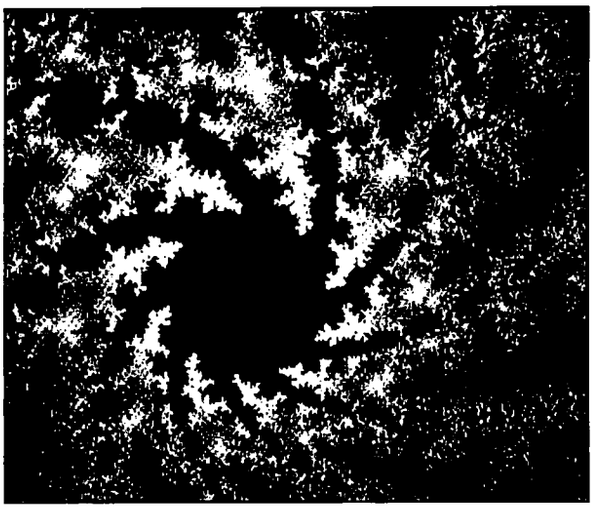
REVIEW

Networks: on a higher plane?

The 'resting' immune system is characterized by extensive lymphoid cell turnover; in the normal, unimmunized animal, the bulk of serum antibodies are reactive with self components. These and similar findings suggest that the immune system possesses an integrated repertoire of characteristics – network properties – that extend beyond simple cellular and molecular interactions. The first of the following two papers reviews the structure and characteristics of natural antibodies, the dynamics of the idiotypic network that they establish and the possible physiological significance of that network. It has been suggested that the network possesses higher order functions, which are widely accepted as properties of many complex biological and nonbiological systems, but these ideas have generally been treated with skepticism by immunologists. The second paper provides a thorough examination of these putative emergent properties and provides a stimulus for debate and further research.



Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'*

S. Avrameas

The immune system of normal unimmunized animals is characterized by the presence of B cells synthesizing and secreting mainly polyreactive, but also monoreactive, IgM and IgG natural antibodies that can react with a variety of self constituents. These antibodies, like the autoantibodies appearing in several immunopathological states, use the same genetic elements as the antibodies directed against environmental antigens, and seem to be encoded by unmutated germ-line genes. Accumulating evidence indicates that these natural autoantibodies exert various biological roles, both related and unrelated to the immune system. In this article, Stratis Avrameas proposes that natural autoantibodies, by interacting with the large number of self constituents present in an organism, establish an extensive dynamic network that contributes to the general homeostasis of the organism.

The observation that antibodies to toxins, bacteria and erythrocytes are present in the sera of normal unimmunized animals goes back to the beginning of immunology[1]. However, it was Landsteiner's demonstration, at the beginning of this century, that physiologically normal subjects with blood group A have anti-B agglutinins in their sera and vice versa that focused attention on this type of antibody. His work firmly established that antibodies that can react with a large variety of cellular and humoral constituents are present in normal sera. These antibodies have been termed 'normal' or 'natural' antibodies but the latter term is now used almost exclusively[1,2]. Studies performed over the last 20 years have shown that several natural antibodies found in normal sera from humans, rabbits, mice, rats and various fish species of different orders can react with self constituents (Table 1 and Refs 3–8).

General characteristics of natural autoantibodies

Human and mouse natural autoantibodies

To study the autoreactivity of human natural autoantibodies (NAA) in a systematic way, it is necessary to study their interactions with a large panel of antigens including highly conserved antigens, such as actin, tu-

*'Horror autotoxicus' was proposed by P. Ehrlich, at the beginning of this century, to express the notion that autoantibodies are prevented from reacting to self constituents, but misinterpretations have gradually altered this notion. At present 'horror autotoxicus' is used to denote that autoantibodies, which are considered to be formed only in pathological situations, are deleterious for the organism. 'Gnothi seauton' comes from the ancient Greek and means 'know yourself', found as 'know thyself' in early English translations. We have proposed that since the immune system can recognize self constituents, it should possess the 'know thyself' (see p. 150 of Ref. 4).

Table 1. Antibodies to evolutionarily conserved components found in normal human sera

Antibody reacting with	Isotype	Polyreactivity reported
Intracellular constituents		
Actin, tubulin, myosin, keratin, DNA, myoglobin, cytochrome *c*, collagen, myelin basic protein	IgM, IgG and IgA	Yes
Protamine	IgM	No
Membrane constituents		
β_2-microglobulin, spectrin, band-3 protein, secretory component	IgG	No
Circulating proteins		
Albumin, transferrin, IgG	IgM, IgG and IgA	Yes
Cytokines		
Interferons, interleukin 1α	IgG	No
Tumor necrosis factor	IgM and IgG	No
Hormones and related molecules		
Insulin	IgG	No
Thyroglobulin	IgM and IgG	Yes
Small molecules		
VIP, cholesterol, Gal α1-3Gal glycosidic epitope	IgG	No
Diphosphatidylglycerol, phosphatidic acid, phosphatidylserine	IgM, IgG and IgA	Yes

bulin, myosin and cytochrome *c*. Analysis of antibodies isolated by adsorption to these antigens shows that they are of the IgM, IgG and IgA isotypes and that most can react with more than two antigens, although monospecific antibodies reacting with a single antigen are also present. In studies using the same panel of antigens, human monoclonal IgM, IgG or IgA derived from patients with immunoproliferative diseases were found to have antibody specificities similar to those of the NAA. A few were specific to only one antigen, whereas most antibodies could recognize two or more antigens[3].

Analysis of human B-cell clones derived from peripheral blood lymphocytes, after human–mouse heterohybridization or by Epstein–Barr virus infection, has shown that up to 33% of the monoclonal immunoglobulins secreted can react with more than two self antigens[3,6,9]. $CD5^+$ B cells have been shown to be preferentially involved in the synthesis of these polyreactive natural autoantibodies (pNAA) but $CD5^-$ B cells also participate in their production[9].

Hybridoma clones derived from the spleens of normal adult nonimmunized BALB/c mice were examined for the secretion of NAA using a panel of self and nonself antigens. Clones that secreted IgM antibodies and reacted with only one antigen were rarely obtained, while 2–20% of all clones, depending upon the study, secreted IgM antibodies exhibiting broad reactivities with various self and nonself antigens[3,6]. Several observations indicate that $Ly\text{-}1^+$ B cells, the mouse counterpart of human $CD5^+$ B cells, are involved in the production of some of the NAA but again there are also opposing results[10]. Recent experiments suggest that most murine B cells that produce these autoantibodies are naturally-activated blast cells[11].

One possible explanation for the NAA found in adult mice is that they correspond to antibodies generated after infection of mice by environmental pathogenic agents that crossreact with self antigens. However, subsequent studies have demonstrated that B cells synthesizing pNAA are also present in newborn, nude, germ-free and antigen-free mice[12,13]. Most of these NAA exhibit similar broad reactivities but, when evaluated against a panel of antigens, they show distinct relative levels of binding to the various antigens. Thus each pNAA possesses a unique pattern of fine specificities[14].

Results from studies using rabbit antisera that recognize the idiotopes of mouse IgM pNAA show a high incidence of crossreactive idiotopes among pNAA from individual mice of different strains. Furthermore, a significant number of human and rat monoclonal polyreactive (but not monospecific) antibodies expressed these crossreactive mouse idiotopes[6]. Indeed, a high proportion of pNAA bear idiotopes in common with antibodies of differing specificities[15].

Origin and structural polyreactivity of NAA

Recent studies have shown that mouse[16] and human[17] NAA are encoded by germ-line genes without, or with very few, mutations. Furthermore, it has been shown that NAA use the same genetic elements as the antibodies directed against foreign nonself antigens while, in general, there is no pronounced predominance among several subgroups in the V_H and V_K gene families[9,10,18,19].

Murine pNAA, encoded by unmutated germ-line genes, are characterized by the presence of many arginine and lysine residues in their hypervariable regions. This raises the question of whether or not induced antibodies of the post-immune repertoire that possess this type of

germ-line configuration can also react with self antigens. Studies using murine monoclonal IgG1 anti-GAT (Glu:Ala:Tyr; 60:30:10 random synthetic terpolymer) antibodies possessing such a configuration have shown that these antibodies do react with a range of autoantigens. In contrast, monoclonal IgG1 anti-alprenolol antibodies, encoded by V_H and V_K genes very similar to the anti-GAT antibody genes but bearing mutations leading to fewer positive charges, did not express autoantibody reactivity[20]. These, and results obtained with anti-DNA antibodies[21], indicate that polar amino acids contribute to the reaction of pNAA with the various, apparently antigenically unrelated, antigens. Kabat *et al.*[22] have proposed that an "... apparent association of similarities and differences in charge distribution can be responsible for immunological crossreactions among what have been generally considered diverse and structurally unrelated substances". Similarly, the polyreactivity of NAA could be due to a particular distribution, on their variable portion of the antigen-binding fragment (Fab) surface, of charged amino acids allowing the formation of salt bridges with complementary charged groups expressed on the surface of the antigen[16]. It is possible that this type of interaction is followed by conformational changes that lead to a more intimate contact between the antibody and the antigen[23].

IgG and IgM NAA

One of the main, misplaced, objections often advanced as a challenge to the relevance of NAA is that they are almost exclusively IgM. It has been shown without ambiguity that, in humans, IgG NAA displaying low (5×10^{-3} M) to very high (5×10^{-11} M) affinity for various self antigens are present (Table 1)[9,24,25]. Initially, it seemed that almost all murine NAA belonged to the IgM isotype. However, since all the relevant studies were based on the hybridization technology, and fusion with splenocytes from unimmunized animals almost exclusively yields IgM hybrids, this was an expected but biased result. When analysis by immunoblotting of sera from various strains of normal mice for IgG antibody reactivity using whole homogenates of the principal mouse organs as the antigen sources was performed, IgG autoreactivity was barely detected but, once separated from IgM, it was found and was active against more than 220 different autoantigens. Enzymes were the most frequently recognized antigens but intracellular constituents, such as keratin, laminin and actin, were also bound. These results strongly suggest that IgG autoantibodies recognize common structures present on phylogenetically conserved proteins. Furthermore, a significant proportion of these IgG autoantibodies could react with apparently cryptic epitopes of major histocompatibility complex (MHC) class I and class II molecules and T-cell receptor antigens. In addition, affinity chromatography has shown that at least 20% of normal BALB/c serum IgG corresponds to pNAA (A. Berneman *et al.*, unpublished).

These results imply not only that IgG autoantibody is present in normal mouse serum but also that it constitutes most of the natural autoantibodies. The IgG autoreactivity found in whole serum is usually low. In this context, it is interesting to note that an IgG antibody possessing proteolytic activity for vasoactive intestinal peptide (VIP) was active only if it was isolated from whole serum[26]. These and additional results suggest that IgG autoreactivity may be present, but is partially or completely masked, in normal sera.

In the case of mouse serum, the inhibition of IgG autoreactivity reported above was due to the presence of polyreactive IgM. To analyse the mechanism of this inhibition, experiments with $F(ab')_2$ fragments were performed. IgM inhibited the binding of IgG $F(ab')_2$ fragments to various immobilized autoantigens in a dose-dependent manner and reacted with immobilized IgG $F(ab')_2$. The IgM antibody isolated on IgG $F(ab')_2$ immunoadsorbent, compared with the initial IgM preparation, was less active against the different autoantigens but much more inhibitory for IgG binding to the autoantigens[27].

These findings suggest that a polyreactive IgM population has sufficient affinity for IgG NAA to exert an important regulatory role. Similarly, treatment of effector cells with normal human IgM induced strong, dose-dependent inhibition of natural killer cell activity[28]. These results contradict the frequently made claims that IgM NAA are of very low affinity and, therefore, cannot play significant biological roles. Moreover, in the few cases in which measurement of natural polyreactive IgM binding was attempted, these antibodies had functional affinities or avidities for conserved self macromolecules ranging from 10^{-5} to 10^{-10} M (Ref. 14). These values are of the same order of magnitude as those found with either monoclonal or polyclonal monospecific IgG antibodies directed against the same self macromolecules and obtained after experimental immunization. Thus IgM NAA apparently do not conform to the normally accepted correlations between low affinity and polyspecificity, and high affinity and monospecificity.

The functional affinity of polyreactive IgM for a single epitope, for example the hapten trinitrophenol (TNP) presented as repeated units of a structure, is high, but the affinity for the same free single epitope is low[14]. In other words, the ability of multivalent IgM to bind antigen and influence biological activity depends on the local density of the epitope expressed on a macromolecule, cell surface or tissue.

The biological significance of NAA

Immunoregulatory roles of NAA

Several different roles have been proposed for NAA. First, because they establish among them, even early in ontogeny, an idiotypic network[11,15], NAA seem to play important roles in the regulation of the immune system. Indeed, it was shown that injection of idiotypically interconnected pNAA into mice resulted in a reduction of the expression of the corresponding idiotypes and either in an enhancement or a suppression of the immune responses related to these idiotypes[29]. Similarly, Sundblad *et al.*[30] have shown that NAA can function in anti-idiotypic suppression. Treatment of newborn mice with either low (100 ng) or high (100 μg) doses of an NAA, recognizing the idiotopes of an NAA with anti-acetylcholine receptor activity, completely suppresses the autoantibody response when treated adult mice are immunized with the acetylcholine receptor. The inhibition

of IgG autoreactivity by IgM, noted in mouse serum and reported above, is also due to an idiotypic interaction. In some pathological situations, modifications of this idiotypic network may lead to the expression and/or expansion of autoreactive IgG-producing clones[27].

Second, NAA may provide a platform for antigen-specific immune responses. It has been proposed that B cells carrying IgM pNAA as receptors can, by interacting with a foreign antigen that is antigenically similar but not identical to an internal self antigen, be induced to undergo proliferation and mutation. Under the selective pressure of the antigen, this would give rise to cells producing antibodies that are strictly specific to epitopes of the foreign antigen[3,6,31,32]. Several lines of evidence seem to support this hypothesis: after immunization with antigen or infection with pathogenic agents, monoclonal IgG antibodies reactive with self components and with the immunizing antigen were found[31,33]. Crossreactive idiotopes were shared by natural and by induced polyclonal and monoclonal antibodies reported to be specific to different antigens, the latter encoded by the same or mutated forms of the germ-line gene that encodes pNAA[16,32].

Third, Cohen and Cooke[34] have postulated that cells carrying self structures resembling epitopes of pathogenic agents will be protected from an autoimmune attack by the binding of NAA.

Nonspecific physiological roles of NAA

It is probable that the closely spaced epitopes found in various intracellular macromolecules allow the natural IgM, and possibly IgG, autoantibodies to bind to catabolic and metabolic substances and thus to contribute, as P. Grabar[35] suggested more than 25 years ago, to their clearance from the organism. For example, the binding of autoantibodies to extracellular keratin filaments increases the removal of insoluble keratin filaments after keratinocyte death[36]. Similarly, by binding to senescent erythrocytes, natural anti-band-3 antibodies facilitate red blood cell phagocytosis by adherent monocytes[37].

Recently, it was shown that IgM from normal human serum enhances monocyte phagocytosis of parasites[38] and it is possible that a similar mechanism could also function in the resistance to tumors of animals with high levels of natural antibodies[39]. Thus, natural antibodies can be considered as a first line of defense against external aggressions that has been phylogenetically conserved. In fact, in trout protected from bacterial infection by previous injection of bacterial extracts, the kinetics of serum anti-bacterial antibodies strictly followed those of the NAA[40]. In fish where one principal class of polymeric immunoglobulins exists, without the possibility of switching to another class, pNAA probably constitute the unique defense mechanism.

Since almost all natural antibodies can react with internal self and external nonself antigens, they may modulate infection by interacting either with the host cell, the pathogenic agent or both. Recently, murine monoclonal IgM pNAA were found to regulate interferon action and, consequently, viral infection, by binding to the cell surface rather than by directly interacting with interferon[41]. Similarly, polyclonal natural anti-TNP autoantibodies isolated from the sera of normal rainbow trout protected trout fibroblasts against viral infection *in vitro*. The protective activity was manifested when anti-TNP antibodies were added after viral infection and, apparently, was not due to their interaction with viruses or cell surface components alone; the presence of both was needed[42]. It has been suggested that the virus-modified cell surface expresses new, or normally cryptic, epitopes that, in conjunction with the virus, are better recognized by the NAA thus enabling them to exert their protective function[42].

It can be concluded that under normal circumstances NAA participate in the nonspecific defense of the organism, but it is evident that in certain pathological situations the opposite effect occurs.

NAA and pathological states

The data currently available indicate that autoantibodies present in autoimmune states, and especially in systemic lupus erythematosus (SLE), have close relationships with NAA. Thus, in lupus mice, the anti-DNA antibodies are encoded by genes similar to those coding for antibodies to foreign antigens; these genes share extensive homologies with germ-line *V* genes, while there is no restriction in their use[5,10,18,19]. Moreover, in lupus-prone (NZB × NZW)F_1 mice, polyreactive IgM and IgG anti-DNA antibodies carrying the common idiotypes of murine IgM pNAA are found in the sera in the circulating immune complexes and can be eluted from the kidney deposits[43]. Treatment of MRL–lpr/lpr newborn mice with polyclonal antibodies recognizing the common idiotopes of pNAA significantly decreases serum anti-DNA antibody titers in the adult[44]. Similarly, in lupus patients, anti-DNA autoantibodies are polyreactive and bear idiotopes similar to those on anti-DNA autoantibodies present in normal humans[5,10]. The sera of lupus patients with nephritis contain considerably increased amounts of pNAA, which contribute to the formation of circulating immune complexes and can be eluted from renal biopsies[45].

The NAA present in normal human sera have been compared with those from patients suffering from various autoimmune and nonautoimmune diseases: IgA nephropathy, autoimmune hemolytic anemia, HIV infection, multiple sclerosis, chronic hepatitis, leprosy, schizophrenia, diabetes mellitus, Alzheimer's disease, Chagas disease, breast cancer and lupus, using the same panel of conserved autoantigens that were applied in previous studies on NAA. In almost all cases, significant modifications in the titers of NAA were noted (Refs 4,6,45,46 and authors' unpublished observations). Similarly, after parasite infection of mice, serum NAA levels increased before the appearance of antibodies to parasite antigens[47]. Two nonmutually-exclusive hypotheses can be advanced to explain these observations. (1) The destruction of tissues and the increased release of self constituents occurring in certain pathological situations disturbs the homeostasis established among NAA and self constituents, and leads to modified NAA titers. It is obvious that damage to different tissues, composed of given constituents, will be reflected by a more or less distinct change of serum NAA titers. In this context, it is interesting to note the frequent increase in antibodies directed against various enzymes in the sera of patients suffering

from different, often unrelated, diseases. Enzymes are present in all tissues and they seem to be the preferential target of IgG NAA. (2) Pathogenic agents carrying antigens similar or identical to self autoantigens induce an increase in NAA.

From the above discussion, it can be concluded that NAA titers are significantly modified in a variety of pathological situations that are not necessarily linked to the immune system. NAA, by forming immune complexes that can be deposited in various tissues, indirectly participate in the pathology of systemic immune diseases and, possibly, of other diseases. Whether NAA participate in a more direct manner in the establishment of pathological processes, or whether autoantibodies with known pathological properties originate from NAA remains to be established, although some evidence supporting these possibilities is beginning to emerge.

Conclusions and hypotheses

Taken together, the findings presented here demonstrate the ubiquity of NAA, indicate that they play important biological roles and suggest that the genes that code for these antibodies are maintained because a positive selective pressure is exerted by the continuous presence of the autoantigens. Furthermore, it can be concluded that, in contrast to the post-immune repertoire, the pre-immune, or natural immune, repertoire is characterized by recognition of self constituents.

NAA, by interacting with the large number of self constituents present in the organism, establish an extensive network. Through this network, which involves multiple interactions of NAA with molecules and cells involved in the functioning of the various biological systems of the organism, the immune system may contribute to the general homeostasis of the organism.

In addition to this general homeostasis, NAA, by interacting among themselves and with the various molecules and cells that constitute the immune system, actively participate in its functioning. Under normal physiological conditions, this natural immune homeostasis is moderately disrupted by environmental nonself antigens and altered self antigens that are structurally similar, but not identical, to internal self antigens. In these cases, a limited number of B cells carrying IgM or IgG polyreactive NAA as receptors will be stimulated and will proliferate to give rise to a small number of cells synthesizing antibodies that gradually become more and more specific to the epitopes of the nonself antigen. The clones that synthesize these antibodies will escape from the autoregulation exerted by the NAA. Thus, to respond to a nonself environmental antigenic stimulus, the immune system of an adult animal can choose between (1) polyreactive, autoreactive clones encoded by unmutated germ-line genes and corresponding to a primary immune response and (2) monospecific clones, most often encoded by mutated germ-line genes and corresponding to a secondary, clonally-expanded immune response. During experimental immunization of animals with foreign antigens or during infection by pathogenic agents or in certain immunopathological states, such normally occurring processes will be focused in a certain direction, while antibody production rates will increase considerably.

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Second generation immune networks

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Network approaches have had little impact on immunology because they have addressed the wrong questions. They have concentrated on the regulation of clonal immune responses rather than on the supraclonal properties of the immune system that emerge from its network organization, such as natural tolerance and memory. Theoretical advances, observations in unimmunized mice and humans, and the success of novel therapeutics in autoimmune diseases have recently promoted a new burst of research on the structure, temporal dynamics and metadynamical plasticity of immune networks.

It is well known that Jerne suggested, more than 15 years ago[1], that immunoglobulin idiotypes are organized as a network of complementary shapes. Yet most of today's immunologists have experienced little or no change in their views and practices as a result of network theory. This is because network research has concentrated on local idiotypes and clonal properties, and neither the theory nor the experiments have addressed the central questions that were left unsolved by previous approaches. Some elementary network ideas have been uncritically 'grafted' onto mechanisms that are proper to clonal selection theory, but the two theoretical frameworks occupy distinct domains and address different levels of description of the immune system.

In our view, conventional immune responses and much of their regulation are satisfactorily explained by clonal selection principles (with adaptations such as somatic mutation[2] and 'induced fit'[3,4]), as shown by the fact that they can be reproduced by isolated clones *in vitro*; clonal selection also contributes the rational basis for anti-infectious protection. Instead, we suggest that the core operation of immune networks has little to do with immune responses, but is fundamental to the understanding of questions that were not solved by the clonal selection theory – internal lymphocyte activities and natural antibody production in unimmunized animals, pre-immune repertoire selection, tolerance and self–nonself discrimination, memory and the evolution of immune systems. Over the last few years, a new burst of interest, ideas and experiments has begun to make some progress in these directions: they can be called second generation immune networks. This re-awakening has come from three sources: theoretical advances, observations in unimmunized mice and humans, and the therapeutic success of novel forms of treatment in autoimmune diseases.

Emergent properties: the uniqueness of network processes

Emergent properties are the core of the explosive field that has been designated alternatively as network dynamics, nonlinear networks or complex systems. They have been found in many fields of study – vortices and lasers, chemical oscillations, statistical physics, genetic networks, developmental patterns, neural and immune networks, population genetics, ecology and geophysics[5]. Immunologists often have difficulties with a network approach because they fail to see it from this wider scientific perspective. What all of these themes have in common are the processes that occur when simple agents are dynamically connected to each other in dense ways. Each such component agent operates strictly on the basis of its local environment but, because of the system's network constitution, there is a global co-operation which spontaneously emerges as the states of all participating components become mutually satisfactory. In such a system, there is no need for a central processing unit to guide the entire operation, and the external impacts do not turn the system's axle. External stimuli modify only the local environment of individual components (which will behave according to conventional stimulus–response patterns), but the system's operation and properties will