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A High Dose of an Idiotype Generates High Levels of Ab2s

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Abstract. *Background: Network perturbations have been reported in autoimmune processes which could cause polyclonal cell activation. Materials and Methods: Hyper-immunization was achieved by the injection of 3 mg of human monoclonal IgM DJ into BALB/c mice. Indirect ELISA was performed to detect induced antibody specificities. Competitive ELISAs were carried out on sera to detect the idiotypes expressed in first (Ab1) and second (Ab2) antibodies in sequence. Results: In the sera of hyper-immunized mice, the concentrations of Ab1 and Ab2 were respectively five and ten times higher than in the sera of normo-immunized mice. Polyclonal lymphocyte activation was demonstrated by an increase in antibodies specific for antigens (cytochrome C) not related to the immunizing antigen, elevated levels of IgM antibodies specific for the immunogen and elevated levels of Ab2s of the IgM isotype. Conclusion: These findings are indicative of antigen-dependent network perturbation being a causative factor in polyclonal lymphocyte activation.*

It is the established view that two organizations coexist within the immune system, the clonal (1) organization and the network (2-4) organization. However, the dividing line between these two is by no means clearly defined or easy to draw. Historically, studies of idiotypes and anti-idiotypic antibodies have been criticized for propounding a clonal theory of immune response (5-7) which allows for no points of commonality with the network theory of immune response (defined as the outcome of interactions between various idiotypes). Whilst monoclonality is the main characteristic of the components of the cellular and humoral immune systems, network studies should include the quantitative measurements of the mutual interactions of its monoclonal counterparts (8).

Because of the variability of the V regions of Abs or T-cell receptors (TCRs), there is still no experimental data to confirm Jerne's hypothesis of a network-regulated immune system. However, the introduction of mathematical modeling (9-12) to network research should facilitate the design of meaningful network experiments. According to the Weisbuch-Boer-Pelerson model (13), immunization-induced perturbations of the immune system may give rise to an immune response, either as a result of percolation (filtration of an idiotypic signal through the network) (14), or through a localized idiotypic memory (15). According to this model, percolation (16) implies polyclonal B-cell activation, or the sequential stimulation of a potentially unlimited number of clones; the network, thus, functions at a higher level because of the activation of multiple idiotypic clonalities. Models of diseases generated by immunization with idiotypes (17, 18) indicated that the intense activation of an idiotypic network (either by polyclonal cell activation or by molecular mimicry) may result in the deregulation of the immune system and in the production of various pathogenic auto-antibodies.

Polyclonal B-cell activation usually precedes an antigen-specific response (19), so that the majority of antibodies specific for auto-antigens detected after polyclonal B-cell activation have low affinities. Lymphocytes with receptors of higher affinities do not respond to polyclonal activation, because they have already been selected by exposure to self-antigen (20) during ontogenesis. Polyclonal lymphocyte activation may be consequential to the loss of appropriate CD4+ T-cell control or to direct stimulation by microbial agents (21). Polyclonal T-cell activation generates effector T-cells capable of providing protection against a range of antigenic variants (viruses), but may simultaneously induce the switching of non-autoreactive T-cells to differentiated T-cell progeny, specific for self-derived, low affinity ligands (22). This T-cell cross-reactivity forms the basis of the molecular mimicry

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hypothesis, according to which T-cells that cross-react with self-antigens initiate and/or perpetuate auto-immune diseases (23).

Here, we report on our experiments to determine whether intense activation of the immune system resulting from hyper-immunization will produce polyclonal cell activation with increased levels of Ab1 and Ab2. Earlier research reported that, during the immune response, expansion of the antigen-activated idiotypic clone was associated with reciprocal expansion (24) or diminution of anti-idiotypic clones (25, 26). The detection of differing Ab1s and Ab2s was facilitated by the use of two monoclonal antibodies interacting with high affinity (10^{-9} M) through their idiotypic regions: the mouse monoclonal Ab of IgG class Y7, and the human monoclonal Ab IgM λ, from patient DJ diagnosed with Waldenstrom macroglobulinemia. The Y7 antibody, (specific for the idiotype on the human IgM DJ antibody), used as the immunogen, was obtained by the BALB/c method (27). Ab1 was detected by using the IgM DJ antibody (28). For detection of the IgM DJ-like Ab2, the mouse monoclonal anti-idiotypic antibody Y7 was used. Polyclonal cell activation was detected from the rise in titer of IgM-class antibodies specific for certain auto-antigens, as well as the immunizing antigen IgM DJ. The presence of IgG anti-ssDNA antibodies of high affinities indicated that, in addition to polyclonal or bystander activation, affinity maturation also occurred, signaling that the specific immune response was under the influence of idiotypic interactions.

Materials and Methods

Purification of immunogen and monoclonal antibody specific for the immunogen's idiotype. Human monoclonal IgM DJ (from the sera of patient DJ diagnosed with Waldenstrom macroglobulinemia) was purified by euglobulin precipitation and, subsequently, by gel chromatography. A Superose-6 column was used and chromatography was performed on the Pharmacia FPLC system (Uppsala, Sweden).

The monoclonal antibody of the IgG1 κ (kappa) isotype, designated as Y7 (27), was used as an anti-idiotypic antibody against the IgM DJ immunogen. It was purified from ascites by affinity chromatography on an IgM DJ column.

Immunization protocol. BALB/c mice, approximately 7 weeks old, were used for immunization. For so-called hyper-immunization, 1 mg of IgM DJ was injected subcutaneously into the pad of a hind foot and to the base of the tail. The antigen was given in FCA (Freund's complete adjuvant) and then in FIA (Freund's incomplete adjuvant) and finally in PBS (phosphate-buffered saline solution) at weekly intervals. Sera samples collected from 20 mice at one week subsequent to the final injection were pooled, complement-depleted at 56˚C for 30 minutes and used for further study. For so-called normo-immunization (controls), the mice were immunized according to the same protocol at 10 μg per dose. The sera from these animals was processed in the same way as the sera from the hyper-immunized mice.

ELISA for the detection of the elicited immune response. Activated polyvinyl chloride (PVC) microtiter wells were coated with 50 μl of a 20 μg/ml solution of human monoclonal IgM DJ, or its $F(ab')_2$ fragments, by overnight incubation at 4˚C. After saturation with 0.1% bovine serum albumin (BSA) and extensive washing in PBS 0.05% Tween 20, different dilutions of mouse sera were added and the plates further incubated for 1 hour. Binding of mouse immunoglobulins from the sera was detected using rabbit anti-mouse IgG or rabbit anti-mouse IgM antibodies, conjugated to horseradish peroxidase (Serolab, Lausanne, Switzerland). The enzyme reaction was developed for 20 minutes using ortho-phenylene diamine (OPD) as the substrate and absorbtion was measured at an optical density (OD) of 492 nm, using a Multiscan Ascent ELISA reader (Labsystems, Helsinki, Finland).

ELISA for detection of anti-cytochrome C and anti-ssDNA antibodies. Cytochrome C, at a concentration of 20 μg/ml (50 μl per well) in PBS solution, was incubated overnight at 4˚C to cover the plates. A single-strand DNA (ssDNA) solution of the same concentration was adhered in the same way to poly-L-lysine (PLL)-precoated plates and subsequently covalently coupled to PLL using 0.5% glutaraldehyde for 15 minutes. The glutaraldehyde was blocked using 0.1 M glycine for 2 hours at 37˚C. In both types of ELISA, blocking was performed using 0.5% BSA in PBS. The detection of mouse antibodies bound to the antigens was performed using rabbit horseradish peroxidase-conjugated antibodies.

Examination of the stability of ssDNA-antibody complexes by their dissociation in NaCl gradient. The relative affinity of the antibodies for ssDNA was determined using a 0.15-0.50 M gradient of NaCl (26). Mouse sera, diluted 1:200, were added to ssDNA-coated plates and, after incubation, the plates were washed with 0.15, 0.30, 0.40 or 0.50 M NaCl in 10 mM Tris-HCl pH 7.2 (TBS) four times and then again three times with TBS. The residual bound antibody was measured by conventional ELISA. Wells washed with 0.15 M NaCl, buffered with 10 mM Tris-HCl pH 7.2, were considered to express 100% binding (maximum amount of bindable antibody).

Competitive ELISA: detection of Ab1s and Ab2s. Standard binding and inhibition curves to calculate the concentrations of the Ab1 and Ab2 antibodies in the sera of hyper-immunized and normo-immunized mice were obtained in three ways.

In the first type of ELISA, 20 μg/ml of IgM DJ immunogen was bound to the plastic overnight (as described elsewhere). Prior to application to the plates, 100 ng of Y7 biotinylated anti-idiotypic antibody was incubated for 1 hour at room temperature with different concentrations of either IgM DJ or Y7 antibodies; this was then incubated on the plate for 1 hour. The competing amounts of Y7 antibodies or IgM DJ antibodies (to block biotinylated Y7 antibody-binding for the antigen immobilized on the plate) were both in the range of 40 - 48×10^3 ng. The standard curve obtained using IgM as a competitor was used to calculate the concentrations of Ab2 antibodies in the sera of immunized mice. In the second type of ELISA, 20 μg/ml of Y7 antibody was bound to the microwell plates. One hundred ng of biotinylated IgM DJ antibodies were incubated in solution with differing amounts (from 50 - 48×10^3 ng) of IgM DJ antibodies or Y7 antibody prior to application to the plates. The inhibition curve, thus, obtained using Y7 as a competitor was later used to calculate the concentrations of Ab1 (immunogen-specific) antibodies in the sera of immunized animals. The sera concentrations of the Ab1 and Ab2 antibodies were calculated from the inhibition curves.

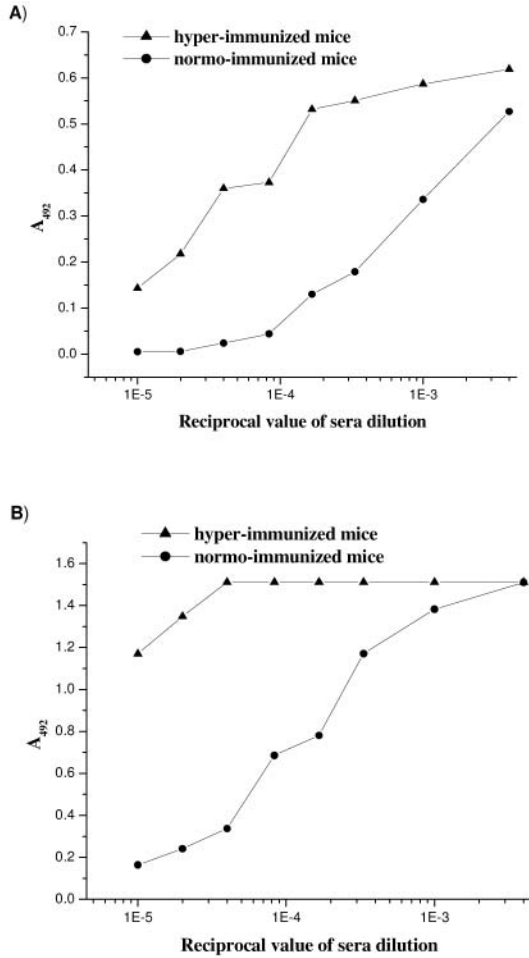

Figure 1. *Titers of the IgM (A) and IgG (B) immunogen-specific antibodies in the sera of hyper- and normo-immunized mice are represented. The x-axis indicates the sera dilution reciprocals. Twenty times higher levels of IgG and IgM antibodies were detected in hyper-immunized sera.*

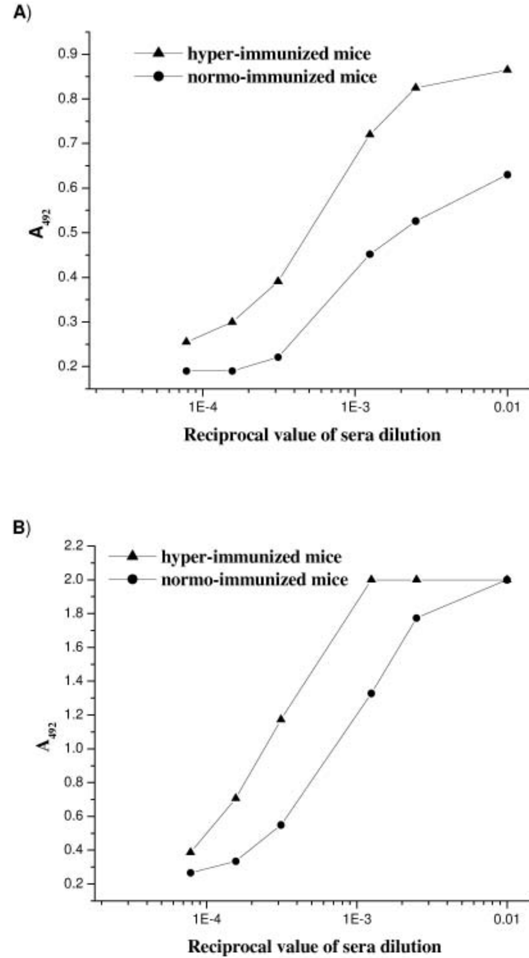

Figure 2. *IgM (A) and IgG (B) antibodies specific for cytochrome C, in the sera of immunized mice.*

Finally, the same ELISAs were performed again; but, instead of non-labelled competitor antibodies (IgM DJ or Y7), serial dilutions of the sera (in triplicates) of immunized animals were used. An inhibition percentage was calculated from the relationship between binding in the presence of an inhibitor and binding without an inhibitor.

Results

Immunogen-specific antibodies in hyper-immunized sera: IgG and IgM antibodies. High levels of antibodies of IgG and IgM classes, specific for IgM DJ, were detected in the sera of hyper-immunized animals (Figure 1B and 1A, respectively). The concentrations of IgG antibodies in the sera from hyper-immunized mice (Figure 1B) were approximately 20 times higher than the concentrations in the sera of normo-immunized mice; the levels of antibodies of the IgM isotype (Figure 1A) were approximately ten times higher. The presence, even three weeks after hyper-immunization, of very high levels of antibodies of the IgM class specific for the immunogen (which can be explained by polyclonal stimulation) indicated that no affinity maturation had occurred.

Detection of antibodies of IgM and IgG classes specific for antigens unrelated to immunogen. As neither IgM DJ nor MoAb Y7 bind cytochrome C, antibodies of IgG and IgM isotype specific for cytochrome C, which were detected in the sera of hyper-immunized mice (Figure 2), indicated polyclonal B-lymphocyte activation as their source.

Antibodies of IgM and IgG classes with immunogen-like paratopic specificities. The concentrations of IgG and IgM anti-ssDNA antibodies detected in normo-immunized sera were of approximately the same magnitude as in normal mouse sera. Conversely, in titers of IgG and IgM anti-ssDNA, the antibody (Figure 3B and A, respectively) levels in hyper-immunized

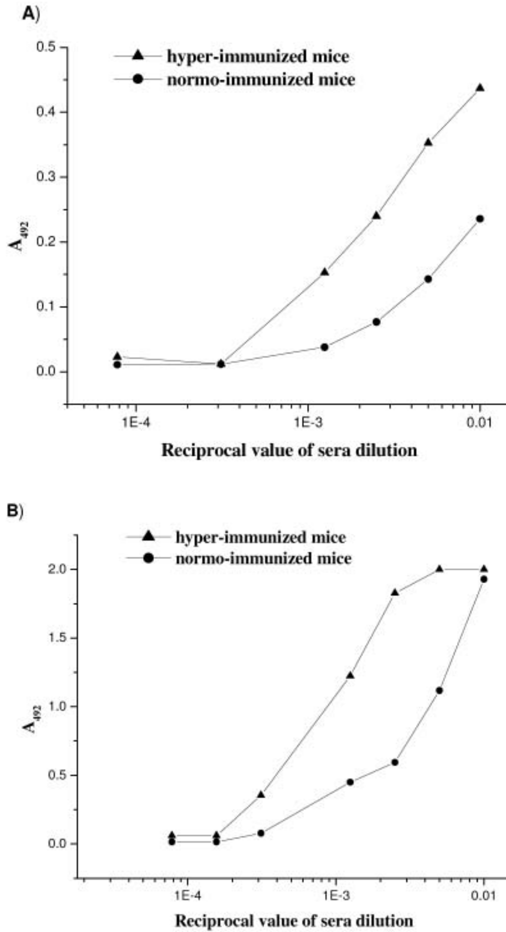

Figure 3. *IgM (A) and IgG (B) Abs specific for ssDNA (immunogen-like) in the sera of mice normo- and hyper-immunized with IgM DJ.*

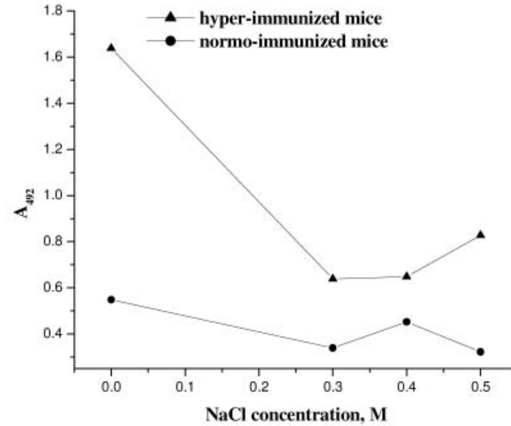

Figure 4. *Binding of IgG anti-ssDNA from the sera of mice normo- and hyper-immunized with IgM DJ to ssDNA in the presence of different NaCl molarities. Dissociation kinetics implied certain affinities of the antibodies toward ssDNA.*

sera were shown to be much higher than in normo-immunized sera. This showed that, by using the hyper-immunization protocol, it was possible to elicit the production of antibodies with immunogen-like paratopic specificity. The high levels of IgM anti-ssDNA present in the sera of hyper-immunized mice indicated polyclonal cell activation (Figure 3A). The affinities of IgG antibodies specific for ssDNA that were detected varied from low to high, as shown in Figure 4. The higher concentrations of anti-ssDNA antibodies of IgG isotype (of relatively higher affinity) in hyper-immunized animal sera may also be taken as a sign of affinity maturation.

Titers of Ab1s and Ab2s in immunized sera. The inhibitory ELISA (Figure 5A) method is based on the use of a relatively high antigen concentration (20 μg/ml) for adsorption on microwell plates. This creates a discrepancy between the concentrations of unlabelled analog (saturant) sufficient to block the binding sites in biotinylated antibodies on the one hand, and concentrations sufficient both for competition with their biotinylated anti-idiotypic antibody (competitor) and for binding to the immobilized immunogen, where the anti-idiotypic antibody is immobilized, on the other hand. Protein bound to the plastic is competed with its protein-like in the sera, and a labelled component is used to detect this binding. Thus, biotin-labelled Y7 antibody binding to IgM DJ immobilized on the plate is competed with IgM DJ-like antibodies from the sera. In network theory, these antibodies are designated as Ab2s. Thus, for example, binding of biotin-labelled IgM DJ antibody to Y7 immobilized on the plate would be competed with Y7-like antibodies (termed Ab1s in network theory) in the sera.

In the competitive ELISA test, 1.4 μg/ml of unlabelled IgM DJ was sufficient to block 50% of the binding of 100 ng/ml of biotin-labelled IgM DJ to the Y7 antibody immobilized on the plate. Its unlabelled anti-idiotypic antibody (Y7), at a concentration of 140 ng/ml, inhibited binding of 50% of the biotin-labelled IgM DJ to Y7 immobilized on the plate. In the assay with biotin-labelled anti-idiotype Y7, the concentration of its unlabelled competitor necessary for 50% inhibition of binding for the plate was 4.5 μg/ml, whilst a 50% blocking of the Y7 binding site was achieved with 270 ng/ml of IgM idiotype. The discrepancy between those concentrations needed for the blockade of binding sites in biotinylated IgM or Y7 antibodies and the concentrations needed for competition of binding to the antigen on the plate may be further explained by different labelling efficacies, since a lower number of biotin molecules per protein molecule requires a higher concentration of unlabelled saturant in the assay. The difference in molecular mass (IgM *versus* IgG) has a similar influence on the assay.

After repeated experimentation, as hyper-immunized (Figure 5B) sera diluted 1:3200 inhibited 50% of biotin-labelled IgM binding, the concentration of Ab1s was calculated to be around 458 μg/ml. This represents only 5% of Id-specific Ab1s (an approximation based on 10 mg/ml of

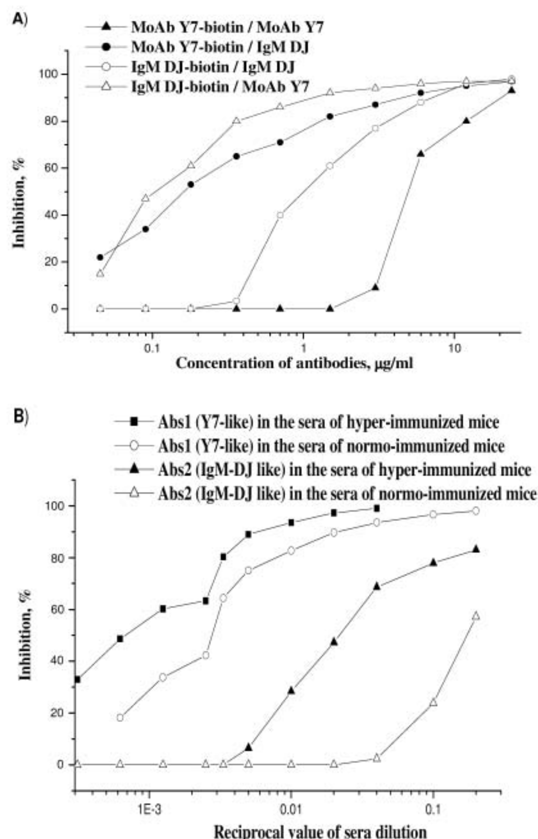

Figure 5. *Competitive ELISA for detection of Ab1s and Ab2s. Standard curves (A) used for calculation of the concentrations necessary for saturation and inhibition of binding. Saturation curves of biotin-labelled IgM DJ, and its biotin-labelled specific anti-idiotypic antibody Y7, were obtained in the presence of their relevant unlabelled forms. In the case where biotin-labelled IgM DJ binding was detected, Y7 as a competitor was used to calculate the concentrations of Ab1, while in the case where biotin-labelled Y7 binding was detected, IgM DJ as a competitor was used to calculate the concentrations of Ab2. Sera dilutions (B) that caused a 50% inhibition of the binding of biotinylated Y7 or IgM DJ for their respective counterparts immobilised on the plate. Inhibition percentages of binding of biotinylated IgM DJ or Y7 caused by Ab2 or Ab1 antibodies from the sera were calculated from the competitive standard curves in (A).*

IgG in mouse serum). The concentration of Ab1s in normo-immunized sera was calculated to be around 84 µg/ml, since 50% inhibition was obtained with a 1:600 sera dilution. If the above approximation is applied to normo-immunized sera, then less than 1% of Id-specific Ab1s were generated.

The calculated concentration of Ab2s in hyper-immunized sera was 13 µg/ml, based on 50% inhibition of biotin-labelled Y7 obtained with a 1:50 sera dilution, representing 0.13% of total serum IgG. In the case of Ab2s, in normo-immunized sera, a dilution of 1:5 gave 50% inhibition leading to a calculated concentration of approximately 1.3 µg/ml, representing 0.013% of the total serum IgG. These concentration values are, of necessity, approximate, based as they are on sera dilution.

Discussion

It was demonstrated that a hyper-immunization regime induced the following: a) high levels of specific antibodies; b) high levels of Ab1s and Ab2s; c) elevation of IgM antibodies with binding specificities typical of natural antibodies; d) stimulation of the production of antibodies specific for antigens other than the immunogen.

The massive elevation of antibodies, with the spectra of specificities described, is indicative of a specific antibody response as well as of polyclonal lymphocyte activation. In our case, the high antigen protein concentration did not induce antigen-dependent suppression, as might have been expected (29). It has already been shown that the high protein concentration used for immunization usually polarizes the immune response towards type Th1 (30). Equally, it has been shown that high doses of parasitic and viral antigens render Th1 clones more susceptible to activation-induced cell death and tip the scales in favor of Th2 clones: this was likely in our experiments.

The quantitative and qualitative aspects of T-cell activation are thought to be controlled by at least three factors: the route of Ag entry; the physical form of the Ag; the association of Ag with immunologic adjuvant (31). There is no data in the literature to permit definite conclusions to be drawn as to whether high or low doses of antigen are best suited to induce Th1- or Th2-type immune responses (32). The failure of our immunization regime to elicit suppression may be partially explained by the nature of the immunogen itself. The immunogen, IgM DJ monoclonal antibody with specificity towards ssDNA, belongs to the class of self-antigens usually recognised by natural antibodies. Thus, it would be expected to find that IgM DJ belongs to the family of natural antibodies. It has been shown that T-cell determinants of protein antigens can possess a different degree of immunogenicity *in vivo* (33), depending on the type, *i.e.,* dominant, subdominant or cryptic.

Measurement of the idiotypic complementarity, either of network or clonal specific interactions, is always limited by the affinity (structure) and concentrations of the idiotypes used, so that only certain high affinity pairs, present in high concentrations, can be measured since the core of the network is composed of natural antibodies (34). We chose to use the natural idiotype as an immunizing antigen in order to measure interactions between clones in the network composed of B-lymphocytes that produce natural antibodies. As a result of hyper-immunization, the sequential stimulation of a potentially unlimited number of clones (percolation) occurred and levels of Ab1's (35) participated in the detected Ab1 concentrations in hyper-immunized sera. The concentrations of Ab1s measured in immune and hyper-immune sera in our experiments were around 84 and 450 µg/ml, respectively. Data reported for Ab1 from other experiments varied between 30 and 240 µg/ml (36) and did not differ significantly from data

obtained in our experiments for hyper-immune sera (450 μg/ml). As the concentration of Ab1 immunogen-specific antibodies, as determined in our experiments, was more than twice as high, it could be suggested that our measuring included Ab1s and Ab3s as Ab1s' (Abs1' – Ab3s with similar properties to Ab1s). Lower levels of Ab2s as compared to Ab1s in both immunization schedules usually characterise the generation of an immune response (12). The levels of Ab2s detected in our hyper-immunization regime might be explained by the immunogen concentration which could, depending on its nature, induce polyclonal cell activation and differentiation of natural memory B-cells (37), which according to mathematical models could also be designated as percolation (15). The detected affinities of the antibodies in our experiments indicated possible percolation and stimulation of a potentially unlimited number of clones. If the enormous rise in Ab1s (Ab1s') and Ab2s, and the rise in antibodies specific for auto-antigens irrelevant to immunogen specificities (cytochrome C), are taken into consideration, then the occurrence of percolation is suggested. However, the rise in IgM anti-ssDNA specific antibodies (natural antibody specificity) indicated polyclonal B-cell activation occurring simultaneously. Polyclonal cell activation precedes affinity maturation, immune response and memory, and the occurrence of percolation confirmed the generation of a specific immune response from natural, self-centered, network-connected antibodies. The exposure of cryptic epitopes on proteins may result in the natural emergence of self-reactive antibodies, indicating lymphocyte activation of otherwise eliminated clones (38). Natural auto-antibodies, directed against a wide range of self-antigens, are present in the sera of healthy individuals (39). The role of these auto-antibodies is, as yet, undetermined. Auto-immune diseases are characterised by the presence of auto-antibodies to self-proteins. The accumulated evidence to date indicates that self-proteins are processed and presented in a similar way to foreign antigens by class I and class II major histocompatibility complex (MHC) molecules. Self-antigenic determinants (epitopes) are presented by the class II MHC. In this context it was shown that most (about 90%) of the class II ligands found so far originate from the cellular proteins of the organism itself, mostly from plasma membrane proteins.

The simultaneous presence of IgM antibodies specific for ssDNA and high affinity anti-ssDNA of the IgG class favors the hypothesis that the polyclonal antibody response against natural antigens is the generator of a pathogenic immune response. Conflicting data exists in the literature regarding the connection between the natural IgM antibodies found before antigen encounter and the early rise in antigen-specific IgM seen shortly after exposure to an antigen (40). Some studies have shown that the innate and the acquired humoral immunities are separate effector arms of the immune system and that high antigen exposure *per se* is not sufficient to increase natural antibody production. Other studies suggested that the production of natural IgM increased after the antigen encounter and provided a “platform for antigen-specific immune responses” (36). The activation of lymphocytes during the innate immune response plays an important role in the efficient recruitment of activated B-cells into antigen-driven adaptive responses that follow the innate response (41).

The discovery of cytochrome C-specific antibodies could suggest, as already stated, that polyreactive antibodies, although different, have partially overlapping V segment structures and can mediate binding to different Ags (42), cross-reacting with a wide variety of unrelated Ags. This cross-reactivity is critical in providing a fast response to the invading pathogen because, instead of looking for adequate specificity, a limited spectrum of polyspecific binding sites could provide the required rapid response.

The elevation of Ab1 and Ab2 antibodies, together with the polyclonal antibodies with specificities detected in our experiment, favors the idea that each idiotype interacts weakly with many idiotypes to attain high connectivity (tolerance), irrespective of its binding site specificity. Contrary to that, the levels and affinities of Ab2s with paratopic specificities identical to the immunogen, as detected in our experiments, may signal the generation of Ab2s in the immune response, at least in those responses elicited by very high concentrations of immunogen, leading to the possibility of immunopathological responses triggering auto-immune diseases.

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