstrated the abrogation or inhibition of myocarditis by short-term administration of anti-Ids prior to virus challenge or by the immunization of susceptible animals with polyclonal or monoclonal anti-Ids (20, 22). Furthermore, our first demonstration, in this report, of the presence of auto anti-Ids during a 42-day period after CVB3 infection suggests that auto anti-Ids are indeed present. However, their effects on the immune response leading to autoimmune myocarditis remain to be resolved.

In addition, recent results from our laboratory suggest the ability of both polyclonal and monoclonal anti-Ids in this system to specifically modulate the chemotaxis of macrophage populations without the release of chemotactic factors from sensitized lymphoid cells (19). While the mechanism(s) of macrophage modulation by anti-Ids in this system remains unknown, the mobilization of macrophage populations seen in the inflammatory tissues of myocytes during the disease may indeed be the result of network activation. Moreover, delayed hypersensitivity to solubilized CVB3-exposed myocyte membrane antigens, as well as to the virus itself, has been well documented (19, 23). Furthermore, the in vitro and

TABLE 2. Micro-ELISA absorbance readings and titers of polyclonal syngeneic anti-anti-Ids (ab-3s) made against anti-Id88 or polyclonal anti-Ids

Ab-3 tested	Absorbance (dilution titer)[a] of ab-3 tested against:				
	Control	$F(ab)_2$ monoclonal anti-Id88[b]	$F(ab)_2$ BALB/c polyclonal anti-Id	CVB3 antigen(s)[c]	Normal BALB/c $F(ab)_2$ IgG
Anti-anti-Id88	0.014	0.217 (1:256)	0.119 (1:64)	0.150 (1:5,120)	0.001 (<1:2)
Anti-anti-polyclonal	0.003	0.339 (1:1,024)	0.073 (1:128)	0.223 (1:1,280)	0.044 (<1:2)
Anti-anti-Id88	0.021	0.171 (1:512)	0.099 (1:64)	0.195 (1:2,560)	0.039 (<1:2)

[a] Highest dilution of antibody having positive absorbance above 0.050.
[b] 2 μg of $F(ab)_2$ protein per test well.
[c] 50 μg of solubilized viral antigen per test well.

in vivo effects of autoanti-Ids on B- and T-cell populations remain to be established; however, both polyclonal and monoclonal anti-Ids seem initially to have suppressive effects on isolated T-cell populations from normal or CVB3-exposed animals in vitro (18a, 28).

There are other possible virus-host interactions that might account for the observed pathogenesis. Antigenic mimicry of mammalian tissue proteins by multiple infectious organisms has been documented many times (2, 15, 16, 23, 27, 30). Mimicries of mammalian tissue epitopes by determinants associated with microorganisms have been postulated to act as stimulatory agents of immunoreactants, i.e., immune cells or antibodies, able to mediate autoimmune disease (14, 15, 23, 25). In addition, the development of autoimmunity associated with the administration of viral vaccines is well documented (4, 9, 17). Our results demonstrate the presence of autoanti-Ids developed during the induction of myocarditis; however, the precise role of these anti-Ids in mediating subsequent inflammatory myocarditis is not yet clear. Paradoxically, our earlier studies suggest that autoimmune myocarditis can be inhibited by the administration of anti-Ids prior to experimental infection or by immunization of susceptible animals with anti-Ids (19, 20, 22). Is it possible that anti-Ids exhibiting mimicry of viral determinants might bind myocytes, thus inhibiting or competing with CVB3 for myocyte attachment? Our results indicating increased binding of anti-Ids to multiple murine tissues, in addition to the selective binding of anti-Ids to KCl-solubilized CVB3-exposed myocyte antigens, suggest that this might be a possibility. Furthermore, polyclonal antiviral idiotypic antibodies bind CVB3 containing the four VP capsid antigens as well as $F(ab')_2$ fragments of both polyclonal or monoclonal anti-Ids. Thus, certain anti-Ids produced by the host's immune system, acting in mimicry of the virus in this instance, might be a natural reservoir for the original, offending antigen. In this posture, the anti-Ids might activate a network cascade that accounts for the multiple immune phenomena that we have seen in our studies and in this system.

It is evident from these studies that autoanti-Ids are indeed present over a 42-day period after virus exposure and selectivity bind to a variety of tissue cell types from CVB3-exposed animals, to solubilized membrane proteins of such animals, and to a major heart cell protein, myosin. The presence of autoanti-Ids raises additional questions as to the almost certain multiple mechanisms operative in the development of autoimmune myocarditis, especially with respect to cellular activation. Further elucidation of the role of autoanti-Ids in the development of autoimmune myocarditis may result in a better understanding of these mechanisms.

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