Role of Repetitive Antigen Patterns for Induction of Antibodies Against Antibodies

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Summary

Antibody responses against antibodies, such as rheumatoid factors, are found in several immunopathological diseases and may play a role in disease pathogenesis. Experience shows that they are usually difficult to induce experimentally. Antibodies specific for immunoglobulin constant regions (anti-allotypic) or for variable regions (anti-idiotypic) have been investigated in animal models; the latter have even been postulated to regulate antibody and T cell responses via network-like interactions. Why and how such anti-antibodies are induced during autoimmune diseases, has remained largely unclear. Because repetitively arranged epitopes in a paracrystalline structure of a viral envelope cross-link B cell receptors efficiently to induce a prompt T-independent IgM response, this study used immune complexes containing viruses or bacteria to evaluate the role of antigen pattern for induction of anti-antibody responses. We present evidence that antibodies bound to strictly ordered, but not to irregularly arranged, antigens dramatically enhance induction of anti-antibodies, already after a single immunization and without using adjuvants. The results indicate a novel link between anti-antibody responses and infectious agents, and suggest a similar role for repetitive self-antigens such as DNA or collagen involved in chronic immunopathological diseases.

Abbreviations used in this paper: HRPO, horseradish peroxidase; IC, immune complex; P.a., Pseudomonas aeruginosa; RF, rheumatoid factors; S.t., Salmonella typhi; T.S., trypic soy; VSV, vesicular stomatitis virus; VSV-G, VSV glycoprotein; VSV-G-huH\(^1\), fusion protein of VSV-G with human IgG1 heavy chain constant regions; VSV-IND, VSV serotype Indiana; VSV-NJ, VSV serotype New Jersey; RF, rheumatoid factors; HRF, homologous rheumatoid factors.

A ntibodies against the constant and the variable parts of immunoglobulins have been investigated in various studies. Anti-allotypic antibodies directed against heterologous and rheumatoid factors (RF)\(^1\) directed against autologous constant immunoglobulin regions have been induced by immunization with immune complexes (IC) that contained antibodies bound to hemocyanin (1), transferrin (2), collagen (3), or LPS (4). Anti-idiotypic antibodies were mainly observed in experimental situations after immunization with heterologous immunoglobulin aggregates together with adjuvants (5), whereas RF (6-8) occur under physiological conditions and were shown to have various beneficial effects, such as clearance of IC from the blood (9), enhanced antigen presentation (10), and neutralization of certain pathogens as shown for herpes simplex virus in vitro (11) and trypanosomes in vivo (12). However, RF may be involved in the pathogenesis of synovitis in rheumatoid arthritis (13, 14) and of some immune complex diseases (15), because they can form immune complexes and efficiently activate the complement system (16). In contrast, anti-idiotypic antibodies have been postulated to play a role in the regulation of antibody (17, 18) and T cell responses (19, 20) via network-like interactions. Experimental induction of anti-antibodies in general is difficult and requires adjuvants plus allotypic or species differences (18); therefore, conclusions from these experiments for the role of anti-idiotypic antibodies are limited, and the biological significance of these antibodies is still unclear.

Because there is good evidence that repetitively arranged epitopes in a paracrystalline structure of a viral envelope cross-link B cell receptors efficiently to induce a prompt T-independent IgM response (21), this study attempted to test whether immune complexes with viruses or bacteria exhibiting highly ordered repetitive antigens on their surface may play a role in the induction of anti-antibody responses.
Materials and Methods

Infectious Agents. VSV serotype Indiana, (VSV-1ND, Mudd Summers isolate) and VSV serotype New Jersey, (VSV-NJ, Pringle isolate) were originally obtained from Professor D. Kolakowski (University of Geneva, Switzerland) and grown on BHK cells in minimal essential medium. UV inactivation was performed as described earlier (22) and monitored by a plaque assay on Vero cells. Recombinant VSV-G protein was produced in a baculovirus expression system as described (23); recombinant baculovirus expressing VSV-G was a gift from Dr. D.H.L. Bishop (NERC Institute of Virology, Oxford, UK). Salmonella typhi strain E83.728 was provided by F. Sadallah (University of Geneva, Switzerland). Pseudomonas aeruginosa strain Fischer IT-2 was obtained from the Swiss Serum and Vaccine Institute. Both bacteria were grown in tryptic soy (TS) broth at 37°C, quantified on TS agar plates and inactivated as a thin layer in a petri dish by UV irradiation for 10 min (Phillips U V lamp, 15 W, distance 8 cm).

Antibodies and IC. Anti-VSV mAb were obtained by fusion of a VSV-immune spleen from BALB/c mice on day 4 after primary (for IgM-secreting hybridomas) or on day 4 after secondary infection (for IgG-secreting hybridomas). The antibodies W N 1 222-5 and WN 4 245-2 (both IgG2a) are broadly reactive anti-LPS-core antibodies derived from NZB mice (24). The antibodies 99-T2 (IgG2b) and 63-T2 (IgM) are highly specific anti-LPS-O-chain antibodies against Pseudomonas aeruginosa strain Fisher IT-2 and were generated in BALB/c mice (25). IC were generated by incubation of a mixture of UV-inactivated virus or bacteria with the respective antibodies for 1 h at room temperature. IC formation in the VSV model could be demonstrated indirectly by reduction of anti-VSV neutralizing antibody titers in mice immunized with IC compared with mice immunized with VSV alone. ELISA for Anti-antibody Detection. We used a sandwich ELISA with the following steps: (a) coating with isotype-specific goat anti-mouse antibody (1 µg/ml; Southern Biotechnologies, Birmingham, AL), (b) blocking with 2% BSA (Fluka, Buchs, Switzerland) in PBS, (c) mAb supernatant (0.2 µg/ml), (d) 20-fold prediluted mouse serum, titrated 1:2 over 11 dilution steps, (e) isotype-specific horseradish peroxidase (HRPO)-labeled goat anti-mouse antibodies (0.5 µg/ml; Southern Biotechnologies), (f) substrate ABTS (2,2'-azino-di-[3-ethylbenzthiazolin-sulfonate (6)], Boehringer Mannheim) and H2O2 (Fluka). Plates were coated over night at 4°C, all other incubations were for 60 to 90 min at room temperature. Between incubations, plates were washed three times with PBS containing 0.5 ml Tween 20 per liter. OD was measured at 405 nm in an ELISA reader. All anti-antibody titers are indicated as -log2 of 20-fold prediluted sera. For Fig. 1, C and D, the dilution step at half maximal OD was determined as shown in Fig. 1 A and then taken as anti-antibody titer. For Fig. 1 E, allotype-specific anti-IgM antibodies (Southern Biotechnologies) were used for coating and detection at the same concentrations as described above.

VSV neutralization assay. VSV IN D neutralizing antibodies (Fig. 2) were determined on the indicated timepoints by a plaque reduction assay on Vero cells as described before (26). Titers are indicated as -log2 of 40-fold prediluted sera.

VSV-G-huH γ1. VSV-G-huH γ1 fusion protein was generated following procedures developed by Traunecker and Karjalainen (27) and will be described elsewhere (Bucher, E., U. Induction of Anti-antibodies by Repetitive Immune Complexes...
Results

Induction of Anti-antibodies by VSV–IgM Complexes. A strictly repetitive paracrystalline order of antigen in a viral envelope with a spacing of 5 to 10 nm has been shown to facilitate B cell responses, even to self-antigen (28). To evaluate whether antibodies bound to such highly organized antigens might also be presented in an orderly and repetitive fashion and therefore could induce anti-antibodies, BALB/c mice were immunized with IC of autologous antibodies bound to vesicular stomatitis virus (VSV) particles exhibiting paracrystalline strictly repetitive glycoproteins (G) in their envelope. Control groups were immunized with irregularly complexed or monomeric antibodies. While in vitro–generated IC containing 1 μg of a monoclonal IgM antibody M1 against VSV-G and 10⁶ PFU of UV-inactivated VSV particles efficiently induced anti-antibodies, IC with the same amount of antibody M1 either irregularly complexed or with the recombinant VSV-G protein spontaneously aggregating in tail-to-tail micelles (28a) or cross-linked with a rat anti-mouse C₅₀ antibody did not (Fig. 1 A). Similar results were obtained in A/J and C57BL/6 mice, but in this situation, IgG anti-IgM antibodies could also be induced by the poorly organized IC containing recombinant VSV-G, although at much lower titers than with the highly repetitive complex (Fig. 1 B). When BALB/c mice were boosted once after 14 d, only those treated with virus/antibody complexes exhibited IgM-specific IgG titers of 1:3,000, which did not drop significantly over a period of 80 d (Fig. 1 C). They were dependent on the antibody dose used for generation of IC, as shown for two different monoclonal IgM (Fig. 1 D). In similar experiments using different VSV-specific monoclonal IgG instead of IgM antibodies, no anti-antibodies were induced. This might be explained as follows: (a) the potentially immunogenic variable domains of IgG molecules binding to the virus surface are not easily accessible to B cells, or (b) clearance and processing of IgG- and IgM-containing IC is distinct, because the latter, exhibit easily accessible Fc domains that could bind to Fc receptors of macrophages, which would then lead to faster clearance of the IC. Also VSV infection itself did not induce anti-antibodies, probably because the virus only abortively replicates extraneuronally in mouse and is rapidly eliminated within 1 d (29), before neutralizing IgM antibodies, which could lead to IC formation, are measured.

Spedifity of Anti-antibodies Induced by VSV–IgM Complexes. To determine the specificity of these anti-antibodies, we immunized BALB/c mice (IgM⁻) or A/J mice (IgM⁺) with IC formed with six different monovalent VSV neutralizing IgM (M1-M6), which were isolated from BALB/c mice. M3 is specific for the distinct serotype VSV- New Jersey (VSV-NJ)), the others for VSV Indiana (VSV-IN D). Analysis of these sera on ELISA plates coated with M1 to M 6 revealed that BALB/c mice produced anti-antibodies exclusively specific for the IgM used for immunization, whereas sera of A/J mice recognized any IgM as long as they were of BALB/c origin (Table 1). A monoclonal IgM of A/J origin as well as IgM of normal A/J serum was not recognized by either of the sera (data not shown). Therefore, the anti-antibodies induced in BALB/c mice are idotype specific, whereas those induced in A/J mice were allotype-specific. Only antibody (M2) failed to induce anti-antibodies (Table 1, Experiment 1); this correlated with its 10–100-fold lower neutralizing capacity and IC formation in vitro compared with the other IgM (data not shown). Both BALB/c anti-idiotypic and A/J anti-allotypic antibodies were IgGs and, therefore, T helper cell dependent; this is demonstrated by the failure of CD4⁺ T cell–depleted or of athymic nude mice to respond with anti-antibodies of IgG isotype (Table 1, Experiment 3). Anti-allotypic antibodies were also found in C57BL/6 mice (IgM⁻), and in this case we were even able to detect an IgM⁺ allotype-specific response on day 4 after primary immunization with IC (titers of 1:100), but not after immunization with virus or antibody alone (Fig. 1 E). This response could be prolonged by additional use of LPS (data not shown).

Next, it was tested whether the specificity of induced anti-antibodies as shown in ELISA could also be demonstrated in an independent in vivo read out. For this purpose, A/J and BALB/c were immunized twice with IC containing 10⁶ PFU inactivated VSV-NJ and 5 μg of the antibody M3 to induce anti-antibodies. 10 d later these mice were treated with a specific IgM anti-VSV-IN D antibody (M5), that did not cross-react with VSV-NJ. This enabled us to measure the half life of M5 by determining the neutralizing titer of the sera against VSV-IN D (Fig. 2). In A/J mice, the half-life of M 5 was reduced from 28 to 4 h, whereas in BALB/c mice it was comparable to uninunized controls; this confirmed the presence of antibodies against the constant region of BALB/c immunoglobulin in A/J, but not in BALB/c mice. Thus, in this model situation anti-allotypic antibodies could be shown to modulate serum IgM antibodies by forming IC in vivo. We were not able to use the same experimental approach to assess modu-
### Table 1. Specificity and Helper T Cell Dependence of Anti-antibodies in BALB/c and A/J Mice

| Experiment | Mice strain | Immunization | Anti-antibody titer on plates coated with | CD4+ T cells |
|------------|-------------|--------------|------------------------------------------|--------------|
|            |             |              | M1 | M2 | M3 | IgM anti-P.a. | M3 | M5 |    |
| 1          | BALB/c      | IND/M1 (G7G9C9) | 7  | <1 | <1 | <1           |
|            |             | IND/M2 (M4C11H12) | <1 | <1 | <1 | <1           |
|            |             | N/J M3 (B3B8H6) | <1 | <1 | 9  | <1           |
|            | A/J         | IND/M1        | 7.5 | 7  | 8  | 6           |
|            |             | IND/M2        | <1 | <1 | <1 | <1           |
|            |             | N/J M3        | 6  | 6  | 7.5 | 4           |
| 2          | BALB/c      | IND/M4 (M3F2B6) | 5  | <1 | <1 | <1           |
|            |             | IND/M5 (H2F1C1) | <1 | 7  | <1 | <1           |
|            |             | IND/M6 (M4E11G8) | <1 | 1  | 7.5 | <1           |
|            | A/J         | IND/M4        | 6.5 | 4  | 2  | 2           |
|            |             | IND/M5        | 8  | 9.5 | 5.5 | 6.5           |
|            |             | IND/M6        | 3  | 6  | 2  | 2           |
| 3          | BALB/c      | IND/M5 | Normal | <1 | 8 |
|            |             | IND/M5 | Depleted | <1 | <1 |
|            |             | N/J M3 | Normal | 7.5 | 5 |
|            |             | N/J M3 | Depleted | 2 | <1 |
|            | A/J         | IND/M5 | Absent | <1 | <1 |
|            |             | N/J M3 | Normal | 7.5 | 5 |
|            |             | N/J M3 | Depleted | 2 | <1 |

*BALB/c (IgM+) and A/J (IgM+) mice were immunized twice with 10⁸ PFU of inactivated VSV complexed with 3 to 8 μg of various anti-VSV-G IgM antibodies (M1-M6) with an interval of 14 d.
†IgG anti-IgM titers are indicated as −log₂ of 20-fold prediluted sera on day 6 after booster injection and are determined as described in Fig. 1A.
‡ELISA plates were coated with a goat anti-mouse IgM as a catcher antibody for the different IgM hybridoma supernatants M1-M6 (anti-VSV) and 63-T2 (anti-Pseudomonas aeruginosa [P.a.]).
CD4+ T cells were depleted in vivo by intraperitoneal injection of 1 mg of the anti-CD4 antibody YTS 191.6 on days −3 and −1 before immunization.

*CD4+ T cells were depleted in vivo by intraperitoneal injection of 1 mg of the anti-CD4 antibody YTS 191.6 on days −3 and −1 before immunization.*
and were independent of B cell activation by LPS (Fig. 3D). In the case of P.a., 38% (18 of 48) of the mice immunized twice with identical complexes died upon the second immunization from a shock-like syndrome compared with only 4% (2 of 48) of the control groups that were immunized with bacteria, purified LPS, or antibody only; this in vivo finding confirmed the presence of anti-antibodies measured in vitro and, in addition, suggested a pathogenic function of RF-like anti-antibodies.

Immunogenicity of Multivalent Versus Bivalent IC. The notion that antibodies bound to repetitively ordered viral or bacterial antigens induced anti-antibodies was further tested with a sort of inversed IC. Complexes were formed between a VSV-G-specific decavalent IgM (M1) or a bivalent IgG2a antibody (VI49; reference 30) as core of the complex that binds a fusion protein of VSV-G plus constant part of the human IgG1 H chain (huHγ1) molecule. These IC display the Fc portions of huIgG1 as repetitive domains and form under optimal conditions decameric (with IgM) or dimeric (with IgG) complexes. In primary and secondary immune responses of BALB/c mice, the fusion protein complexed with the decavalent IgM induced much higher titers of anti-antibodies to huHγ1 than bivalent IgG-complexed or the noncomplexed fusion protein alone (Fig. 4). These anti-antibodies were of IgG isotype and T-help dependent, as shown by the negative effect of in vivo CD4+ T cell depletion. Although a very rigid IC structure cannot be assumed in this model, the results show that antibody responses to a foreign constant IgG region, which involves species differences, can be markedly enhanced by multimeric aggregation compared with dimers or monomers. Importantly, they may even suggest that IgM-bound antigen in turn binding IgG may be able to induce anti-IgG antibodies.

Figure 3. IgG anti-IgG titers after immunization with antibodies complexed to Salmonella typhi (S.t.) and Pseudomonas aeruginosa (P.a.). (A) BALB/c mice were immunized twice (interval 14 d) with 10^8 CFU of S.t. complexed with 20 μg (closed squares) or 2 μg (closed triangles) of the anti-LPS antibody WN1 222-5 (IgG2a) or with 10^6 (open squares) or 10^5 (open triangles) CFU of S.t. alone or with 20 μg of antibody WN1 222-5 alone (open diamonds). 6 d after the second injection, IgG2a plus IgG1 anti-IgG2a titers in 20-fold prediluted sera were determined in a sandwich ELISA. (B) BALB/c mice were immunized twice with 10^6 PFU of P.a. complexed with 7 μg of the anti-LPS antibody 99-T2 (IgG2b) (closed squares), with 10^3 CFU of P.a. alone (open triangles) or with 7 μg of 99-T2 alone (open diamonds). IgG2a plus IgG1 anti-IgG2b titers of 20-fold prediluted sera were determined in a sandwich ELISA. (C) Sera of BALB/c mice immunized twice with IC or with bacteria alone as described in A or B were tested on ELISA plates coated with different antibodies of the corresponding isotype, and anti-antibody titers were determined as described in Fig 1. (D) BALB/c, LPS-responsive C3H/HeN, and LPS-nonresponsive C3H/HeJ mice were immunized twice with IC, and anti-antibody titers were determined 6 d after booster injection as described in the legend to Fig 1. Bars in C and D represent geometrical means of 2–3 animals per group. Standard deviation was within ± one dilution step. Results of one out of three comparable experiments are shown.
IgM or IgG antibodies. BALB/c mice were immunized with 10 μg of the fusion protein VSV-G–huH1 alone or complexed with anti-VSV-G antibodies. However, when allotypic differences (Fig. 1A) or species differences (Fig. 4) were the target, anti-idiotypic antibodies were only induced with highly organized repetitive IC (Fig. 1A). Induction of RF could only be demonstrated in the Pseudomonas model (Fig. 3B), where in addition to repetitive antigen order the B cell stimulator LPS was necessary (LPS alone or IC containing purified LPS were not sufficient). These findings confirm that autoreactive B cells including those with specificity for membrane-bound self-antigens (e.g., immunoglobulin) may not be deleted (28, 31) and perhaps exert biological effects. It will be interesting to evaluate the relevant differences to various other membrane-bound self-antigens that cause deletion of autoreactive B cells (32–34).

Our findings indicate a novel link between anti-antibody responses and infectious agents or repetitive self-antigens. The role of highly ordered multimeric antigen patterns for efficient B cell activation has been tested systematically in several model situations involving linear (flagellin [35]; haptenated polymers [36]) or particulate antigens (Hepatitis B core antigen [37] or VSV [26]). Most infectious agents including viruses, bacteria, and parasites expose highly ordered repetitive antigenic epitopes on their surface. They are able to induce efficient T-independent IgM responses by cross-linking B cell receptors (21, 26, 38, 39). This study now demonstrates that the same rules seem to apply for induction of anti-antibodies. These anti-antibodies were readily induced after only two injections of repetitive IC involving mAbs, but without using adjuvants. In view of the observation that protective anti-Hemophilus influenzae (40) and anti-viral antibodies (30) are of restricted or virtually monoclonal specificity, our results with mAbs against infectious agents can probably be generalized. These findings may explain the secretion of RF during virus infections (influenza [41], rubella, CMV, EBV, HIV) (6) and how the same configuration together with or without LPS may sustain anti-antibody responses in the course of bacterial infections (such as bacterial endocarditis, tuberculosis, or syphilis for review see 42). Especially chronic infections with persistence of the pathogen during an ongoing antibody response would fulfill the conditions for IC formation in vivo, as it is described for herpesvirus (43, 44), HIV (45, 46), or mycobacteria (47, 48). In addition, the results in BALB/c mice could suggest that, instead of RF, anti-idiotypic antibodies may enhance immunopathology found in reactive forms of arthritis, which are apparently RF negative. Also, repetitive self antigens (e.g., collagen II [49] in rheumatoid lesions, DNA [50] in SLE) exposed after primary or secondary tissue injury may induce anti-antibodies by formation of highly repetitive immune complexes. This may explain why RF secretion is such a typical manifestation accompanying these diseases (15).

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1790 Induction of Anti-antibodies by Repetitive Immune Complexes
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