Warm Autoimmune Hemolytic Anemia: A Clinical Model to Study Mechanisms of Immunoregulation*

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Key Words

Self-tolerance · Natural autoreactivity · Warm autoimmune hemolytic anemia · Immunoregulation · Immune complex

Summary

The study of immune hemolytic anemias has contributed significantly to the understanding of the immune response in the human system in the past. Thus, the concept of receptor specificity – becoming the rationale of the clonal selection theory when trying to explain how the organism avoids autoaggression – has been developed originally upon the study of immune hemolytic anemias, and the question whether the antigen-antibody reaction is determined primarily by chemical or by physical aspects of interaction of molecules has been discussed originally taking immune hemolytic anemias as an example. Today, immunohematology still provides excellent models to study immune mechanisms that are of both clinical relevance and fundamental significance for the understanding of mechanisms that determine the appropriate recognition of self- and non-self-antigens in the context of complex dynamic interactions at the cellular and humoral level of the human immune system. The data summarized in this review focus on the aspects that the study of warm autoimmune hemolytic anemia may contribute to the understanding of immunoregulation in the human system.

Schlüsselwörter

Immuntoleranz · Natürliche Autoreaktivität · Wärmeautoimmunhämolyse · Immunregulation · Immunkomplex

Zusammenfassung

Wesentliche Konzepte zum Verständnis immunologischer Mechanismen wie das Konzept der Rezeptorspezifität – die Grundlage der klonalen Selektionstheorie – wurden in der Vergangenheit am Beispiel der Immunhämolysen entwickelt, und grundlegende Diskussionen wie die Diskussion, ob die Antigen-Antikörper-Reaktion primär chemischer oder primär physikalischer Natur ist, wurden bereits zum Ausgang des 19. und zu Beginn des 20. Jahrhunderts am Beispiel der Immunhämolysen geführt. Das Studium der Immunhämolysen ist auch heute noch in hervorragender Weise geeignet, klinisch relevante Fragen mit immunologisch-konzeptionellem Potential exemplarisch anzugehen. Die vorliegende Übersichtsarbeit fasst Daten zusammen, die am Beispiel der Autoimmunhämolyse vom Wärmetyp zeigen, wie die Immunhämatologie zum heutigen Verständnis immunregulatorischer Mechanismen beitragen kann.

Generating and Maintaining Self-Tolerance

That autoreactivity is equivalent to autoaggression and therefore forbidden under physiological conditions has been a cen-

tral dogma of immunology since Paul Ehrlich established the concept of the ‘Horror autotoxicus’: An autotoxin that may destroy the cells of the organism that originally formed it does not exist under physiological conditions [1–3]. Karl Landsteiner’s observation that plasma of healthy individuals contains natural antibodies directed exclusively at ABO blood group antigens that the individual’s red blood cells do not express,

*Dedicated to Prof. Dr. Peter Hanfland, Bonn, on the occasion of his 65th birthday.

gave strong support to Ehrlich's concept of a 'Horror autotoxicus' and became the rationale for compatible red blood cell transfusion [4].

Since Burnet and Medawar published their groundbreaking work, it is widely accepted that deletion of autoreactive lymphocyte clones underlies immunotolerance [5–7]. Deletion of autoreactive lymphocyte clones occurs during lymphocyte development at a structural level (i.e. central tolerance, achieved by receptor editing or apoptosis of self-reactive clones). Once lymphocyte maturation is complete but has resulted in the formation of autoreactive lymphocytes that have escaped central tolerance mechanisms, immunotolerance is achieved in the periphery mainly at a functional level by a variety of mechanisms that render autoreactive cells anergic – although recent evidence in mice indicates that receptor editing may still occur in mature antigen-activated peripheral lymphocyte populations [8–16].

However, immunotolerance – seen from the angle of a functional concept – results from a variety of additional regulatory mechanisms in the context of complex interactions at the cellular and humoral level of the human immune system: Complement proteins are involved in the selection of B cells [17]. The outstanding significance of CD4⁺ CD25⁺ regulatory T cells – not yet fully understood in its different subsets – for the generation of immunotolerance has become clear during the last decade [18–24]. Dendritic cell homeostasis is central to the regulation of self-reactivity [25–26], although views differ on the molecular details [27–31]. Furthermore, the interaction of T cells and macrophages as well as self-versus non-self-discrimination by natural killer (NK) cells – in particular in the setting of pro-inflammatory conditions – points to a close linkage between functions of innate and adaptive immunity when it comes to maintaining immunotolerance [32, 33]. Likewise, immunoglobulins use a variety of mechanisms to regulate immunotolerance, involving F(ab')₂ regions, Fc regions, isotypes and immunoglobulin subclass-dependent functions, as well as the capability to penetrate the membrane of living cells and exert intracellular functions [34–47].

Natural Autoreactivity Contributes to the Maintenance of Self-Tolerance

Already in 1900, Ehrlich's concept of the 'Horror autotoxicus' was opposed by members of the group around Elie Metchnikoff who observed the existence of autoantibodies under physiological conditions and suggested the concept of autoimmunization [48, 49]. The concept of autoreactivity existent under physiological conditions has subsequently been taken up by the team of Niels Jerne [50–55] and that of Irun Cohen [56–60]. Meanwhile, it is established knowledge that autoreactive T cells, B cells and antibodies (i.e. natural autoantibodies) are present under physiological conditions, and are of substan-

tial significance for the generation and maintenance of immunotolerance [61–75].

The critical time period for establishing natural tolerance toward self-antigens is the fetal and perinatal period, and it is widely dependent on a functional thymus [54–76]. Once established, immunotolerance must be maintained throughout life. Although scientific evidence indicates that plasma cells as well as B and T memory cells may be characterized by a significantly longer survival period and may therefore contribute much more efficiently to the maintenance of immunological memory than originally assumed [77–81], there is a constant need to educate newly developing lymphocytes in the process of differentiation of hematopoietic progenitor cells to discriminate self and non-self, throughout life and despite of thymus involution [54]. How does the organism meet this challenge, in particular with regard to the maintenance of natural autoreactivity?

A genetically determined natural autoreactivity, active at the onset of development of natural tolerance toward the immunological self, would implement an outlasting internal image of the immunological self. Such an internal image would allow newly developing lymphocytes to align throughout life with the original demand on how to define self. Such genetically determined natural autoreactivity requires that T lymphocytes as well as B lymphocytes are selected for survival during their development at the basis of their autoreactivity (i.e. by positive selection) and are maintained in an active state on the basis of their autoreactivity. Current experimental data indeed point to the existence of a genetically determined natural autoreactivity at the onset of self-tolerance [54, 82–87]. Positive selection of T cells, tightly regulated by the affinity of the TCR for self-antigen, characterizes T cell development in fetal and perinatal life [88]. Recent evidence in mice indicates that self-reactive B cells may undergo positive selection due to recognition of self-antigen, proliferate in the presence of the corresponding self-antigen and are maintained on the basis of their self-reactivity [67]. In the periphery, such a genetically determined internal image of self may be mediated by natural autoantibodies ('the immunological homunculus') [56, 89, 90].

Repertoires of human naturally self-reactive antibodies have been intensively investigated during the last decade [72, 91]. Natural self-reactive antibody repertoires are directed toward a limited subset of immunodominant autoantigens, arise early in development and remain conserved throughout the aging process [92–96]. The selection of natural self-reactive IgG antibody repertoires requires normal interaction between T and B cells [97, 98]. Although the epitopes participating in the selection of human natural self-reactive B cells have not been identified yet, it has been shown that many self-molecules with immunoregulatory function – such as CD4, CD5, MHC class I molecules, Fcγ receptors, idiotypes of antibodies and of surface immunoglobulin on B cells, and parts of the TCR – are recognized by natural autoantibodies [72]. Natural self-re-

active antibodies regulate immune responses by several mechanisms [72]. Such mechanisms include the interference with the selection of B cell repertoires [41, 42, 99, 100] – depending at least in part on antibody variable regions [41] – and the control of the proliferation of CD4⁺ T cells specific for autoantigens recognized by natural autoantibodies [101]. Natural autoantibody repertoires of IgM and/or IgG are altered in a variety of autoimmune disorders, immunodeficiency syndromes and lymphoproliferative diseases [91], and intravenous immunoglobulin (IVIg) – a therapeutic preparation of pooled normal poly-specific human IgG obtained from large numbers of healthy blood donors that contains homologous immune antibodies as well as homologous natural autoantibodies – has been shown to prevent or attenuate autoimmune diseases efficiently in the experimental as well as in the clinical setting [41, 72, 102]. Taken together, scientific evidence indicates that natural autoreactivity, present and active under physiological conditions, contributes significantly to the generation and maintenance of immunotolerance in the human system.

Warm Autoimmune Hemolytic Anemia: Dysregulation of Natural Autoreactivity Contributes to Disease

The same mechanisms that generally contribute to immunotolerance are supposed to account for self-tolerance toward red blood cells (RBC), although development of self-tolerance toward RBC under physiological conditions in the human system has not been investigated in particular. Intensive research in the past 10 years, however, has led to an altered understanding of components of RBC membranes, such as blood group antigens, which are now appreciated as biomolecules with functional significance [103]. Within this context, components of RBC membranes, such as the cell surface glycoprotein CD47 (integrin-associated protein/IAP), linking the interaction of band 3 complex and the Rh complex in the maintenance of RBC membrane integrity, have also gained attention as markers of self [104, 105]. Thus, a decreased expression of CD47 on murine RBC has been described to be involved in enhanced extravascular clearance of RBC, possibly by altered CD47-SIRP α interactions [106]. Natural autoantibodies against RBC do exist. They are directed toward structures of the RBC membrane and the cytoskeleton, and in recent years it has been demonstrated that natural autoantibodies against ABO blood group antigens exist in the plasma of healthy individuals [107–109].

In warm autoimmune hemolytic anemia (WAIHA), self-tolerance toward RBC is broken. WAIHA accounts for 50–70% of cases of autoimmune hemolytic anemia [110], and is characterized by the occurrence of antibodies binding to RBC. Autoantibodies are typically of polyclonal origin, belong to the IgG isotype and arise idiopathically or in the context of lymphoproliferative disorders or other autoimmune diseases.

IgG-coated RBC undergo accelerated Fc γ receptor mediated extravascular clearance [111, 112]. The mechanisms of Fc γ receptor mediated clearance of IgG-coated RBC have been studied intensively [45, 113–118], with research focussing on the effector mechanisms resulting in RBC destruction. Mechanisms at the origin of broken tolerance toward RBC in human WAIHA remain unclear. The typical approach to understand an autoimmune disease is to analyze the nature of the autoantigen and to investigate the effector mechanisms finally resulting in tissue destruction. Whereas the effector mechanisms leading to RBC destruction in WAIHA became evident very quickly, the characterization of the autoantigens has proved to be more difficult. Although some RBC structures, such as rhesus antigens, glycophorin A or the band 3 structure, may be particularly recognized by autoantibodies, a review of the literature reveals that nearly every RBC antigen characterized by serological studies has been described as target structure in WAIHA [112, 119–121]. In addition, warm autoantibodies typically ‘broaden’ their specificities and show reactivity with autologous and allogeneic RBC [112]. Autoantibodies eluted from autologous RBC have been described that have a well-defined specificity, although the patient’s RBC lacked the corresponding antigen [122]. Such antibodies can be absorbed by RBC both positive and negative for the corresponding antigen. The nature of the epitopes against which such mimicking antibodies are directed is yet unclear [111, 112]. The specificity of RBC-bound IgG fails to discriminate between natural and WAIHA-related autoreactivity of IgG toward RBC [123]. Overall, investigations to characterize the autoantigen in WAIHA did not yet reveal a general recognition pattern of antibodies on the RBC membrane.

Natural IgM critically contributes to the maintenance of self-tolerance [100, 124, 125]. Experimental models indicate that deficient serum IgM predisposes to the development of IgG-mediated autoimmune diseases [126–128]. In 1994, first data appeared indicating that a dysregulated control of natural autoantibodies might be involved in the pathophysiology of spontaneous autoimmune hemolytic anemia of NZB mice [129]. So does a failure of control of natural anti-RBC specific IgG contribute to development of warm autoimmune hemolytic anemia in humans?

Choosing an approach of screening antibody reactivities in complex antibody mixtures, such as serum, toward a large panel of antigens derived from tissue extracts by quantitative immunoblotting, followed by interpretation of the data by multiparametric statistical analysis using principal component analysis and linear discriminant analysis, it could be demonstrated that self-reactive antibody repertoires of IgG purified from plasma and of IgG purified from RBC eluates do not differ between healthy donors and patients with IgG-mediated WAIHA. In contrast, autoreactive repertoires of the patients’ IgM exhibit broadly altered patterns of reactivity compared to those of healthy controls [130, 131]. IgG purified from eluates of RBC of healthy donors agglutinate RBC to a similar

Fig. 1. Surface plasmon resonance analysis of binding affinities of IgM of WAIHA patients toward normal IgG. The binding affinities of IgM purified from plasma of 5 WAIHA patients toward **a** IgG purified from plasma of 3 different healthy individuals and toward **b** anti-RBC IgG purified from RBC eluates of 2 different healthy individuals were compared with the binding affinities of IgM purified from plasma of 5 healthy individuals. The figure depicts the mean relative binding intensities $\pm$ standard deviation of IgM (150 μ g/ml) of WAIHA patients in comparison to the mean binding intensities $\pm$ standard deviation of IgM (150 μ g/ml) of healthy individuals (mean, healthy individuals = 100%). A p value of ≤ 0.05 was considered to represent differences of statistical significance. Reprinted from Stahl D, Sibrowski W: Warm autoimmune hemolytic anemia is an immune complex disease. *J Autoimmun* 2005;25(4):272–282, with permission from Elsevier Inc.

extent as IgG purified from eluates of RBC of patients with WAIHA. However, the agglutinating ability of IgG in RBC eluates is suppressed in unfractionated eluates of RBC of healthy donors, whereas it can be readily found in unfraction-

Fig. 2. Plasma of WAIHA patients contains an enhanced amount of IgM-IgG immune complexes. IgM was purified from plasma of WAIHA patients (n = 15) and healthy individuals (n = 15) and analyzed for IgG content. The figure depicts the content of IgM-IgG immune complexes within purified IgM. Given are mean contents $\pm$ standard deviation for WAIHA patients in comparison to mean contents $\pm$ standard deviation for healthy individuals (mean, healthy individuals = 100%). A p value ≤ 0.05 was considered to represent differences of statistical significance. Reprinted from Stahl D, Sibrowski W: Warm autoimmune hemolytic anemia is an immune complex disease. *J Autoimmun* 2005;25(4):272–282, with permission from Elsevier Inc.

ated eluates of RBC of patients. Non-IgG molecules, including IgM, are essential in controlling the agglutinating capability of IgG in unfractionated RBC eluates. Taken together, these observations indicate that anti-RBC IgG autoantibodies of patients with WAIHA share extensive similarity with natural anti-RBC autoantibodies of healthy donors, suggesting that a defective control of IgG autoreactivity by autologous IgM is an underlying mechanism for autoimmune hemolysis in WAIHA [130].

Warm Autoimmune Hemolytic Anemia is an IgM-IgG Immune Complex Disease

Recent data indicate that altered IgM-IgG immune complexes are at the origin of such altered antibody repertoire of plasma IgM of WAIHA patients [132]. IgM in plasma of WAIHA patients exhibits a significantly increased binding affinity toward IgG purified from plasma of healthy individuals and eluted from the RBC surface of healthy individuals, compared to the binding affinity of IgM of healthy individuals (fig. 1 a, b), resulting in a significantly higher amount of IgM-IgG immune complexes in plasma of WAIHA patients than in plasma of healthy individuals (fig. 2). The occurrence of an enhanced amount of IgM-IgG immune complexes in plasma of WAIHA patients is independent of the etiology of the disease and can

Fig. 3. Pathophysiological model of WAIHA.

The original pathological event in WAIHA is a disturbed balance between IgM specific for IgG (i.e. anti-IgG IgM) and IgG. The disturbed balance of IgM-IgG interactions starts at the level of secretion of altered anti-IgG IgM (1.). Such altered anti-IgG IgM is characterized by an enhanced affinity for normal IgG. Future investigations on anti-IgG IgM at the molecular level will clarify the structural basis for the enhanced affinity of IgM for normal IgG. The secretion of altered anti-IgG IgM results in loss of control of IgM over the level of proliferation of IgG secreting B cells, thereby withdrawing these B cells from the efficient control by anti-IgG IgM (2.). Proliferation of IgG secreting B cells leads to the enhanced formation of soluble IgM-IgG immune complexes (2.). Such IgM-IgG immune complexes adhere to RBC via the IgG component of the immune complex (2.). Binding of IgM-IgG immune complexes to RBC via the IgG component is most likely to occur via the Fab fragments of IgG of the immune complex, i.e. via RBC antigen specific binding. This mechanism implies that the IgG secreting B cells exhibit specificity of the BCR for RBC structures (2.). IgG coated RBC are recognized by Fcγ receptor bearing macrophages and cleared from the circulation resulting in extravascular hemolysis (3.). In order to depict this concept plastically, the IgM BCR is shown as a pentamer, and not as a monomeric molecule. Reprinted from Stahl D, Sibrowski W: Warm autoimmune hemolytic anemia is an immune complex disease. *J Autoimmun* 2005;25(4):272–282, with permission from Elsevier Inc.

be observed in primary WAIHA as well as in secondary WAIHA. IgM-IgG immune complex formation in plasma might simply represent an epi-phenomenon of WAIHA. However, several lines of evidence suggest that IgM-IgG immune complexes in plasma of WAIHA patients primarily contribute to the disease: 1. IgG1 and IgG3 are actively enriched in IgM-IgG immune complexes in plasma of WAIHA patients, in accordance with IgG subclasses typically detected onto the RBC surface of WAIHA patients [123]. 2. IgM-IgG immune complexes are present in the immunoglobulin fraction eluted from RBC of patients with WAIHA. 3. IgM in plasma of WAIHA patients exhibits binding affinity to Fc fragments as well as toward F(ab')₂ fragments derived from IVIG, whereas IgM derived from RBC eluates from WAIHA patients binds preferentially to F(ab')₂ fragments of IVIG, suggesting active enrichment of altered IgM-IgG immune complexes onto the RBC membrane. To understand how an enhanced IgM-IgG immune complex formation may originally contribute to anti-RBC autoimmunity in WAIHA, the following hypothetical model may be taken as a basis for aligning future experimental work in this field (fig. 3). The original pathological event in WAIHA is a disturbed balance between IgM specific for IgG (i.e. anti-IgG IgM) and IgG. The disturbed balance of IgM-IgG interactions starts at the level of secretion of altered anti-IgG IgM (fig. 3, 1.). Such altered anti-IgG IgM is characterized by an enhanced affinity for normal IgG. Future investigations on anti-IgG IgM at the molecular level will clarify the

structural basis for the enhanced affinity of IgM for normal IgG. The secretion of altered anti-IgG IgM results in loss of control of IgM over the level of proliferation of IgG secreting B cells, thereby withdrawing these B cells from the efficient control by anti-IgG IgM (fig. 3, 2.). Antibody-mediated feedback regulation of antibody production has been principally described as a regulatory mechanism of B cell proliferation [133, 134] and might be operating not only at the IgG-FcγRIIB level, but also at an IgM-FcμR level. A Fc receptor for IgM, constitutively expressed by the majority of B lymphocytes, has been described recently [135]. Proliferation of IgG secreting B cells leads to enhanced formation of soluble IgM-IgG immune complexes. Such IgM-IgG immune complexes adhere to RBC via the IgG component of the immune complex (fig. 3, 2.). IgG coated RBC are recognized by Fcγ receptor bearing macrophages and cleared from the circulation resulting in extravascular hemolysis (fig. 3, 3.).

The Impact of Immune Complexes on the Regulation of Immune Responses

Taken together, altered IgM-IgG immune complexes in plasma and associated with the RBC membrane are the characteristic feature of WAIHA, independent of the etiology of the disease. The data suggest that not the RBC itself but autologous immunoglobulin of isotype IgG with a specificity for

RBC structures might be the autoantigen in WAIHA [132]. To identify the epitope specificity of IgM and IgG within the IgM-IgG immune complexes of WAIHA patients and to characterize the mechanism of immune complex-RBC interaction, is at the center of further investigations. The molecular and structural characterization of IgM of WAIHA patients will be of particular significance for the further understanding of the pathophysiological processes leading to WAIHA. The results of these studies may define targets for therapeutic intervention in WAIHA at a causal disease-specific level.

However, the observation of altered IgM-IgG immune complexes in WAIHA may also be of conceptual interest for the understanding of the impact of immune complexes on the regulation of immune responses. Immune complexes typically consist of antigens bound to antibodies specific for the corresponding antigen. Depending on the immunoglobulin isotype, an immune complex may activate complement. Immune complexes contribute critically to the regulation of normal immune responses toward self- and non-self-antigens under physiological conditions. They mediate increased uptake and processing of antigen by antigen presenting cells [101, 136, 137], influence signal transduction in antigen presenting cells, such as B lymphocytes, by receptor cross-linking [138–140] and may contribute to T cell tolerance by binding to immature dendritic cells in the absence of co-stimulation [141]. Immune complexes exert immunoregulatory functions by binding to receptor structures, such as Fc γ receptors, the B cell receptor (BCR) or complement receptors. IgG2a-chromatin immune complexes in mice activate autoreactive B cells by synergistic engagement of the BCR and a Toll-like receptor, positioning immune regulation by immune complexes in the central interface of the innate and the adaptive immune systems [142]. Immune complexes consisting exclusively of immunoglobulin molecules, i.e. Ig-Ig immune complexes, may be formed either according to the principles of an idiotype-anti-idiotype reaction [51], or – if the antigenic determinants of the Ig molecule belong to the Fc region of the molecule – represent rheumatoid factors [143]. Ig-Ig immune complexes might use all of the above mentioned mechanisms to shape the immune response.

Besides patients with IgG mediated WAIHA, an enhanced frequency of IgM-IgG immune complexes in plasma has so far been described in patients with Graves' disease and in patients with hepatitis A. A decreased frequency of IgM-IgG immune complexes in plasma has been demonstrated in patients with gastric cancer [144]. In WAIHA patients, IgG3 and IgG1 are actively enriched in IgM-IgG immune complexes, despite of a normal IgG subclass distribution in plasma (IgG 1 > IgG 3 > IgG 2 > IgG 4) [132]. The IgG subclass distribution in IgM-IgG immune complexes of human plasma has not yet been systematically investigated. However, one should expect that the different physicochemical and sterical properties of the

various IgG subclasses, which are due to different hinge regions [145, 146], favor a selective enrichment of IgG subclasses in IgM-IgG immune complexes under physiological conditions. Such selective enrichment of IgG subclasses in IgM-IgG immune complexes of plasma might differ from the normal IgG subclass distribution in plasma itself and contribute to the physiological functions of IgM-IgG immune complexes. Taken together, the future structural and functional analysis of IgM-IgG immune complexes may reveal novel regulatory mechanisms for immune responses to self-immunoglobulin under physiological conditions in humans. Of particular interest will be the question of how pro-inflammatory conditions contribute to the generation and function of altered IgM-IgG immune complexes, given that such conditions may lead to a subpopulation of anti-F(ab')₂ antibodies that cover a part of the physiologically active Fc domain of their counterpart and are nevertheless able to immobilize the Fab arms [36, 38].

Mechanisms of Immunoregulation in WAIHA as a Model to Investigate Regulation of Complex Dynamic Biological Systems

The data summarized in this review focus on the aspects that the study of warm autoimmune hemolytic anemia may contribute to the understanding of immunoregulation in the human system and demonstrate that immunohematology contributes to transfusion safety much more than diagnostic strategies. Immunohematology provides a conceptual understanding of compatibility underlying diagnostic strategies. Beyond-genome biology strongly indicates that the specificity of functional modules of the immune response contributes to the overall outcome of immune responses. Thus, specificity – and consequently compatibility – may be defined by functional aspects of the immune system in its respective condition as much as by structural components of immune cells and immune receptors. Immunohematology in the context of the current state of the art in medicine and biology still provides excellent models to study mechanisms that are both clinically relevant and of fundamental significance for the understanding of principles of the immune response.

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