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# Population dynamics of natural antibodies in normal and autoimmune individuals

(natural autoantibodies/immune networks/population dynamics/autoimmune disease)

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ABSTRACT **We have measured the quantities of naturally occurring autoantibodies in the serum of normal, unmanipulated individuals. These change over time following broad-band complex dynamical patterns that are similar in mouse and man. The patterns more likely reflect the network architecture of the natural antibody repertoire, regulating the activation and decay of individual clones. The temporal changes of both disease-specific and nonspecific autoantibodies are consistently modified in autoimmune individuals.**

Each individual's immune system, even if secluded from environmental antigens, contains a diverse repertoire of antibody species circulating in the blood (1–3). Such natural antibodies (NAb) constitute a selected sample of the available B-lymphocyte repertoire (4). Their frequent reactivity with autoantigens in normal individuals (1, 5) and the observations that autoantibodies of similar specificity and idiotype can be found in patients with autoimmune disease (6) have led to the suggestion (7, 8) that pathological autoimmunity may result from abnormalities in NAb production. Little is known, however, about the cellular and molecular mechanisms selecting, activating, and regulating the cells that secrete NAb. While the total immunoglobulin content of serum is kept constant, the concentration of the few NAb species previously studied was found to change over time in complex patterns (9).

We have now studied the temporal fluctuations of the serum titers of six NAbs for periods up to 3 months in normal and autoimmune mice and the fluctuations of autoantibody activity in the serum of three healthy subjects and two patients with Hashimoto thyroiditis. We report here that most antibodies in healthy individuals present a frequency power spectrum (FPS) that increases approximately as 1/frequency, often with a peak of activity around one to two cycles per month, and a variability in titers that increases with the observation window. The patterns of fluctuation detected in patients with autoimmune disease shared similarities with those observed in autoimmune mice and differed significantly from those observed in healthy individuals. These results are best explained by the hypothesis that natural antibodies and the cells that produce them form a dynamical network (10–12), which is altered in autoimmune disease (13).

## MATERIALS AND METHODS

**Mice.** BALB/c, C57BL/6, and B6.*lpr*/*lpr* mice were bred in our colonies at Pasteur Institute and University of Umeå. They were used between 3 and 6 months of age.

**Serum Antibody Determinations.** Serum antibody concentrations in normal BALB/c mice were determined as before (9). ELISA plates were coated (5 $\mu$g/ml) with each of four monoclonal anti-idiotypic antibodies, all reactive with BALB/c idiotypes: H81-98.21 ($\gamma$2a/$\kappa$) (14), identifying an Ia.7-crossreactive idiotype frequently isolated in newborn hybridomas (15); A11 ($\gamma$1/$\kappa$;) (9) directed to the BA.N 1:1.8 idiotype (16); (SP6)2–4 ($\gamma$1/$\kappa$) specific for the SP603, anti-trinitrophenyl hybridoma protein (17, 18); and F23.1 ($\gamma$2a/$\kappa$) (19), identifying TCRV$\beta$8-crossreactive idiotypes. Specific IgM binding was quantitated by a wide-spectrum serum titration with enzyme-labeled anti-$\mu$ chain antibodies (Southern Biotechnology Associates, Birmingham, AL) in parallel with the appropriate standards (idiotype-positive IgM hybridomas of known concentration); the slopes of the titrations were used to calculate the serum idiotype concentrations in microequivalents/ml to the standard. In each experiment, assays for a given idiotype were carried out with all individual serum samples at the same time on parallel plates, each containing the same standard titration, so that variations in serum idiotype concentrations cannot be ascribed to variability of the assay system. Similar conditions were used to determine serum concentrations of autoantibodies in C57BL/6 mice and their *lpr*/*lpr* congenics. Serum samples (a series of 11 bleedings at 3-day intervals) were assayed on ELISA plates coated with single-stranded DNA (100 $\mu$g/ml), prepared from salmon testes DNA (Sigma). Specific antibody concentrations were derived by comparison of the titration slopes with that of a standard monoclonal IgM anti-single-stranded DNA (ssDNA) antibody (20). Determinations of autoantibodies in human sera also were carried out in a similar manner, but only IgG antibodies were studied. ELISA plates were coated with purified human thyroglobulin and calf thymus DNA as described (21). IgG preparations with known autoantibody activity were added to each ELISA plate and served as references for autoantibody titrations.

**Treatment of the Data.** Analyses were performed by using the NAG Fortran Mark 11/15 Library routines; after the discrete Fourier transforms were computed, the first data point [constant or dc level] was always removed. Results from different animals have been normalized relative to their dc level for ease of comparison.

## RESULTS

**Dynamical Patterns of NAb in Normal Mice and Man.** The variations over time in the concentrations of four idiotypes, naturally expressed in BALB/c mice, were determined in

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Abbreviations: FPS, frequency power spectrum; NAb, natural antibody; SDL, standard deviation of the logarithm; ssDNA, single-stranded DNA.

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ELISA tests. Groups of mice were regularly bled, either every second day for 25 days or every week for 98 days, aiming at recording high frequencies (up to six cycles per month) and low frequencies (up to one-third cycle per month) of fluctuations, respectively. Examples of the raw data for two idiotypes over time are shown in Fig. 1. To analyze the fluctuations we computed their FPS, which provides a quantitative approach to the dynamical patterns present in the data. However, because of limitations in the frequency of bleedings, the number of data points gathered are insufficient for a full spectral analysis. Thus, we also computed the variability of concentration changes over the number of days considered in the measurement. A useful indicator in this respect is the standard deviation of the logarithms of the concentration (SDL), a measure originally introduced for the study of the variability of animal populations (22, 23).

Using these two quantitative criteria (FPS and SDL), we have analyzed the patterns of dynamical behavior of four clonotypes studied in eight individual mice. We have classified the patterns in three main groups (Table 1). In a first group (9 of 31 total cases analyzed), the amplitude of the spectra increased with lower frequencies, following approximately an inverse law $1/f^{\delta}$, with $\delta$ ranging between 0.8 and 1.8 (Fig. 2). We call these spectra "red-shifted," following Steele's metaphor for the time variations of physical variables at lower frequencies (24). In the second group (16 of 31 cases), one or more frequency peaks were markedly superimposed onto the broad band; we call these spectra "mixed." The peaks in mixed spectra varied from one idiotype to another, most commonly between one and two cycles per month. In virtually all cases for both red-shifted and mixed spectra (22 of 25 cases), the variability (SDL) increased over time, whereas it remained constant for relatively few cases (3 of 25). These observations considerably extend those previously reported on a different set of mice and antibodies (9). A third small group (6 of 31 total cases) showed FPSs that were less consistent, usually containing an important contribution near the maximal (Nyquist) frequency measured, thus showing evidence of possible artifacts. The variability in the latter group showed an equal tendency to increase or to remain constant. We refer to this third group as "flat." Representative examples of each of the three dynamical patterns of idiotype expression are shown in Fig. 2. Any particular idiotype followed different dynamical patterns in

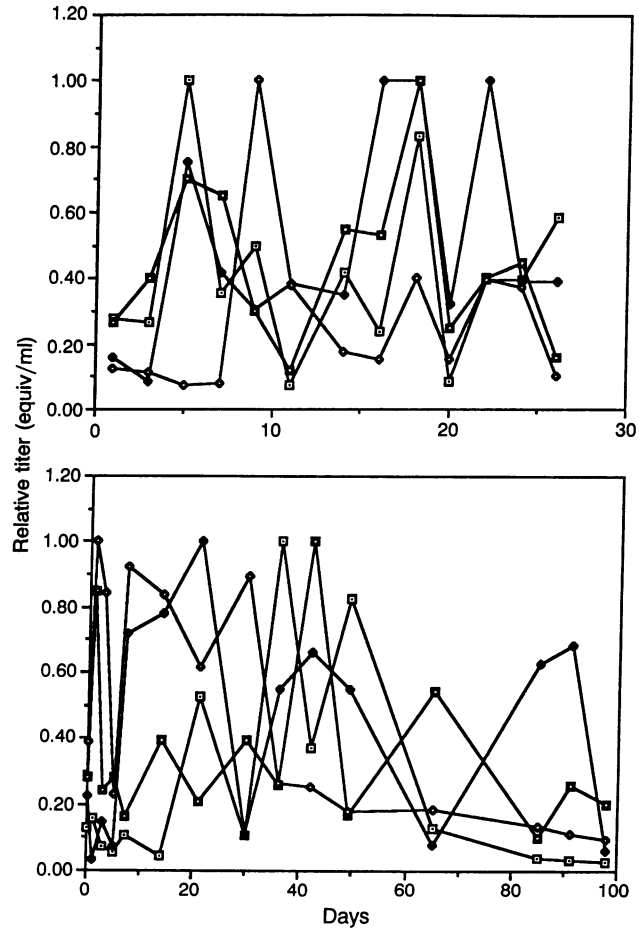


FIG. 1. Representative examples of the time course of expression of two naturally expressed idiotypes, H81 (*Upper*) and A11 (*Lower*), in four normal nonmanipulated BALB/c mice during a shorter (*Upper*) and a longer (*Lower*) time span. Titers were normalized between groups of animals for comparison.

Table 1. Classification of fluctuation patterns in NAb concentrations according to spectra and variability

| Spectra | | Variability | | | |
|---|---|---|---|---|---|
| | | Increasing SDL | | Constant SDL | |
| Class | *n* | Clonotype | Animal | Clonotype | Animal |
| Red-shifted | 9 | SP603 | II, III, VIII | A11 | 6 |
| | | F23.1 | 1, 2, VII, VIII | | (*n* = 1) |
| | | H81 | 2 | | |
| | | | (*n* = 8) | | |
| Mixed | 16 | SP603 | VIII | SP603 | 2, 6 |
| | | A11 | 1, 5, II, III, VII, VIII | | (*n* = 2) |
| | | F23.1 | II, III | | |
| | | H81 | 1, 5, III, VII, VIII | | |
| | | | (*n* = 14) | | |
| Flat | 6 | SP603 | 1, 5 | A11 | 2 |
| | | H81 | II | F23.1 | 5, 6 |
| | | | (*n* = 3) | | (*n* = 3) |
| Total *n* | 31 | | (25) | | (6) |

Arabic numerals refer to experimental animals ($n$ = 6) sampled at 0, 1, 3, 5, and 7 days and every following week up to day 98; they provide data for the lower frequencies. Roman numerals refer to animals ($n$ = 8) sampled at 0, 1, 3, and 5 days and every following 2 days up to day 25; they provide data for the higher frequencies. Numbers in parentheses are totals in each category. Only those time series containing reliable results for all days measured were included in this table (31 time series analyzed; >56 total). Spectra (FPS) are classified in reference to a 1/frequency law, and the variability (SDL) was computed in nested periods (see the legend to Fig. 2).

different mice; the extreme counterexample was idiotype H81-98.21, for which five of eight animals fell within the same category. Thus, dynamic patterns do not seem to be signatures of a particular molecular species but rather highly contextual phenomena that vary with the individual's history.

We also have calculated whether the different idiotypes coexisting within a given individual mouse show a high degree of cross-correlation. A four-way Pearson first-moment product correlation for all four antibodies in the same animal yielded few significant values: the average correlation was 0.2, with a maximum of 0.83 and a minimum of 0.02 (for 11 degrees of freedom, significance at the $P < 0.1$ level is reached only in two instances). We thus conclude that there is only a weak correlation of temporal patterns for any pair of idiotypes without a strong connectivity such as those studied here.

It is well known that normal human serum contains IgM autoantibodies. However, immunoglobulins of the IgG class, which react with autoantigens, are also readily detected by using purified IgG. Fig. 3 *Left* depicts the patterns of dynamical behavior of anti-thyroglobulin and anti-DNA activity in the IgG fraction of the serum from three healthy individuals studied weekly for a period of 3 months. The patterns were strikingly similar to those observed for NAb idiotypes in normal mice: four of six patterns showed a red-shifted law, and two exhibited mixed spectra; variability was not considered, since the time series was too incomplete.

**Altered Dynamics of Autoantibodies in Autoimmune Diseases of Man and Mouse.** The origins of autoimmune disease remain a subject of debate. The boundaries between normality and disease are difficult to establish simply by the presence or titers of autoantibodies, since a physiological autoimmunity does exist (4). Because autoimmune disease could reflect a network disarray (7, 8), we have contrasted the above results obtained in normal individuals with the dynamical behaviors measured in autoimmune conditions.

Analysis of the dynamical behavior of anti-thyroglobulin IgG activity in the serum of two patients with Hashimoto's thyroiditis revealed a distinct peak of oscillation around two cycles per month, indicating a major shift in the temporal pattern of behavior compared with healthy individuals (Fig. 3 *Right*). This could suggest alterations in network dynamics associated with disease, but "clonal" explanations of autoimmunity are still prevalent today. Thus, in contrast with NAbs in normal subjects, IgG autoantibodies in the serum of these patients exhibit a restricted epitope specificity on the thyroglobulin molecule (25, 26) and a disease-associated immunodominant crossreactive idiotype (21, 27).

Together with demonstrations of extensive somatic mutation in disease-associated autoantibodies (28, 29), this led to suggestions that disease-associated antibodies may represent a mutated species of NAbs. To examine whether the perturbation in the dynamic behavior of serum antibodies was specific of anti-thyroglobulin IgG, we also studied anti-DNA IgG in the patients with Hashimoto's disease. The spectra of anti-DNA activity were also clearly distinct from those in normal individuals, one patient showing also a dominant peak of activity, while the other showed a flat spectrum (Fig. 3 *Right*).

To evaluate the generality of those differences in autoantibody dynamics between normal and autoimmune individuals, we compared normal C57BL/6 animals with their congenics at the *lpr* locus, which develop an autoimmune syndrome with similarities to human systemic lupus erythematosus (30). This has also allowed us to analyze several murine NAb species on the basis of their autoantibody reactivities, as had been done in humans, rather than as idiotypes. The titers of anti-IgG1 and anti-IgG2a rheumatoid factors, and anti-DNA IgM and IgG autoantibodies were followed in five normal and

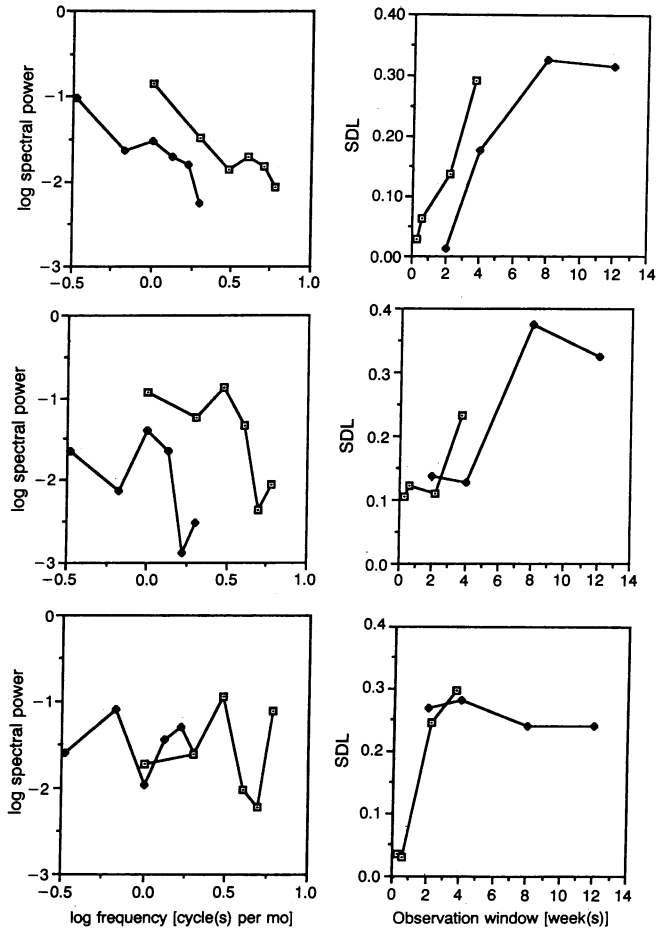


FIG. 2. Patterns of fluctuations for NAbs in normal BALB/c mice. The three groups of dynamical behavior described, red-shifted (*Top*), mixed (*Middle*), and flat (*Bottom*) (see Table 1), are displayed here through representative examples, covering the lower and higher frequencies of variation. (*Left*) FPS of the changes in antibody concentrations expressed in double-logarithmic scale. ◆, 8198-2 (*Top*), SP603-II (*Middle*), F231-5 (*Bottom*); ⊡, A11-III (*Top*), 8198-1 (*Middle*), A11-VIII (*Bottom*). (*Right*) Measure of the variability for each class, as the SDL of the antibody concentrations for nested windows of time 2, 4, 8, and 12 weeks (◆) and 2, 4, 16, and 26 days (⊡), respectively, depending on the sampling interval. Cases with no increase in variability were nearly all found for the "flat" class.

five *lpr* mice. For these specificities, control animals displayed predominantly (18 of 20 cases) red-shifted and mixed spectra. In contrast, diseased animals for all antibodies analyzed showed a marked tendency (17 of 20 cases) to display either flat spectra or a marked peak around two cycles per month. It should be noted that such differences are found even for IgM autoantibodies of those specificities, which are abundant in normal animals and, in contrast with IgG species, are not extensively mutated in diseased individuals (28, 29). Fig. 4 shows a typical example, namely the FPSs for IgM anti-ssDNA antibodies in C57BL/6 and B6.*lpr*/*lpr* mice, which are clearly separable, although they cannot be distinguished on the basis of their titers (data not shown).

## DISCUSSION

**Temporal Changes in NAb Concentrations and Network Architecture.** Variations in NAbs titers could simply represent ongoing immune responses to environmental antigens in unmanipulated animals and in healthy individuals. If such environmental impacts have some degree of consistency in time, that is, a degree of autocorrelation, their spectra would

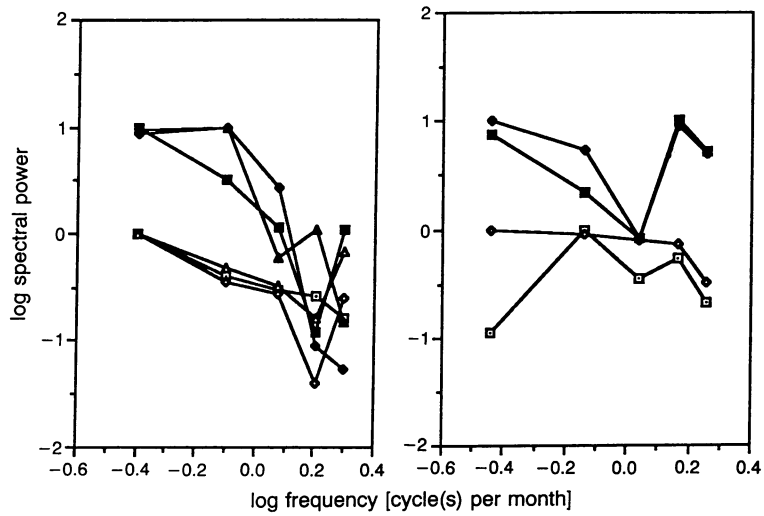


FIG. 3. Patterns of fluctuation for NAbs in serum of three normal donors (*Left*) and two Hashimoto's disease patients (*Right*). FPS are shown for the expression of anti-thyroglobulin (filled symbols) and anti-ssDNA (open symbols) IgG. Values between individuals have been normalized; anti-thyroglobulin results have been displaced upwards 1 logarithmic unit. Data from the same individual for both antibodies are matched by symbols.

also tend to be red-shifted. Thus, the longer we observe a given antibody population, the more likely it would be to find extreme excursions in quantity. However, natural antibody production does not seem to reflect the sum of multiple ongoing immune responses. It occurs at near control levels in "antigen-free" animals (2, 3), it does not involve extensive clonal amplifications (4), it is not accompanied by somatic mutation of antibody genes (3, 31), and natural antibody-producing clones are very resistant to external antigenic stimulation (32). Therefore, an alternative explanation to our observations is that natural antibodies result from self-recognition and stimulation, as suggested by their frequent reactivities with autoantigens and variable regions (1, 5, 33). In this case, the observed autocorrelation would result from interactions between the antibody populations themselves. The concentrations of each NAb species in serum vary with the respective rates of production and decay. The former is essentially a function of the number of high-rate immunoglobulin-secreting plasma cells, and the latter differs for antibody molecules that are free or associated in complexes. Thus, both parameters determining serum concentrations of NAbs and rates of production and decay are functions of connectivity of the same clonal components in these repertoires. It has been shown (9) that injection into mice of minute amounts of a given NAb induces marked transients on the levels of the same idiotype and of other connected species, demonstrating the network behavior of this repertoire.

Such network interdependence of free and cellular signals has been made explicit in a model (11, 34), simulations of which reproduce complex patterns of fluctuations similar to those described here. A phase-space analysis confirms the presence of cyclic and fractal attractors for reasonable ranges of parameters (35). For such a network architecture, perturbations can then be white noise, and an increase in variability still appears with red-shifted spectra because of its autoregressive properties (36, 37). A more complete set of data, where longer autocorrelograms can be computed, could further differentiate between the intrinsic and extrinsic origins of such variability (38). Furthermore, a $1/f^{\delta}$ ($\delta \approx 1.5$) spectrum also suggests (but does not establish) a fractal dimension or chaotic attractor for the time series (39). Such attractors are known in other biological systems (40), and multispecies population models can produce a chaotic regime with red-shifted spectra (41). For the immune network, we suggest that such chaotic regimes do not represent a dysfunctional condition but rather a reservoir of dynamic diversity from which the system can bifurcate to a distinctly oscillatory or steady state, since the three main spectral classes described above are obviously a continuum. In other words, we suggest that indeed the immune system operates "at the edge" of a chaotic attractor (42).

**Autoimmune Disease May Represent a NAb Network Disarray.** Our observations in autoimmune conditions are remarkably parallel in humans and mice: serum antibody concentrations in disease follow dynamical patterns that tend either to resemble random fluctuations or to exhibit a more marked rhythmicity than as yet detected in healthy individuals. This supports the view that autoimmune disease represents a basic perturbation in network regulation (7, 8, 13), affecting the expression of a wide range of organ-specific and

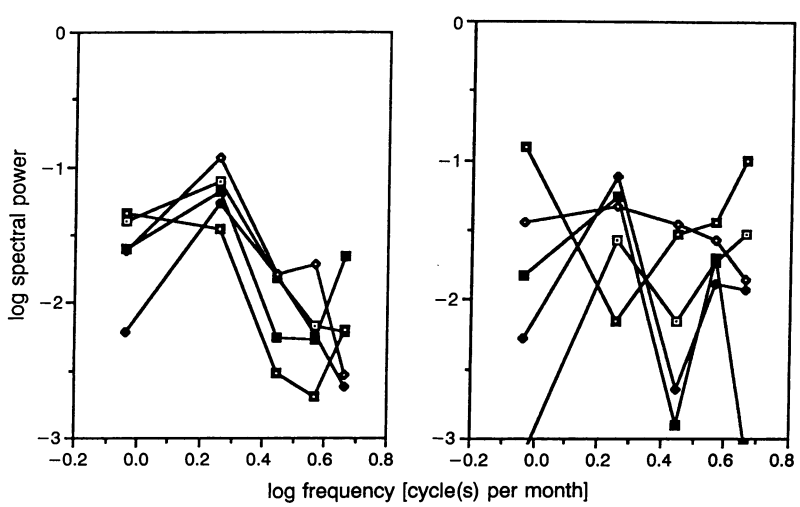


FIG. 4. Contrast in spectral patterns (FPS) for serum autoantibodies in normal (*Left*) and autoimmune (age- and sex-matched B6.*lpr/lpr*) (*Right*) C57BL/6 mice. Data for IgM anti-ssDNA autoantibodies are shown as a representative example of the four autoantibody species studied. In this case, all normal animals displayed a similar spectral behavior of the "mixed" type. In diseased mice, spectra differed markedly and were either "flat" or with peaks of marked dominance around 2 cycles per month.

disease-anchored autoantibodies as well as normal autoantibodies that are not directly associated with the pathology. In other words, autoimmune disease is associated with a general perturbation in the dynamics of the self-referential network, rather than with a clonally localized escape of organ-specific autoantibodies. This view is reinforced by the finding of similar dynamic alterations in two conditions generally classified in separate groups (organ-specific and generalized) of autoimmune diseases. Given that the dynamics of NAbs is determined by variable-region interactions of cell-bound and free immunoglobulins of each clone, this autoimmune behavior could be explained by a generalized defect in connectivity (43). This change in connectivity can be seen expressed in the power spectra described here, since autoimmune individuals show either a strong oscillatory or a close-to-random pattern ("flat"). These represent deviations to either extreme of the intermediate conditions of red-shifted or mixed spectra typical of NAbs. It remains to be established whether the putative connectivity defect is the cause or the consequence of the localized clonal alterations. The extreme oligoclonality of the disease-associated autoantibody repertoire noted by others (28, 29) would be more compatible with the latter possibility.

## CONCLUSION

One main conclusion from this study is that immunoglobulin populations that are "naturally" produced consistently manifest temporal changes in their circulating levels. This characteristic of NAbs has been ignored so far in immunology. The patterns of fluctuations were similar in all of the clonotypes examined in mice and for two autoantibody activities studied in man. A second important finding is that autoantibody dynamics were altered in the autoimmune patients and mice studied. From these observations, we conclude that temporal fluctuations in NAb concentrations are functionally significant. The importance of this conclusion for the understanding of pathological autoimmunity remains to be explored. These hypotheses suggest therapeutic approaches directed at reestablishing normal autoantibody dynamics, possibly through modifications in connectivity. This might be possible by using the very components of the normal immune system—i.e., the pool of NAbs, embodying normal connectivity levels. Such preparations of IgG obtained from normal donors have indeed already been shown of therapeutic value in some autoimmune diseases (44).

We have taken here an "ecological" view to the study of expression of specific antibodies present in the serum of unimmunized individuals. We feel that this is justified since they constitute, like animal species, a web of interrelated populations within the environment of the body and the larger milieu of the animal. Also, like in ecosystems, these populations of antibodies and plasma cells undergo a high rate of replacement by similar but not identical components (45). The variability of populations in both cases seems to follow the same trend (22, 23). Further, the matrix of connectivity in both immune networks (46) and ecosystems (47) appears to be constituted by compartments or blocks, which play an important role in their dynamics. Further homologies between these two complex biological networks should prove to be informative.

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