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Evidence for a functional idiotypic network among natural antibodies in normal mice

(natural idiotypes/antibody repertoires/network dynamics)

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ABSTRACT **We monitored in normal adult BALB/c mice the serum concentrations of four natural IgM antibodies, two of which show idiotypic complementarity in *in vitro* assays. In each individual, serum concentrations of all four idiotypes were found to fluctuate in complex dynamical patterns with low correlation. The spectral power of some such patterns was found to be compatible with the existence of a chaotic regime. Groups of normal adult mice were injected intravenously with low (10 ng) or moderate (10 μg) doses of either of the two complementary idiotypes in saline. This treatment resulted in a pronounced inhibition of the fluctuation in the serum concentration of both complementary idiotypes for periods up to 3 months. Such compensations were not detected for the two unrelated natural idiotypes and were specifically induced, for they did not occur following the injection of unrelated antibodies. These results indicate the functional operation of an idiotypic network among natural antibodies.**

Two alternative theoretical frameworks are prevalent in immunology. One, derived from the clonal selection theory, is based on the exclusion of self-reactivities from mature antibody repertoires (1, 2). The other, developed from the idiotypic network concept (3), considers (idiotypic) autoreactivities as the basis of immune repertoires and functional activities (4–6). In spite of extensive experimentation on idiotypic regulation of immune responses and the early demonstration of *potential* antibody networks (7, 8), evidence for widespread connectivity in the *actual* repertoire (9) of activated lymphocytes and natural antibodies of normal individuals has been obtained only recently (9, 10). These observations, however, fall short of indicating the dynamical properties and functional significance of such idiotypic molecular interactions detected *in vitro*. Thus, serologic assays, usually carried out after immobilization of one of the two antibody partners on a solid phase, may well detect positive interactions with affinities that are too low to be relevant in the operation of an idiotypic network *in vivo*.

This question has been extensively investigated by injection into normal mice of "artificially" prepared anti-idiotypic antibodies (11). These treatments have drastic consequences on the B-cell repertoire that is expressed upon subsequent antigenic challenges. Although such experiments have shown that idiotypic selection of available antibody repertoires takes place under these particular conditions, the high doses of antibodies injected, and the fact that these had not been isolated from normal individuals, could cast doubts upon the operation of a network under physiological conditions. Furthermore, none of those experiments addressed actual antibody repertoires and they provided no information on network dynamics.

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We have approached these questions by analyzing the dynamics of two naturally expressed idiotypes known to be connected. We find that their circulating levels normally vary in individually characteristic ways with dynamics fundamentally different from those observed during immune responses. Furthermore, injection of either antibody in minute amounts—much below the preexisting concentrations—has drastic and long-lasting consequences for the production of both. These results indicate the dynamic operation of an idiotypic network among natural antibodies.

MATERIALS AND METHODS

Mice. BALB/c mice, kept under specific-pathogen-free conditions, were obtained from our own facilities and used at 8–10 weeks of age.

Antibodies and Treatments. The IgM monoclonal antibodies BA.N 1:1.8, BA.N 4:4.57, BA.N 1:6.30, and BA.N 3:2.50 were isolated from normal BALB/c mice (9, 12). Purified IgM was obtained from culture supernatants or ascitic fluids by ammonium sulfate precipitation followed by dialysis against 5 mM phosphate buffer. III-A11-C7 and (SP/6)2-4 (IgG1, κ) are conventional syngeneic monoclonal anti-idiotypic antibodies to the "natural" antibody BA.N 1:1.8 or to the anti-trinitrophenyl IgM antibody SP/603. H-81.98.21 (IgG2a, κ) is an Ia.7-specific monoclonal antibody isolated from A.TH mice (13), previously characterized as an anti-idiotype to BA.N 4:4.57 (14) and was a gift from M. Pierres (Marseille-Luminy). F23.1 (IgG2a, κ) is a monoclonal antibody specific for $V_{\beta 8}$-chain variable regions of the T-cell receptor (15). All IgG antibodies were purified from ascitic fluid on protein A-Sepharose columns (Pharmacia). Mice were bled from the retroorbital venous plexus (0.2–0.3 ml) at several time points before and after being injected i.v. with 10 ng or 10 μg of purified antibodies in saline or with saline alone. Each experimental group consisted of six mice assayed individually.

Assays. The serum titers of BA.N 1:1.8, BA.N 4:4.57, SP/603 (16), and F23.1$^+$ IgM (P. Pereira and A.C., unpublished observation) equivalents were determined in ELISAs where wells of polyvinyl microtiter plates (Nunc) were coated with III-A11-C7 (250 ng per well), H-81.98.21 (250 ng), (SP/6)2-4 (500 ng), or F23.1 (500 ng) antibodies, respectively. After saturation of nonspecific sites with 1% (wt/vol) gelatin, mouse serum was appropriately titrated and bound IgM molecules were detected by addition of peroxidase-labeled goat anti-mouse IgM antibodies (Southern Biotechnology Associates, Birmingham, AL) followed by substrate. The absorbance was read at 450 nm. The titers were estimated by comparing the serum dilutions to known concentrations of the prototype idiotypes (BA.N 1:1.8, BA.N 4:4.57, SP/603, and BA.N 1:5.24 antibodies, respectively) titrated in the same assay plates. These were included in every experiment so that the idiotype concentrations could be converted to equivalents (ng/ml) of the respective prototype antibody.

Quantitative Analysis. Calculations were performed with routines available from the NAG FORTRAN Mark 11/15 mathematical library. The Fourier transforms were computed either to the discrete set of data points (NAG C06AEF) or by previously interpolating a Bezier polynomial (NAG E02AGF). Correlations were computed by using the Pearson first moment–product correlation (NAG G02ABF). Some data were examined under the nonparametric sign correlation by using the Wilcoxon matched-pairs signed-rank test (NAG G08ABF).

RESULTS

Patterns of Fluctuations of Natural Idiotypes in Normal Mouse Serum. Throughout these experiments we have studied adult BALB/c mice for serum levels of two idiotypes that we previously isolated from normal 6-day-old donors and that were found to react with each other (9). Fig. 1 shows the serologic complementarity of BA.N 4:4.57 and BA.N 1:1.8 antibodies, as well as the specificity of the anti-idiotypic antibody reagents used to detect them. Appropriate titrations of either prototype antibody in each ELISA plate allowed us to derive the equivalent concentration of idiotype from the normal serum titrations.

Normal adult BALB/c mice showed considerable individual variation in the serum concentrations of these complementary idiotypes. More interestingly perhaps, the fluctuation of these levels with time was characteristic for each mouse (Fig. 2 *Left*). With serum from the first bleeding as reference level, it becomes apparent that the range of normal variations fluctuates 2- to 3-fold above or below the reference level with few exceptions. That is, natural production of these antibodies is kept roughly bounded in a dynamic pattern that looks very different from an immune response to an antigenic challenge (even when autoantigens or idiotypes are used), which shows peaks of production that often reach orders of magnitude above preexisting levels.

The two complementary antibodies measured here appear indeed to behave in a complementary and functionally relevant manner—that is, their respective levels fluctuate in apparent interdependence. To pursue this point, we calculated the (linear) covariance between pairs of the four (two connected and two nonconnected) antibodies for the entire time studied here. As shown in Table 1, correlation values between the two (*in vitro*) connected antibodies are far from reaching values over ±0.80 necessary to be robustly significant. In fact, the overall values can hardly be distinguished from those for the two nonconnected idiotypes. Similarly, nonparametric tests, such as sign correlation, gave equally weak results throughout. Thus, the superficial appearance of correlation does not resist quantitative analysis.

A more revealing picture can be obtained through a Fourier analysis of these time series (Fig. 3). For example, idiotype BA.N 1:1.8 appears to have a pattern of oscillation around a few cycles per month. However, for a subset of mice (3/6) the power spectra for this idiotype are best described as containing all lower frequencies, following a $1/f^{\delta}$ law, with a slope of $\delta \approx -0.7$ in the log–log plots (Fig. 3*A*). (For other idiotypes we obtained similar profiles with $-0.7 \leq \delta \leq -2.5$). In two other animals, the power spectra show a slight dominant component at around 2 cycles per month, but for another animal (I.2) the spectrum approaches a bell-shaped spread (Fig. 3*B*). These conclusions apply equally well to the data obtained for all four idiotypes analyzed (data not shown), in spite of the fact that F23.1$^+$ and SP/603 show fluctuations within a range that is 1 order of magnitude smaller (see below, Fig. 6).

Consequences of Small Perturbations in Serum Idiotype Concentrations. In search of evidence for the functional dynamics of these two natural antibodies within physiological conditions, we evaluated the consequences of a rapid but quantitatively small variation in their serum concentrations. Adult mice were injected with very low (10 ng) or moderate (10 μg) doses of idiotype and the serum levels of both BA.N 4:4.57 and BA.N 1:1.8, as well as of the two other, "unrelated" idiotypes, were measured over the next 3 months. In spite of the extremely low dose of antibody injected (0.1–10% of the preexisting levels in serum, as measured in idiotype equivalents), striking effects were recorded in the pattern of fluctuations of both interconnected idiotypes upon modification of the concentration of either one. Fig. 2 shows some of these results, in groups of six mice injected with either saline (control, discussed above) or with each of the two complementary idiotypes. In contrast to the control group, where each individual showed a characteristic variation, idiotype-treated mice behaved in a concerted fashion as they

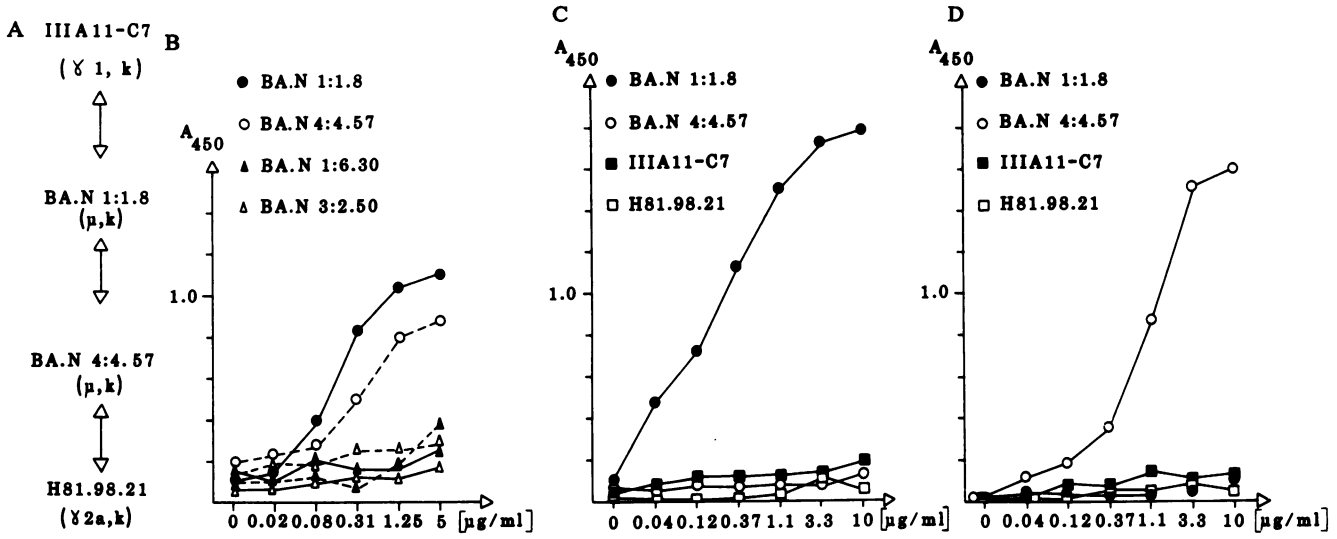

FIG. 1. (*A*) Schematic representation of interactions between complementary idiotypes as established *in vitro*. (*B*) The connected idiotypes BA.N 1:1.8 and BA.N 4:4.57 specifically react with each other. ELISA plates were coated with limiting concentrations of either BA.N 1:1.8 (- - -) or BA.N 4:4.57 (—). After saturation and washing, the plates were incubated with various amounts of the indicated IgM antibodies and bound IgM was detected by the addition of anti-μ antibodies. (*C* and *D*) Specificity of the assays detecting idiotype concentrations. Plates coated with either III-A11-C7 (*C*) or H-81.98.21 (*D*) were incubated with various amounts of each of the four IgM antibodies described above, and bound IgM subsequently was detected.

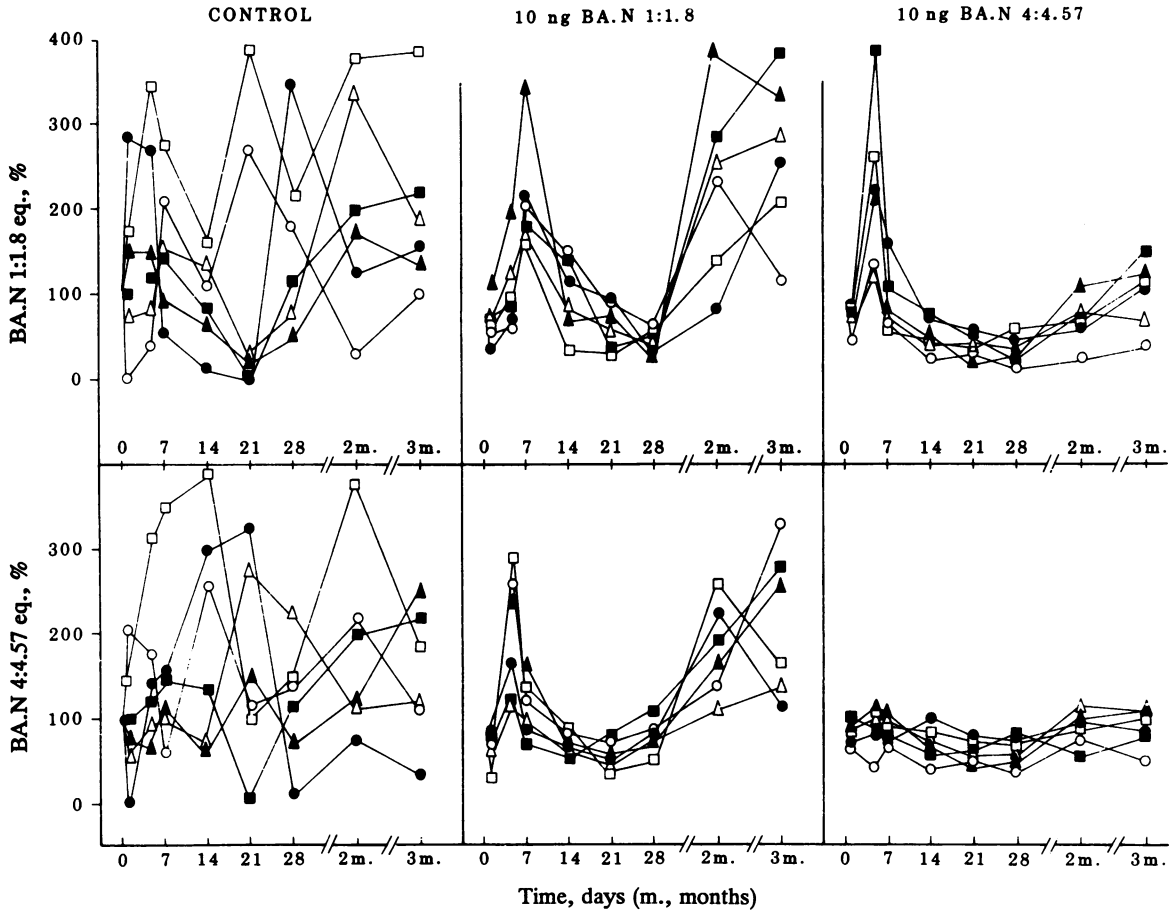

FIG. 2. Time course of expression of two connected idiotypes (BA.N 1:1.8 and BA.N 4:4.57) in adult BALB/c mice that had been injected with 10 ng of either idiotype or had been left untreated. Repeated bleedings from six individual mice, per group, at the indicated times were analyzed for the expression of BA.N 1:1.8 (*Upper*) and BA.N 4:4.57 (*Lower*) equivalents. The concentrations obtained on the day of the first bleeding (day 0) were normalized to 100% for each mouse, and all other titers are expressed as a percentage of this initial value.

were brought, with variable delays, to a minimal level of production. This appeared to be the case with either low (10 ng; Fig. 2) or moderate (10 μg; data not shown) doses of idiotype. Thus, after BA.N 4:4.57 injection, for example, the concentration of BA.N 1:1.8 decreased slightly in every mouse, to increase again in all of them on day 5 and decrease 5–7 days later. In contrast, treatment with BA.N 4:4.57 immediately suppressed the production of this idiotype for many weeks.

A few points are for us remarkable in these results. First, injection of antibodies induces a steady suppression of the production of both idiotypes, after a transient peak (16, 17). These dynamics are in contrast with the natural behavior described above. Second, the effects persist for a long time and, therefore, cannot reflect the persistence of the injected molecules, which are, in any case, identical to those already present at much higher concentrations in the mouse before treatment. Third, the induced, concerted variations are kept within the normal range of variations in concentration; that is, if a "response" to the idiotype injection exists, it is not expressed in the form of an immune response, by the production of increased, high concentrations, either of complementary or of similar idiotypic profiles. Rather, a change in idiotype concentration, even when extremely small, is manifested at the level of network dynamics.

Table 1. Correlation matrices for the concentrations of four natural idiotypes in normal mouse sera

	B.AN 1:1.8	B.AN 4:4.57	F23.1	SP/603
Mouse no. 1				
B.AN 1:1.8	1	−0.62	0.46	0.22
B.AN 4:4.57		1	−0.29	−0.37
F23.1$^+$			1	0.23
SP/603				1
Mouse no. 3				
B.AN 1:1.8	1	0.09	0.63	0.10
B.AN 4:4/57		1	−0.19	−0.55
F23.1$^+$			1	0.08
SP/603				1

Values correspond to the Pearson first-moment product correlation. For simplicity, matrices are shown for only two of the six control mice in Fig. 2, but the other four showed the same pattern.

The effects on expression of both idiotypes by perturbing the normal concentration of one appear specific, as shown by the unaltered patterns of expression of two idiotypes that are not directly connected with the injected antibodies (Fig. 4*A*).

A number of other groups of mice were analyzed in this experiment, after treatment with idiotypes that are not directly connected with either natural or induced antibodies. Some of these results are shown in Fig. 4*B* to demonstrate the specificity of these interactions. Thus, treatment with a natural antibody not directly connected to these only reduces the amplitude of the normal fluctuations in idiotype concentrations. Similarly, treatment with an induced antibody of the same class results in none of the altered patterns observed after treatment with BA.N 4:4.57 or BA.N 1:1.8 (data not shown). There is, therefore, a clear specificity in the consequences of minute alterations in the concentrations of a given idiotype for the expression of that idiotype and of its connected subset.

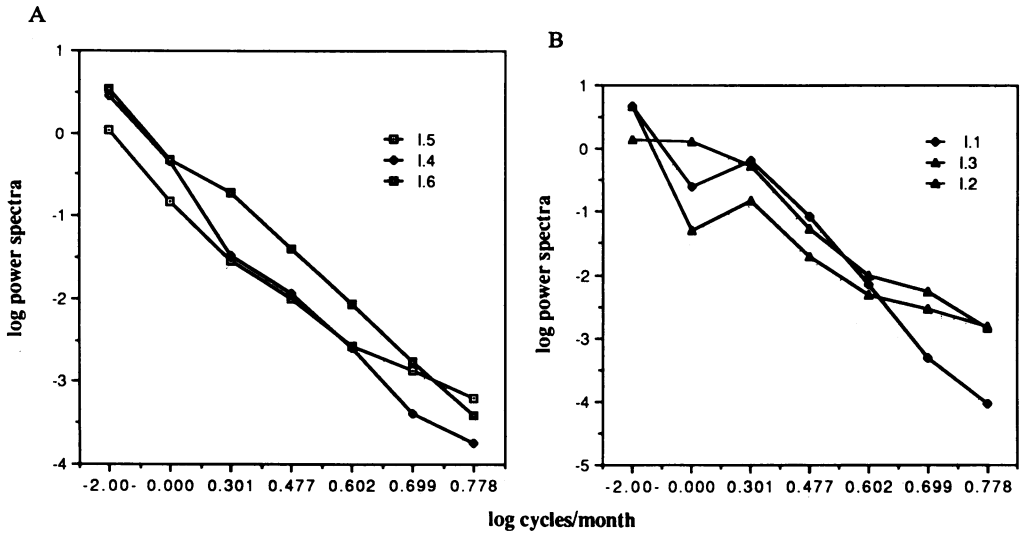

FIG. 3. Fourier analysis of the time series shown in Fig. 2 for idiotype BA.N 1:1.8 for all six normal, untreated mice (group I). (*A*) Double logarithmic plot clearly shows a $1/f^{\beta}$ power distribution (slope, -0.7) for three animals. (*B*) For two other animals the power spectra deviate slightly from the previous pattern, with a peak at about 2 cycles per month, whereas animal I.2 presents a bell-shaped distribution.

DISCUSSION

This work addressed the general question of the network organization of vertebrate immune systems. More precisely, we investigated whether or not idiotypic affinities detected *in vitro* operate in a functional manner in the normal individual. We chose two connected idiotypes—previously isolated from newborn, unmanipulated BALB/c mice—whose respective anti-idiotypes detected idiotype-bearing immunoglobulin molecules in every adult syngeneic individual tested thus far. We quantitated the variations with time in serum concentrations of each idiotype, in either normal donors or mice treated with minute doses of either antibody, and from these data we infer that the production of each of these molecules apparently correlates with the serum concentrations of both.

The functional interdependence of these idiotypes was first suggested by the finding that fluctuations in both idiotypes were drastically altered after i.v. injection of either one in doses that are much below the amounts detected in serum

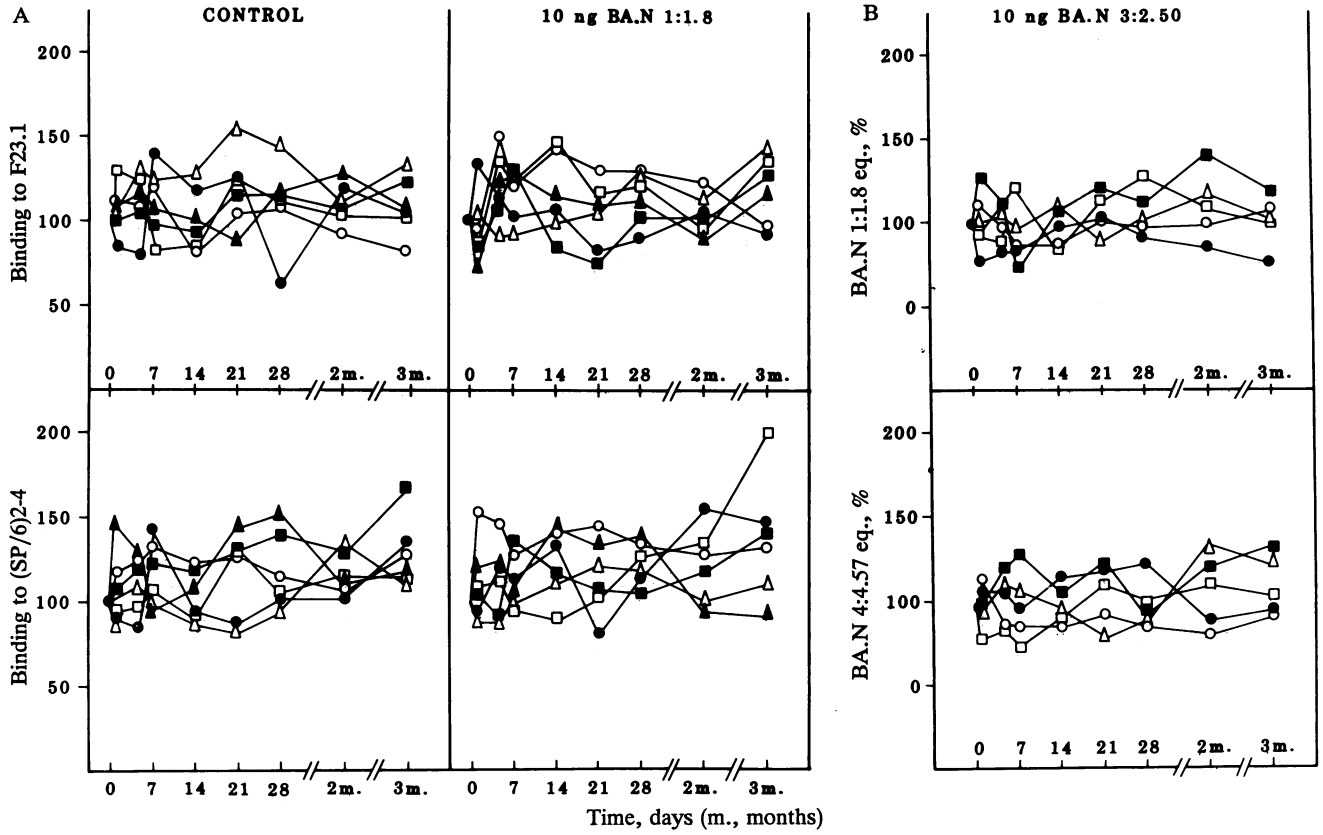

FIG. 4. (*A*) Serum levels of SP/603 and F23.1$^+$ idiotypes in the mice shown in Fig. 2. (*B*) Expression of BA.N 1:1.8 and BA.N 4:4.57 idiotypes in mice treated with an "unrelated" idiotype. Sera obtained at the indicated times after injection of 10 ng of BA.N 3:2.50 antibody, as indicated, were analyzed for idiotype concentration; results are expressed as in Fig. 2. Similar results were obtained after injection of 10 μg of these antibodies (data not shown).

before treatment. Because the appropriate controls appeared not to affect the patterns of idiotype fluctuation, we concluded that internal idiotypic connectivity regulates the production of the corresponding idiotypes. These conclusions were supported by the pattern of idiotype fluctuation in normal mice. A good indication that such patterns are not merely random variables was given by the time-series analysis of the idiotypes. These did not show a simple oscillatory behavior; we found instead spectral patterns of various complexities. This suggests that the underlying dynamics are complex, as is to be expected in a highly connected biological network. Elsewhere, we have proposed an explicit mathematical model of the immune network based on experimental observations (6). The nonlinear nature of the equations, as well as computer simulations of the model, corresponds well with the dynamics found in the data presented herein. The low correlation values of Table 1, even for (*in vitro*) highly connected idiotypes, also point in the same direction. In fact, most nodes in the immune network are subject to multiple simultaneous influences from many other nodes, and hence we do not expect any two of them to be highly correlated. In contrast, transient effects on neighboring nodes can be quite dramatic, as the present evidence also indicates.

In other complex systems, a $1/f^{\delta}$ power spectrum, as found here for several idiotypic temporal fluctuations, is usually associated with chaotic regimes (attractors of fractals dimension; ref. 18). Such attractors are known to exist in other biological systems (19, 20), and they may represent not a dysfunctional condition but rather a reservoir of diversity from which the system can bifurcate to simpler oscillatory or steady states. This seems to be the case, for instance, in the normal operation of the olfactory bulb for every breath cycle (21). Clearly, our data only suggest that possibility; many more data points would be needed to ascertain more precisely that chaotic regimes are present in normal immune networks. In any case, these initial results highlight the importance of further studies on the dynamical patterns of natural antibody populations.

These conclusions are only valid for the compartment of natural antibodies and the cells participating in their production: the "naturally" activated lymphocytes of normal individuals. Such a functional network does not operate in the small lymphocyte pool, as these cells are resting, and they simply cannot be engaged in functionally relevant interactions. This argument has been developed in some detail (22). In brief, newly produced immunocompetent lymphocytes that find productive internal connections—other lymphocyte receptors or antibodies, or other molecular patterns in the internal milieu—are activated into the large-cell pool, becoming, together with their complementarities, members of a functional network. This obviously implies a sharp selection from *available* to *actual* repertoires (23), and evidence has been produced that the latter are enriched in self-related, idiotypically connected specificities (24, 25). Lymphocytes that display specificities with no internal complementarities remain resting and rapidly decay (26). In contrast, activation results in prolonged life-spans and, eventually, in the production of serum antibodies (27). Because of their concentration—as compared to cell-bound receptors—natural antibodies appear to be fundamental in accounting for recursivity and for the history of the network in each individual. It is indeed one of the most striking aspects in the present results that the system keeps, for periods up to 3 months, the "memory" of a single injection of 10 ng of an autologous idiotype.

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