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An experimental model of cryoglobulin-associated vasculitis in mice

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Introduction

Cryoglobulins are immunoglobulins (Ig) that precipitate at temperatures lower than 37°C with resolution upon warming [1–3]. Since their initial characterization by Lerner et al. [1], it has been recognized that they are not a single distinct group of Ig, but heterogeneous with respect to their composition and physical properties. Cryoglobulins can be classified into three types [3] according to the presence in the cryoprecipitates of: monoclonal Ig (type I), both monoclonal and polyclonal Ig (type II), or polyclonal Ig with or without other serum proteins or exogenous antigens (type III). Monoclonal type I cryoglobulins are mostly associated with various lymphoproliferative disorders. Mixed type II or type III cryoglobulins are often found in serum from patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis or with a variety of chronic infectious diseases.

Clearly, the interaction between Ig molecules plays a critical role in the formation of cryoglobulins, since the sole or major components of a vast majority of cryoglobulins are Ig. The demonstration of rheumatoid factor (RF) and/or anti-idiotypic-like activity in IgM present in mixed cryoglobulins suggests the involvement of immune mechanisms in the generation of mixed cryoglobulins [2–6]. However, one should be aware that mixed cryoglobulins could be generated following in vitro, but not in vivo, interaction of low-affinity IgM RF with IgG complexes during the incubation of sera in test tubes at 4°C to isolate cryoglobulins.

The molecular mechanisms responsible for the generation of type I monoclonal cryoglobulins are less clear. Available data suggest that several different mechanisms may be operative depending upon the particular cryoimmunoglobulins. Certain monoclonal Ig appear to form cryoprecipitates by self-association of Ig molecules via nonspecific physicochemical interaction [7–9], although a common physicochemical mechanism underlying such interactions has not been elucidated. An alternative, intriguing hypothesis is that cryoglobulins represent temperature-sensitive immunological interactions between the Fab region of the cryoprotein and antigenic determinants on the same molecules, i.e. an autoantibody-like reaction, as shown in

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the two cases of monoclonal IgM cold agglutinins [10, 11]. It is of interest to note that similar cold agglutinins with cryoglobulin activity can be induced in mice after immunization with bacterial polysaccharide antigen [12].

The presence of cryoglobulins could result in a wide range of vascular, renal and neurological complications, depending on their relative concentrations, their temperature-dependent solubility behavior, and the nature and type of proteins involved [3]. However, the precise cellular and molecular mechanisms involved in the induction of cryoglobulin-associated pathologies have not yet been well defined. This situation is at least partly due to the scarcity of relevant experimental models. Models of cryoglobulinemia should reproduce the appearance of one or other type of cryoglobulins, and some of the main pathological features usually observed in severe human cryoglobulinemia. In this article, we will focus on a unique physicochemical property of murine IgG3 that exhibit cryoglobulin activity, and discuss the pathogenesis of IgG3 cryoglobulin-associated vascular lesions in mice.

Cryoglobulin activity of murine IgG3

IgG3: the major source of cryoglobulins in mice

MRL-*lpr/lpr* autoimmune mice spontaneously develop a lupus-like syndrome characterized by unique immunopathological manifestations such as arthritic-like lesions, necrotizing vascular lesions of the skin of ears and footpads, and severe glomerulonephritis [13]. In parallel, they produce the most striking amounts of cryoglobulins among several lupus-prone mice like (NZB × NZW)F1 or BXSB mice [13, 14]. Cryoglobulins from MRL-*lpr/lpr* mice are composed almost exclusively of IgG of polyclonal origin. Strikingly, the IgG3 content of cryoglobulins is markedly enriched, as compared with other IgG subclasses [14]. This preferential participation of IgG3 in the formation of polyclonal cryoglobulins is not an exclusivity of autoimmune MRL-*lpr/lpr* mice, but can also be found in non-autoimmune mice after polyclonal stimulation of B lymphocytes with bacterial lipopolysaccharides (LPS) or malaria parasites [14]. The absence of specific concentrations of anti-LPS, anti-malaria or anti-DNA antibodies in the respective cryoprecipitates excludes a possible involvement of idiotype-anti-idiotype interaction in the generation of these cryoglobulins.

Since IgG3 is selectively concentrated in spontaneous and induced cryoglobulins, it is likely that the self-association and subsequent cryoprecipitation of murine IgG3 molecules is the principal mechanism responsible for the generation of cryoglobulins in mice. To address this question, we have assessed the ability of IgG3 monoclonal antibodies (mAb) from autoimmune MRL-*lpr/lpr* and (NZB × NZW)F1 mice to generate cryoglobulins. Out of 32 IgG3 mAb, independent of their immunological specificities, 28 are capable of generating cryoglobulins composed exclusively of each IgG3 mAb without the intervention of any antigens, and are thus classified as type I monoclonal cryoglobulins [14–16]. The importance of the γ3 constant (Cγ3) region for cryoglobulin generation has been most directly demonstrated by Ig class-switch experiments: the cryoglobulin activity is gained following the Ig class switch of mAb from IgM to IgG3, but lost following the class switch from IgG3 to IgM or IgG1 [14, 17–19]. The cryoglobulin activity of IgG3 is not a unique phenomenon in autoimmune mice, because 6 of 8 IgG3 anti-dinitrophenyl (DNP) mAb derived from non-

autoimmune mice similarly develop cryoglobulins [20]. Such an activity of IgG3 mAb has also been noticed by others [17, 21–23].

Fc-dependent self-association of IgG3

Clearly, murine IgG3 have a unique physicochemical property which allows them to self-associate via their Fc-Fc interactions, independently of their specificities and origins. However, this unique property of murine IgG3 to self-associate is not limited to cryoprecipitating mAb. In fact, both cryoprecipitating and non-cryoprecipitating IgG3 mAb are able to interact with each other in a quantitatively similar manner, but not with other IgG subclasses [14, 20]. This nonspecific IgG3-IgG3 interaction would account for the microaggregate formation observed in the first described murine IgG3 myeloma protein, J606 [24]. The complete identity of the nucleotide sequence of the Cγ3 region between the non-cryoprecipitable J606 protein and a cryoprecipitable 6–19 IgG3 RF mAb derived from MRL-*lpr/lpr* mice rules out any abnormality in the Cγ3 region of either cryoprecipitable or non-cryoprecipitable IgG3 protein [25]. Thus, it is clear that the Cγ3 domain is necessary for the self-association of IgG3 mAb, but not sufficient in itself to determine cryoprecipitation.

The molecular basis for the peculiar self-associating activity of murine IgG3 is still unclear. Because of the presence of a potential asparagine (*N*)-linked glycosylation site on the C_H3 domain uniquely present in murine IgG3 [26], it has been speculated that the carbohydrate side chain possibly attached there may play a role in the formation of self-associating IgG3 complexes. This possibility has been explored by analyzing the self-associating ability of a 5–7B IgG3 RF mutant mAb lacking the potential glycosylation site in the C_H3 domain [27]. Although the author has claimed a significant reduction of the self-associating capacity of the mutant mAb, our recent study with the 6–19 IgG3 RF mAb has clearly shown that the self-associating ability is indistinguishable between wild-type and similarly mutated 6–19 RF mAb (unpublished observation), thus excluding the possible role of oligosaccharide chains in the C_H3 domain in the IgG3 self-association. It should be mentioned that human IgG3 have physicochemical properties similar to those of murine IgG3. All the human IgG3 myeloma proteins studied undergo a concentration- and temperature-dependent aggregation, though not always cryoprecipitation [28–31]. Although this aggregating site is apparently localized in the Fd fragment of the human IgG3 heavy (H) chain [29], it remains to de determined whether this is also the case for murine IgG3.

Role of the electrostatic charge in the H-chain variable region for IgG3 cryoglobulin activity

Studies demonstrating inhibition of cryoprecipitation by anti-idiotypic antibodies [32] implicate some role of the variable (V) region. More significantly, it has been shown that the cryoprecipitation of anti-DNP IgG3 mAb is completely inhibited after the binding of anionic DNP-amino acid conjugates, and could be enhanced by the binding of cationic conjugates [20]. This is consistent with the idea that electrostatic interactions are a significant factor in the precipitation process, as suggested by studies on human monoclonal cryoglobulins [8, 33, 34]. It is not surprising, however, that isoelectric point determinations of human monoclonal cryoglobulins and murine

Table 1. Role of the V_H amino acid sequences in the cryoglobulin activity of murine IgG3 mAb

mAb[a]	H-chain[b]	L-chain	Cryoglobulin
6–19	6–19 (Q/K)	6–19	++
6–19/9A6	6–19 (Q/K)	9A6	++
9A6	9A6 (E/A)	9A6	–
9A6/6–19	9A6 (E/A)	6–19	–
$6\text{–}19_{Q6E/K23A}$	6–19 (E/A)	6–19	+
C11M	C11M (E/S)	C11M	++
DN6	DN6 (Q/K)	DN6	–

[a] 6–19/9A6 and 9A6/6–19 mAb are hybrid IgG3 antibodies. $6\text{–}19_{Q6E/K23A}$ mAb is a 6–19 mutant mAb at the two V_H residues 6 (glutamine to glutamic acid) and 23 (lysine to alanine)

[b] Amino acid residues at positions 6 and 23 are indicated in parentheses (*Q* glutamine, *K* lysine, *E* glutamic acid, *A* alanine, *S* serine)

IgG3 cryoglobulins fail to demonstrate differences between cryo- and non-cryoimmunoglobulins, because the presence of only a few electrostatic contacts may be all that is necessary to produce abnormal precipitation.

Modulation of cryoglobulin activity as a result of the binding of charged hapten strongly suggests the implication of the V region sequences in cryoprecipitation of self-associating IgG3 complexes, probably by providing additional positively charged residues. This idea is further supported by the demonstration that the cryoglobulin activity of IgG3 hybrid mAb composed of H-chains derived from either cryoglobulins or non-cryoglobulins is associated with the presence of H-chains derived from cryogenic IgG3 mAb (Table 1). More specifically, residues 6 and 23 of the H-chain V domain (V_H) have been postulated to play a role in cryoprecipitation in a study comparing a panel of cryogenic and non-cryogenic monoclonal IgG3 [35]. Neutral glutamine and cationic lysine are found at positions 6 and 23 in cryoglobulins, respectively, while their counterparts in non-cryoglobulins are more negatively charged (anionic glutamic acid at position 6 and a neutral amino acid at position 23). Further studies on the 5–7B IgG3 RF monoclonal cryoglobulin mutated at position 6 or 23 have shown that V_H residue 6 is an important factor in the cryoprecipitation of this IgG3 mAb [35]. However, although the residues 6 and 23 of V_H are consistently observed to be more positively charged in the cryoprecipitating populations than in the non-cryoprecipitating populations, there are exceptions. C11M anti-DNP mAb exhibits a strong cryoglobulin activity, despite the fact that the residues at V_H positions 6 and 23 are identical to those seen in non-cryoglobulins (Table 1). To the contrary, DN6 anti-IgG1 mAb fails to cryoprecipitate, although glutamine and lysine are present at positions 6 and 23, respectively, like cryogenic IgG3. In addition, we have observed that mutation of the 6–19 anti-IgG2a RF IgG3 mAb to non-cryoglobulin-like residues at positions 6 and 23 significantly, but not completely, reduces its cryoglobulin activity (Table 1). All these results indicate that the cryoglobulin activity of murine IgG3 is additionally determined by a factor other than the V_H amino acid sequence.

Role of galactosylation in IgG3 cryoglobulin activity

IgG are glycoproteins, in which the C_H2 domain of each H-chain bears an *N*-linked biantennary carbohydrate side chain. Each of these oligosaccharide chains, most of

Table 2. Role of the IgG galactosylation in the cryoglobulin activity of murine IgG3 mAb

IgG[a]	Galactosylated glycoforms[b]				Cryo
	G0 (%)	G1 (%)	G2 (%)	$\frac{G1 + G2}{G0}$	
6–19	49.3	35.9	14.7	1.03	+++
L8D	36.4	49.3	14.3	1.75	++
6–19J	21.9	55.4	22.7	3.57	+

[a] L8D mAb made of 6–19 IgG3 H-chains and J558 L-chains is obtained following the fusion of 6–19 hybridoma cells with J558L H-chain loss mutant myeloma cells [43]. 6–19J mAb made of the same H- and L-chains is generated following the transfection of J558L cells with the $VDJ_{H6\text{-}19\text{-}C\gamma3}$ plasmid [39].
[b] The molar ratios of G0 (no galactose, terminating in *N*-acetylglucosamine), G1 (one terminal galactose and one terminal *N*-acetylglucosamine) and G2 (two terminal galactose residues) glycoforms

which are non-sialyated and neutral, ends either by two terminal galactose residues (G2), one terminal galactose and one terminal *N*-acetylglucosamine (G1) or two terminal *N*-acetylglucosamines (G0) [36]. Since the level of terminal galactosylation is highly heterogeneous and substantially diminished in some diseases [36–38], differences in the structure of carbohydrate side chains present in the C_H2 domain of IgG may play an important role in determining the cryoglobulin activity of self-associating IgG3 complexes. We have thus explored the possibility of a relationship between the cryoglobulin activity and the content of galactose residues in the oligosaccharide side chains on the H-chains. For this purpose, we have generated by two different means (cell fusion and transfection) two cell lines secreting hybrid IgG3 mAb made of the same H-chains (derived from 6–19 RF) with a potentially different galactosylation pattern (because of their synthesis in different myeloma cells) and of the same light (L) chains from J558 anti-α1-3 dextran antibodies [39]. The assessment of cryoglobulin activity of these two IgG3 mAb, identical in the amino acid sequence of their H- and L-chains, has demonstrated that the cryoglobulin activity is significantly reduced (approximately four times less) in the antibody having an increased proportion of galactosylated H-chains (Table 2). More recently, we have established an IgG3 mAb from mice expressing a 6–19 H-chain transgene, which bears the transgenic 6–19 IgG3 H-chain and an endogenous L-chain, which is by chance identical to the 6–19 L-chain. Most strikingly, this IgG3 "6–19" mAb completely fails to exhibit cryoglobulin activity, and has again an increased content of galactosylated H-chains (manuscript in preparation). This clearly indicates that the galactose content of oligosaccharide chains plays a key role in determining the cryoglobulin activity of IgG3 mAb. Although at present we do not have a good explanation for the role of galactose content in the IgG3 cryoglobulin activity, one likely explanation may be that the conformation of IgG3 molecules is somehow modified as a result of an increased content of galactose residues, thereby reducing their cryoprecipitating activity, as suggested by our recent study [39].

The observation that more galactosylated IgG3 have a lesser cryoglobulin activity raises an important question: what controls IgG galactosylation? It has been reported that B lymphocytes from patients with rheumatoid arthritis and from MRL-*lpr/lpr* mice have a reduced β-1,4-galactosyltransferase activity [40–42], which may account for an increased proportion of IgG lacking galactose in parallel to the progression of their diseases [36, 38]. However, a marked, but not complete reduction in the

proportion of galactosylated IgG in mice deficient in β-1,4-galactosyltransferase indicates the involvement of additional galactosyltransferase(s) for IgG galactosylation (manuscript in preparation). In addition, we have observed an increased percentage of non-galactosylated IgG in MRL-*lpr/lpr* mice, but not in MRL-+/+ mice or in C57BL/6 mice bearing the *lpr* mutation (manuscript in preparation). It thus appears that a decrease in the level of galactosylated IgG, putatively resulting from a decreased level of galactosyltransferase in B lymphocytes, involves the *lpr* (Fas) mutation in combination with the MRL genetic background. This mutation probably results in an increased life-span of activated B lymphocytes, because of functional defects in Fas-mediated apoptosis. In an autoimmune-prone genetic background such as MRL, expansion of some autoreactive B cell clones may be accompanied by a progressive decrease in galactosyltransferase activity of these aging cells, thus favoring the appearance of undergalactosylated autoantibodies, which could be more pathogenic because of a higher cryoglobulin activity. If so, the dysregulated expression of galactosyltransferase involved in IgG galactosylation could be a significant genetic factor predisposing to the development of autoantibody-mediated tissue lesions.

Pathogenic potential of murine IgG3 monoclonal cryoglobulins

Induction of cryoglobulin-associated glomerular and cutaneous vascular lesions by IgG3 monoclonal cryoglobulins

Since most IgG3 mAb, independently of their specificities, formed cryoglobulins, we have explored whether cryoprecipitable IgG3 mAb are able to provoke tissue lesions in normal mice after intraperitoneal injection of IgG3-secreting hybridomas. The 6–19 IgG3 anti-IgG2a RF mAb, derived from MRL-*lpr/lpr* mice, induces a remarkable pathology [15, 16]. At 5–7 days after the injection of 6–19 IgG3 RF hybridoma, vascular purpura, which is the most common manifestation in patients with cryoglobulinemia [3], develops in the skin of the ears, tails and footpads, which are not well protected by hair from thermic variations. Histological examination of the skin lesions shows an intracapillary precipitation of cryoglobulins associated with an extensive infiltration of polymorphonuclear leukocytes (PMN) around small vessels and into subcutaneous tissue, and a massive extravasation of erythrocytes (Fig. 1). Moreover, the 6–19 mAb induced a severe acute glomerulonephritis. In the initial phase, the predominant glomerular lesions are characterized by exudation of PMN and a mild proliferation of glomerular cells (Fig. 2A). A progressive accumulation of cryoglobulins in the subendothelial spaces of glomerular capillary walls leads to the formation of glomerular lesions resembling the "wire-loop" lesion typical of lupus nephritis (Fig. 2B). In contrast, IgM and IgG1 class-switch variants of the 6–19 IgG3 mAb, which lose the cryoglobulin activity, fail to generate skin and glomerular lesions (Fig. 2C) [18, 19]. This indicates that the cryoprecipitating activity of IgG3 RF mAb is critically involved in the development of skin vasculitis and glomerulonephritis induced by the 6–19 IgG3 RF mAb. This is consistent with the finding that among a panel of anti-IgG2a RF mAb of different Ig isotypes, including clonally related mAb bearing five different Ig isotypes, only the RF mAb able to cryoprecipitate – all from the IgG3 subclass – can induce skin and glomerular lesions [25].

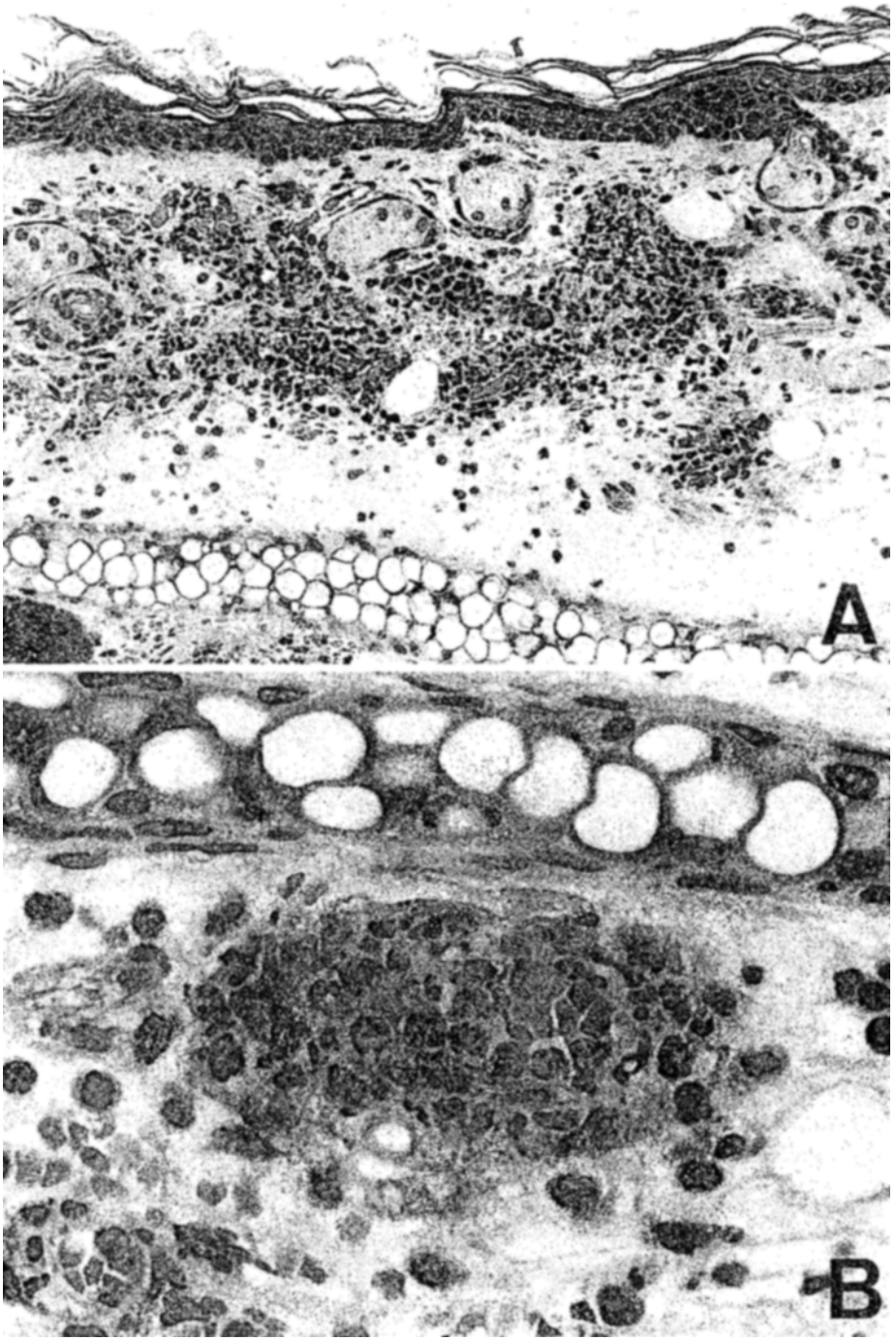

Fig. 1A, B. Representative histological appearance of skin vascular lesions of ears 7 days after the intraperitoneal implantation of the 6–19 IgG3 RF hybridoma cells into (BALB/c × MRL)F1 mice. Leukocytoclastic vasculitis is characterized by the infiltration of PMN and the extravasation of erythrocytes (*PMN* polymorphonuclear leucocytes). H+E; **A** ×100, **B** ×400

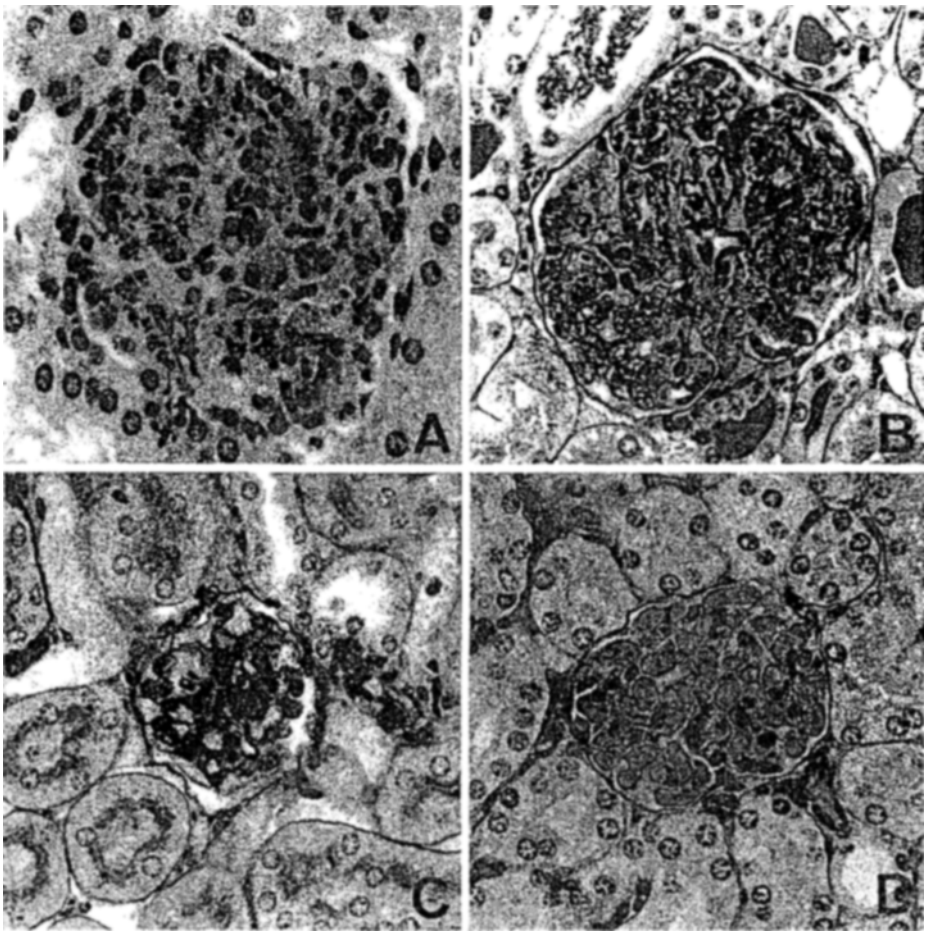

Fig. 2. **A** Representative histological appearance of glomerular lesions in mice sacrificed 5 days after the injection with 6–19 hybridoma cells. Note the infiltration of PMN and increased glomerular cellularity. **B** Representative histological appearance of glomerular lesions in mice killed 8 days after the 6–19 hybridoma injection. Note the presence of PAS-positive deposits along the glomerular capillary walls, resembling "wire-loop" lesions. **C** Representative histological appearance of glomerulus from mice injected with hybridoma cells secreting 6–19 IgG1 class-switch variant. In contrast to mice receiving hybridoma cells secreting 6–19 IgG3 mAb, no significant glomerular changes are observed. **D** Representative histological appearance of glomerular lesions in PMN-depleted mice 8 days after the injection with 6–19 hybridoma cells. Their glomerular lesions are characterized by the voluminous intracapillary deposition of PAS-positive material which nearly occlude the capillary lumen, but the absence of "wire-loop" lesions (*PAS* periodic acid-Schiff). **A** H+E, **B–D** PAS; **A–D** ×200

Since the 6–19 mAb has both anti-IgG2a RF and cryoglobulin activities, it is important to determine the role of the RF activity in the pathogenicity of the 6–19 mAb. To answer this question, we have determined the pathogenic effect of the 6–19 RF mAb in mice depleted of B lymphocytes by treatment with anti-IgM antibodies from birth or in SCID (severe combined immune deficiency) mice [43, 44]. Both B lymphocyte-deficient mice, thus lacking the corresponding IgG2a autoantigen, develop

Table 3. Development of glomerular and skin vascular lesions in mice injected with 6–19 hybridoma cells

Mice	GN	Skin lesion
Wild-type	+	+
Ig-deficient	+	–
C3-deficient	+	+
FcγR-deficient	+	–
PMN-depleted	(+)[a]	–

GN: Glomerulonephritis

[a] PMN-depleted mice fail to develop "wire-loop" glomerular lesions, but present a different type of glomerular lesion, characterized by voluminous intracapillary thrombi [49]

glomerular lesions as severe as those induced in immunologically normal mice, but completely fail to develop skin vascular lesions (Table 3). This strongly suggests that different pathological mechanisms govern the development of each tissue lesion. Both RF and cryoglobulin activities, and hence IgG3–IgG2a cryoglobulin immune complexes (IC) are required to induce cutaneous vascular lesions, while the renal pathogenicity is dependent on the cryoglobulin activity, but not on the anti-IgG2a RF activity. This conclusion is further supported by the finding that glomerular, but not cutaneous, lesions are similarly induced by a hybrid IgG3 mAb made of the 6–19 IgG3 H-chains and the L-chains from J558 anti-α1-3 dextran antibodies, devoid of the RF activity [43].

It should be emphasized, however, that only a fraction of IgG3 monoclonal cryoglobulins with other specificities are able to provoke significant glomerular lesions, independently of serum levels of cryoglobulins [15, 16, 22]. This would well explain the fact that the quantity of circulating cryoglobulins does not always correlate with the degree of nephropathy in patients with cryoglobulinemia [45]. Since there is no mutation in the Cγ3 region of the pathogenic 6–19 mAb [25], it may be either that a particular V_H sequence by itself is sufficient or that a unique combination of H- and L-chains is required for the expression of the renal pathogenicity of IgG3 cryoglobulins. Our preliminary results on transgenic mice expressing either the H-chain or both H- and L-chains of the 6–19 IgG3 RF mAb have shown similarly increased production of IgG3 cryoglobulins in both 6–19 H single and 6–19 H/L double-transgenic mice, despite their differences in L-chain composition of transgenic IgG3. Interestingly, only the 6–19 H/L double-transgenic mice expressing high levels of 6–19 IgG3 RF spontaneously develop severe chronic glomerulonephritis. This result suggests that the majority of IgG3 cryoglobulins resulting from a heterogeneous combination of the transgenic 6–19 H-chains with endogenous L-chains in the 6–19 H single transgenic mice are poorly pathogenic. This strongly argues for the potential role of the ligand-binding property of the 6–19 RF mAb in the generation of glomerular lesions. An attractive hypothesis is that the 6–19 RF mAb may recognize a cross-reactive antigen present or implanted in glomeruli, thereby provoking glomerular inflammation. The presence of autoantibodies reactive with glomerular antigens has been reported in lupus-prone mice [46, 47], and the nephritogenic activity of some IgG3 anti-DNA mAb may be mediated through their interaction with nucleosomal antigens implanted in glomeruli [48]. However, a recent study has shown that glomerular lesions similar to those induced by the 6–19 RF mAb are provoked by an IgG3 (8A4) mAb against a hydrogenase from *Wolinella succinogens*, but not by its mutant

lacking three amino acids in the L-chain V region, though maintaining the same ligand-binding specificity [23]. This could raise another possibility that the pathogenicity of IgG3 cryoglobulins can be determined by physicochemical properties, probably related to the V region amino acid sequences, irrespective of their antigen-binding specificity.

Role of mast cells expressing IgG Fc receptors, but not complement, in the development of skin vasculitis induced by 6–19 IgG3 RF monoclonal cryoglobulins

PMN are the principal inflammatory cells in skin vascular lesions caused by 6–19 RF mAb. As expected, no skin purpuric lesions develop in mice depleted of PMN by the combined treatments of irradiation and anti-PMN mAb (Table 3) [49]. Notably, the treatment of mice with mAb against LFA-1 (CD11a) or ICAM-1 (CD54) almost completely suppresses the development of 6–19 RF cryoglobulin-induced skin lesions. Strikingly, mice depleted of C3 after treatment with cobra venom factor or genetically deficient in C3 still develop typical cutaneous vascular lesions (Table 3) [49]. Thus, complement activation does not play a major role in the pathogenesis of PMN-dependent skin vascular lesions induced by 6–19 RF monoclonal cryoglobulins, despite the fact that IgG3–IgG2a IC are critically involved in the generation of cryoglobulin-associated skin vascular lesions. This suggests the involvement of other types of PMN chemoattractants in this model of cutaneous leukocytoclastic vasculitis.

It should be also stressed that among a number of IgG3 monoclonal cryoglobulins studied, anti-IgG2a RF monoclonal cryoglobulins are the only mAb capable of inducing skin vascular lesions. The failure of non-RF IgG3 cryoglobulins to generate skin vasculitis may be due to insufficient amounts of the IC formation by certain mAb or related to different properties of IC generated. However, we have recently observed that mice injected with IgG3 anti-DNA hybridoma cells fail to develop skin vascular lesions, despite the presence of substantial amounts of circulating DNA-anti-DNA IC and the development of severe glomerulonephritis (unpublished observation). The IC formation is probably a result of a massive release of nuclear antigens from implanted hybridoma cells. This raises the possibility that IgG2a, present in IgG3 RF IC, may play a crucial role in the pathogenesis of skin vascular lesions. Since the IgG2a, but not IgG3, isotype is known to efficiently interact with IgG Fc receptors (FcγR) [50], FcγR-dependent activation of effector cells, such as mast cells, may be responsible for the generation of skin vasculitis, as in the case of the Arthus reaction [51, 52]. In fact, mice deficient in FcγR are unable to develop skin vasculitis following the implantation of 6–19 RF hybridoma cells (Table 3), but are capable of doing so after the transfer of wild-type FcγR-expressing mast cells [53]. We speculate that the activation of mast cells, through the interaction of FcγR with IgG2a-containing IgG3 cryoglobulin IC, may result in the release of inflammatory mediators that recruit and activate PMN, thereby generating leukocytoclastic vascular lesions. Further analysis with mice deficient in tumor necrosis factor (TNF) has demonstrated that TNF released from activated mast cells is one of the mediators responsible for triggering this type of vasculitis [53], illustrating the clinical significance of FcγR and TNF in IC-mediated autoimmune vascular syndrome.

Absent role of FcγR and complement activation in the development of glomerulonephritis induced by 6–19 IgG3 RF monoclonal cryoglobulins

In a marked contrast to the pathogenetic mechanisms responsible for cutaneous leukocytoclastic vascular lesions, the development of glomerulonephritis induced by 6–19 RF monoclonal cryoglobulins is not dependent on the activation of FcγR-expressing cells (Table 3) [53]. As FcγR does not have any significant affinity to the IgG3 isotype [50], the lack of any inhibition of glomerular lesions in FcγR-deficient mice is consistent with the fact that the development of these glomerular lesions is independent of the formation of IgG3–IgG2a IC [43, 44]. It has been recently reported that FcγR-mediated inflammatory responses play an important role in the pathogenesis of lupus nephritis occurring in (NZB × NZW)F1 hybrid mice [54]. However, MRL-*lpr/lpr* mice deficient in FcγR still develop lethal glomerulonephritis as severe as that in conventional MRL-*lpr/lpr* mice (T. Saito, personal communication). Considering that MRL-*lpr/lpr* mice spontaneously generate extremely large amounts of IgG3 cryoglobulins [13], these data suggest that lupus nephritis occurring in MRL-*lpr/lpr* mice might be more dependent on the production of IgG3 cryoglobulins, and thus less dependent on FcγR, unlike (NZB × NZW)F1 hybrid mice. This is consistent with the observation that the progression of lupus nephritis in MRL-*lpr/lpr* mice is correlated with the production of IgG3 autoantibodies [55–57].

It should be stressed that C3-depleted or C3 null mutant mice still develop typical glomerular lesions following the implantation of 6–19 RF hybridoma cells (Table 3) [49]. The presence of substantial PMN infiltration in glomerular lesions in C3-deficient mice receiving 6–19 hybridoma cells indicates that the complement activation and subsequent C3 deposition do not play a major role in the PMN recruitment and subsequent generation of "wire-loop" glomerular lesions. This is in agreement with the lack of PMN infiltration and of "wire-loop" lesions in IgG3 anti-DNP cryoglobulin-induced glomerular pathology, despite the presence of substantial C3 deposits [58].

Role of PMN in the development of "wire-loop" glomerular lesions induced by 6–19 IgG3 RF monoclonal cryoglobulins

Like in skin vascular lesions, PMN are also the major inflammatory cells in the initial phase of 6–19 RF-induced glomerular lesions. Significantly, mice depleted of PMN fail to develop "wire-loop" lesions normally induced by the 6–19 mAb, but present a different type of glomerular lesion (Table 3) [49]. Predominant glomerular changes are characterized by voluminous intracapillary thrombi that almost completely obstruct the glomerular capillary lumen (Fig. 2D). Examination by electron microscope reveals a diffuse plugging of glomerular capillaries by amorphous deposits, while subendothelial deposits resembling "wire-loop" lesions are hardly detectable in the PMN-depleted mice. It is of interest to note that these glomerular lesions are essentially identical to those induced by IgG3 anti-DNP monoclonal cryoglobulins, which are unable to provoke glomerular infiltration of PMN [58]. These results indicate a critical role for PMN in the generation of "wire-loop" glomerular lesions induced by the 6–19 RF monoclonal cryoglobulins. IgG3 RF mAb are capable of producing "wire-loop" lesions in the absence of corresponding IgG2a autoantigens or C3. Therefore, the development of these two different types of glomerular lesions

is unlikely to be related to the different capacities of IgG3 cryoglobulins to induce IC formation and subsequent complement activation.

It remains to be determined how glomerular deposits of 6–19 RF cryoglobulins lead to PMN infiltration and how PMN infiltration is involved in the generation of "wire-loop" glomerular lesions. The ability of cryoglobulins to precipitate in the subendothelial space and/or in the glomerular capillary lumen is likely to be related to physicochemical properties or ligand-binding properties of each individual cryoglobulin. Since mesangial cells are the initial site of cryoglobulin accumulation in glomeruli, a unique conformation of IgG3 cryoglobulins, either resulting from a particular pair of V_H and V_L amino acid sequences or from the specific binding to target antigens, may promote a direct interaction with and subsequent activation of mesangial cells. An attractive hypothesis is that mesangial cells may express a receptor specific for the IgG3 isotype, but their interaction may be strongly influenced by the conformation of IgG3 cryoglobulins. The presence of IgG3-specific "FcγR", different from conventional FcγR, has been suggested by several studies [59, 60], although this has not yet been formally proved. If this is indeed the case, IgG3 uptake through the putative IgG3-specific "FcγR" by mesangial cells may be critical for determining the renal pathogenicity of IgG3 cryoglobulins. Consequently, proinflammatory mediators released following the activation of mesangial cells probably play an active role in the PMN recruitment and subsequent activation, which leads to glomerular endothelial cell damage, ending up with "wire-loop" lesions. In this respect, we have recently observed a marked glomerular induction of chemokines in mice injected with 6–19 hybridoma cells, but not in those injected with anti-DNP hybridoma cells (unpublished observation).

Future directions

The development of a murine model of acute cryoglobulinemia associated with vascular and glomerular pathology following the implantation of hybridoma cells secreting IgG3 mAb represents a good opportunity to further define the pathogenetic mechanisms leading to the lesions, and also to evaluate various therapeutic approaches based on an interference at different levels of essential pathogenic pathways. In addition, in view of the wide range of pathological manifestations seen in human patients with cryoglobulinemia, the generation of a chronic model of cryoglobulinemia might further help determine immunopathological consequences as a result of the persistent and chronic presence of cryoglobulins, which cannot be studied in hybridoma transplantation experiments. For this reason, we have recently established the transgenic mice expressing either H-chains or H- and L-chains of the IgG3 6–19 RF mAb. These transgenic mice expressing the IgG3 6–19 RF mAb indeed develop severe chronic glomerulonephritis, whose lesions are highly heterogeneous similar to those seen in lupus-prone mice, but distinct from those acutely induced by the implantation of 6–19 hybridoma cells. More interestingly, these mice develop necrotizing arteritis, affecting small to medium-sized arteries in kidneys and skeletal muscle, but fail to develop cutaneous leukocytoclastic vasculitis, a typical vascular lesion induced by the implantation of 6–19 hybridoma cells. This indicates that a single cryoglobulin is able to induce different types of glomerular and vascular lesions, depending on the levels and kinetics of its production. Clearly, comparative analysis on the 6–19 H/L double transgenic mice and 6–19 hybridoma-injected mice would allow us to better define pathogenic mechanisms leading to the spontaneous

development of glomerular and vascular lesions as a consequence of the acute and chronic presence of pathogenic cryoglobulins. A better understanding of molecular and cellular events involved in the generation of cryoglobulin-mediated tissue lesions could have clinical implications in the design of future therapeutic strategies.

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