Human Rheumatoid Factor Cross-idiotypes. IV.
Studies on WA XId-Positive IgM Without
Rheumatoid Factor Activity Provide Evidence that
the WA XId Is Not Unique to Rheumatoid Factors
and Is Distinct from the 17.109 and G6 XIds

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Summary

The WA cross-idiotype (XId) is the major XId among human monoclonal rheumatoid factors
(mRF) and is almost always associated with the light (L) chain XId, 17.109, and the heavy (H)
chain XId, G6. A cell line, 35G6, was cloned that bears the WA XId, but shows no reactivity
with immunoglobulin G (IgG) and is negative for the 17.109 and G6 XIds. The 35G6 L chain
appears to be derived from the same V,,IIIJKI genes as most WA mRFs L chains. In contrast
to the WA mRFs H chains in which V 1 genes are used, the 35G6 IgM expresses a V.3 gene.
Sequence comparisons with other WA XId-positive mRF suggested several common structural
features that may be related to the WA XId and differences that may relate to lack of IgG reactivity.

Cells similar to 35G6 have previously been described in pokeweed mitogen-stimulated cell lines
of peripheral blood lymphocytes from normal individuals and patients with rheumatoid arthritis
and type II mixed cryoglobulinemia. These observations were confirmed, and in addition, it
was shown that the majority of WA XId-positive cells in these cultures were negative for the
17.109 and G6 XIds. The presence of the WA XId in the absence of IgG reactivity suggests
that the WA XId is more directly associated with an antigen specificity other than IgG, and
its association with RF activity may be incidental. It is postulated that these WA XId-positive
RF-negative antibodies may serve a physiologic role as natural antibodies to a pervasive pathogen,
and that IgG reactivity is a consequence of somatic diversification accompanying proliferation
of the WA XId-positive RF-negative cell.

The WA cross-idiotype (XId)1, the major cross-idiotype
among human monoclonal rheumatoid factors (mRF),
is a conformational antigenic determinant involving both H
and L chains and appears to be located in the antigen binding
site (1, 2). The L chain–associated XId identified by the mAb
17.109 and the H chain–associated XId detected by the G6
mAb have been reported to occur in almost all WA XId-
positive mRFs (3) and has led to the notion that there is re-
stricted expression of these germline genes with little or no
somatic mutation in the WA mRFs. Earlier, it had been postu-
lated that the V„IIIb L chain detected by 17.109 mAb and
encoded by the Humkv325 gene was the structural basis for
the mRF WA XId and was unique to IgM with RF activity
(4); however, this hypothesis was disproved (2), and it has
been shown that the 17.109 and G6 XIds occur separately
(5) and together (6) in IgM without RF activity.

Although the WA XId occurs in high frequency among
mRF in serum cryoglobulins from patients with type II
cryoglobulinemia, it appears to occur in only small amounts
among polyclonal RFs in patients with rheumatoid arthritis
(7, 8). Although the WA XId has not been reported among
monoclonal IgM immunoglobulins without RF activity,
among polyclonal RFs, WA XId-positive RF-negative Igs
have been reported. WA XId-positive RF-negative plasma
cells were detected in PWM-stimulated PBL cultures from
normal individuals and patients with rheumatoid arthritis

Footnotes

1 Abbreviations used in this paper: mRF, monoclonal RF; XId, WA cross-
idiotype.

The sequences reported in this paper have been deposited in the GenBank
data base under accession numbers M97268 and M87269.
and were also found in synovia of patients with rheumatoid arthritis (10). Moreover, a high incidence of WA XId-positive RF-negative mRF and 35G6 were prepared by standard methodologies. The Wa-biotin and 35G6 were prepared by standard methodologies. The F(ab')2 goat anti-McD as the ligand. Biotin-labeled Wa and 35G6 were used for inhibition. Either 25 μl of Wa-mAb or 35G6-biotin at 2 μg/ml was incubated with 25 μl of varying concentrations of inhibitor at room temperature in the coated plate for 2 h. The plates were then processed in routine manner. Percent inhibition was determined as follows: 100/OD x (no inhibitor) [OD (no inhibitor) – OD (inhibitor)]. The amount of inhibition that yields 50% inhibition is the I50. Comparison of relative I50 is made by setting the I50 of WA mRF at 1.0.

The competitive inhibition assay for V,Illb was performed as previously described (15). The assays for the 17.109 Xld, V,1-associated Xlds using the G6 or G8 mAbs, and the V,3-associated Xlds using the B6 or D12 mAbs and Glo antigens using Glo 86.3 mAb were performed in the same way. In addition, Glo 86.3 was tested directly for reactivity with 35G6. The WA mRF was used as the ligand for the V,1-Xld assay; the Glo protein was used as the ligand for the 17.109 Xld, V,3-Xld, and Glo assays. Assays for viral Ab activities (cytomegalic, hepatitis A, B, and C, and HIV-1)
and various autoantibodies (antinuclear, thyroglobulin, microsomal, cytoplasm, DNA, DNA histones, and BSA Abs) were performed by routine methods.

PWM-induced plasma cells and staining of plasma cells for IgG, WA XId, and aggregate IgG binding has been previously described in detail (9). Staining with mAbs 17.109 or G6 were done in the same way except that a fluorescein-labeled F(ab')2 anti-mouse was used as a second Ab. Slides of cell samples from the original cultures and additional cultures prepared in the same laboratory at about the same time as the original were stored at -70 °C until used in this study.

Oligonucleotides. The following oligonucleotide primers were used: V3 FR1, TCCCTGAGACTCTCCTGTGC; V H3 Leader, GCTGGCTTTTTCTTGTGGCT; constant (CM-31), GGA-ATTCTCACAGGAGACGA; μ constant (CH1-24), TCACAG-GAGACGGGGAAGAA; V3 FR1, GCCACCCTGTCTTTGCTCCA; V JII Leader, TCTCCTTGCTACTCTGGCTCC; κ constant, TCTCCAGATGCGCCGAAGAT; and Vr-D junction, TCTATTACTGTGCCGCCC.

RNA-PCR and DNA Sequencing. Total RNA was prepared by standard methods. Reverse transcription, using 20 pmol κ- or μ-C region primer or 100 pmol random hexamer primer, and 200 U MoMuLV reverse transcriptase (Superscript RNase H-; Bethesda Research Laboratories, Gaithersburg, MD), and PCR amplification for 30 cycles with a thermal profile of 1 min at 94°C, 1 min at 51°C for H chain primers or 1 min at 55°C for L chain primers, and the Vr-D junction/μ constant (CH1-24) primer pair, and 1 min at 72°C, was performed (16). Asymmetric PCR (17, 18) was performed similarly except that a 1:20 ratio of primers was used for 40 cycles. Both strands of each cDNA were sequenced with Sequenase (US. Biochemical, Cleveland, OH) in replicate with each primer. Direct sequencing was employed rather than sequencing cloned PCR products to minimize potential PCR artifacts (18). 35G6 DNA sequences were compared with sequences found in GenBank (release 69).

Results

Characterization of the 35G6 Cell Line and IgM

Protein Analysis. The 35G6 clone produced only IgMx immunoglobulin. By NH2-terminal amino acid analysis, the first 21 amino acids of the L chain were identical to the product of the VJII gene Humkv325 (19), and the first 19 amino acids of the H chain were identical to the product of the VH3 gene, Vn26 (20).

Sequence of the L Chain. The sequence for the L chain mRNA/cDNA (Fig. 1 A) revealed a close homology with the VJIIb germline L chain gene Humkv325 (19) (97.9% homology). The six point mutations in the coding region resulted in four changes in the amino acid sequence; each of the CDR has a change in amino acid, as does FR2. No mutations were found in the J1 gene. Compared with Humkv325, an additional codon was found at the V-J junction.

Sequence of the H Chain. The sequence for the H chain mRNA/cDNA (Fig. 1 B) revealed a close homology with the V3 germine gene VH26 (96.6% homology) (20). Ten base changes were observed and three 3' terminal nucleotides of the V gene appear to have been deleted. Four of the changes coded for amino acid changes: one in FR1, two in CDR1, and one in CDR2. The D region could not be assigned to a specific D gene and a JD gene was utilized, which contained two point mutations.

Further confirmation of the sequence for the V-D junction of 35G6 H chain mRNA was obtained by performing an RNA-PCR with an oligonucleotide spanning the Vr-D junction and the μ constant primer (CH1–24) at stringent annealing conditions which would permit amplification only if the Vr-D junction primer annealed along its full length without interruption or mismatch. The anticipated 130-bp product (data not shown) was obtained.

Comparison of 35G6 and WA mRFs V Genes. Previous studies (21–23) of genes associated with the WA XId have noted the essential role of VJIIb genes, the preferential use of J1, Vn1, and J4 genes, and a 9–10 amino acid beginning with glutamic acid and ending with proline. 35G6 similarly utilizes VJIIb-J1 genes and a nine-amino acid Dn ending in proline linked to J4 but lacks the initial glutamic acid in Dn and uses a V3 instead of a Vn1 gene (Table 1).

The NH2-terminal J4 residue of 35G6 (amino acid 100)
is tyrosine rather than phenylalanine, but Wol, a WA mRF, also has a tyrosine at this position. The only WA mRF reported to use a J\(_h\) gene other than J\(_h4\) is RF-TS1, which uses a J\(_h3\) gene. J\(_h3\) is the same length as J\(_h4\) and bears the closest homology to J\(_h4\) of the J\(_h\) genes. The presence of J\(_h3\) does not affect the reactivity of RF-TS1 with G6 or G8 (24) or with the WA XId reagent in this study.

Another difference between 35G6 IgM and other WA proteins is that the arginine (position 94) at the \(\text{V}_\alpha\)-D junction has been deleted (Fig. 1 B; GenBank, release 69). The arginine at 94 is also common in PO mRFs (25).

**Serologic Studies of the 35G6 IgM.** The 35G6 IgM was shown to have the WA XId antigen by both ELISA (Fig. 2) and immunoblot analysis (Fig. 3). Quantitative WA XId analysis showed 1 \(\mu\)g/ml of 35G6 IgM to give the same inhibition as 0.85 \(\mu\)g/ml of WA mRF (Table 2). Considering the 10% variation of the assay, essentially all of the 35G6 IgM bears the WA XId. Direct comparison of the presence of WA XId antigen using either WA mRF or 35G6 IgM binding to the same anti-WA XId reagent (goat anti-McD) showed essentially the same relative inhibition (Table 2).

35G6 IgM showed no RF activity in concentrations < 100 \(\mu\)g/ml in an IgG binding assay that could detect as little as 0.01 \(\mu\)g/ml of mRF and also lacked the L and H chain XId associated with WA mRFs. The 35G6 IgM had ~10% of the reactivity of WA mRFs with the 17.109 mAb by inhibition assay. The weak reactivity of 17.109 with 35G6 IgM was confirmed by the failure to demonstrate reactivity by di-

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**Table 2.** Comparison of Inhibition of WA mRF, 35G6 IgM Binding to Anti-WA XId (F\(\text{Fab}^\prime\) goat anti-McD), Monoclonal 17.109 to Glo mRF, and Monoclonal G6 Binding to WA mRF by Various Monoclonal RFs

| Inhibitor | WA mRF | 35G6 IgM | 17.109 | G6 |
|-----------|---------|----------|--------|-----|
| 35G6      | 1.2*    | 0.8      | 9.2    | >200|
| Wa†       | 1.0     | 1.0      | 1.0    | 1.0 |
| Glo†      | 0.5     | 0.5      | 0.4    | >200|
| Gol       | 0.6     | ND       | 4.6    | 135 |
| McD†      | 0.6     | 0.6      | 2.5    | 4.6 |
| Bla‡      | >10.0   | >10.0    | >16    | >200|
| Pu‡       | >10.0   | >10.0    | >16    | >200|
| Sha‡      | >10.0   | >10.0    | >16    | >200|

* Ig, WA mRF = 1.0.
† WA mRF.
‡ Bla mRF.
§ Non-RF mIgM.

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rect binding ELISA or immunoblot assays. Glo, Gol, and 35G6 L chain sequences differ from that of Sie (the immunogen for 17.109) by 4, 8, and 11 residues (GenBank, release 69), respectively. Comparison of reactivities of these proteins with 17.109 and anti-WA XId (Table 2) indicates that a few amino acid changes in the V_{III} L chain can greatly reduce reactivity with 17.109, although they do not affect reactivity with the anti-WA XId. For example, Glo and Gol had almost identical reactivities with anti-WA XId, but Gol had <10% of the reactivity with 17.109 shown by Glo. As expected, both 35G6 IgM and Glo mRF did not inhibit G6 binding because both possess V_{α3} H chains, but it is notable that, of five WA mRFs, only two were G6 positive, and one of these (McD) was only weakly positive (Table 2). The 35G6 IgM did not inhibit the B6 and D12 V_{α3}-associated XId assays and did not react with mAb Glo 86.3, which has been shown to have reactivity with the WA mRFs and Po mRF, a V_{α3} protein (26) (data not shown). No viral or autoantigen reactivity was found with 35G6 IgM. The 35G6 IgM did react with staphylococcal protein A, as do most IgM with V_{α3} H chains (27).

Immunofluorescence Studies of PWM-stimulated PBLs from Normal Individuals and Patients with RA. WA XId-positive RF-negative plasma cell cultures from PWM-stimulated PBL from normal individuals and patients with RA (four HL, BB, NA, and BB from the original study [9] and seven additional cultures) were reexamined for WA, 17.109, and G6 XId antigens, and aggregated IgG binding (Table 3). The previous findings that WA XId-positive cells were negative for aggregated binding were confirmed. In the entire study, only a rare WA XId-positive cell was found with aggregated IgG binding. The percentage of cells that were positive for 17.109 and G6 was similar but substantially less than the observed percentage of WA XId-positive cells. Cells from patient DE were also tested with anti-McD and mAb 17.109 absorbed in the same manner and with the same normal human serum (NHS) used in the original study, i.e., 12% NHS was added to each antiserum (9). WA XId-positive cells decreased from 24 to 18%. There was no change in 4% positive 17.109 cells. The relatively small drop in the percentage of WA XId-positive cells in this study (6%) compared with the sample in the original study (45%) is most likely attributable to the more complete absorption of the antisera in this study, resulting from the more sensitive assays used for the detection of contaminating Abs, i.e., ELISA versus hemagglutinin.

By double-staining analysis, the majority of WA XId-positive cells were negative for both 17.109 and G6; all cells positive for 17.109 or G6 were also positive for WA XId (data not shown). The percentages of cells reactive with WA XId and either 17.109 or G6 were not altered by the order of

Table 3. Cytoplasmic Igs and WA XId Detected in RA and Control Cells after 6-d Culture of PBL with PWM

| RF Patient | Percent plasma cells of total cells | Percent WA XId positive cells of total plasma cells | Percent 17.109 positive cells of all plasma cells | Percent G6 positive cells of all plasma cells | Percent cells binding aggregated IgG of all plasma cells |
|------------|------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| DU         | 14.5%                              | 33%                                         | 7%                                           | 7%                                           | 0%                                           |
| HL         | 12.6%                              | 15%                                         | 6%                                           | 11%                                          | 0%                                           |
| BB         | 10.6%                              | 10%                                         | 4%                                           | ND                                           | 0%                                           |
| FE         | 11.2%                              | 46%                                         | 4%                                           | 8%                                           | 0%                                           |
| DE         | 25.0%                              | 24%                                         | 4%                                           | 1%                                           | 0%                                           |
| QT         | 10.0%                              | 8%                                          | 0%                                           | 3%                                           | 0%                                           |
| AL         | 36.0%                              | 33%                                         | 14%                                          | 13%                                          | 0%                                           |
| VI         | 11.8%                              | 27%                                         | 3%                                           | 10%                                          | 0%                                           |
| Control    |                                    |                                              |                                              |                                              |                                               |
| NA         | 13.3%                              | 4%                                          | <1%                                          | <1%                                          | 0%                                           |
| BP         | 15.0%                              | 3.3%                                        | 1.5%                                         | 1.0%                                         | 0%                                           |
| VB         | 14.2%                              | 3.5%                                        | 1.3%                                         | 0.98%                                        | 0%                                           |

* Data indicate the percentage of cells showing cytoplasmic staining with fluorescent anti-human Igs (see Materials and Methods), as counted among 100 total cells identified by phase contrast.  
† Data indicate the percentage of cells showing WA XId stained with anti McD and fluorescein-conjugated second Ab (see Materials and Methods), as counted among 100 Ig-containing cells identified by staining with rhodamine-conjugated antihuman Ig Abs.  
§ Data indicate the percentage of cells stained with either mouse monoclonal 17.109 or mouse monoclonal G6 and fluorescein-conjugated second Ab (see Materials and Methods), as counted among 100 Ig-containing cells identified by staining with rhodamine-conjugated antihuman Ig Abs.  
∥ Data indicates the percentage of cells binding fluorescein-conjugated IgG, as counted among 100 Ig-containing cells identified by staining with rhodamine-conjugated antihuman Ig Abs.
staining with the anti-McD and mAb 17.109 mAb G6. An example of the paucity of 17.109-positive cells among WA XId-positive cells is illustrated in Fig. 4.

**Discussion**

These studies indicate that the conformational H-L chain antigen that constitutes the WA XId is not restricted to RFs and does not require the presence of the 17.109 or G6 XIds. Furthermore, certain Vn3 as well as Vn1 H chains can generate the WA XId.

The data presented in this study, which demonstrate that relatively few mutations in the _Humk325_ gene markedly decrease the expression of the 17.109 XId without affecting the WA XId, indicate that previous conclusions regarding the prevalences of WA mRFs based on serologic studies with mAb 17.109 are not valid. Also, from dual staining immunofluorescent studies with the WA XId reagent and either 17.109 or
Comparison of 35G6 L and H chain structure with those of WA and other mRFs suggests that the WA XId is determined by structural motifs contributed by conserved FR3 present on V\(_{\lambda}1\) and V\(_{\lambda}3\) H chains, a discrete-sized D region, and use of J\(_{\lambda}3\) or J\(_{\lambda}4\) in combination with specific V\(_{\lambda}I\)Hb L chains. Of interest regarding the discrete-sized D region requirement is the recent report that 17.109 positive G6 positive IgM without RF activity produced in transfecotomas have marked variability in the length of the D region (6). Because all 17.109, G6 mRFs previously studied have been WA XId-positive, the requirement of the discrete-sized D region can be tested by assaying these transfecotoma IgMs for WA XId.

From comparative studies of 35G6 with Wa mRF, IgG reactivity appears to be related to the presence of arginine at position 94 and glutamine at position 95 in Wa mRF H chains. The presence of an extra proline at the V\(_{\lambda}3\)-J\(_{\lambda}\) junction in 35G6 is against the hypothesis that such an additional proline is important for RF activity (27). An additional amino acid at the V\(_{\lambda}3\)-J\(_{\lambda}\) junction is relatively uncommon, and it has been proposed that the mechanism for generation of such additional amino acids may be an abnormal process (28). The additional amino acid at this position in a human Ab from normal individuals (29) and in the 35G6 IgM that was generated from a normal individual indicates that the mechanism involved is more likely a normal one.

The original explanation (9) for the observation that WA XId-positive RF-negative cells are present in PWM-stimulated (PBL) cultures was that pentameric IgM capable of binding aggregated IgG was not present in these cells. Our finding that the 35G6 cell line produces a WA XId-positive RF-negative IgM suggests an alternative explanation that the WA XId-positive RF-negative cells are progenitors of the cells that do produce WA XId-positive RF-positive IgM. The high prevalence of WA XId-positive cells in PWM-PBL cultures compared with the prevalences of 17.109-positive and G6-positive cells may be due to somatic mutation of the germline genes that encode the 17.109 and G6 XIds or may indicate that other genes encode the WA XId. The data demonstrating that relatively few mutations in the Humkv325 gene greatly decrease the expression of 17.109 XId without affecting the WA XId, and the rarity of expression of genes other than Humkv325 in Wa mRF, favor the former possibility.

It was initially thought that increased prevalence of WA XId-positive plasma cells in patients with rheumatoid arthritis may be related to the disease process, but small amounts of 17.109 XId and G6 XId found in the blood were interpreted as against this possibility (4). The finding that with somatic mutation the 17.109 XId is lost and the WA XId retained, coupled with studies that demonstrated overexpression of somatically mutated Humkv325 genes in synovial tissue (28), indicate that the absence of 17.109 and G6 XIds does not exclude Wa mRF.

Pernis et al. (10) have suggested that the WA XId-positive RF-negative cell represents a regulatory cell type. The presence of a molecule with the WA XId but without IgG reactivity would permit modulation of anti-WA XId without the presence of RF in the circulation. The identification of WA XId-positive RF-negative cells in normal individuals and in patients with rheumatoid arthritis may be consistent with the hypothesis that the WA XId-positive RF-negative IgM cell modulates suppression of RF production. The detection of WA XId-positive RF-negative IgM cells in patients with juvenile rheumatoid arthritis may reflect this suppression (11). However, if this hypothesis were correct, the WA XId-positive RF-negative cell would be absent or reduced in patients with essential mixed cryoglobulinemia; this is not the case (9).

An alternative hypothesis is that the WA XId-positive RF-negative cell has a physiologic role, which is the secretion of natural Ab to a common pathogen, and that RF activity is a cross-reactivity that develops with somatic mutation accompanying proliferation of these cells. Both the serologic data on Wa mRFs and the immunofluorescent studies on the PWM-stimulated cells indicate that the WA XIds are preserved in the presence of mutation of the germline genes that encode the 17.109 and G6 XIds. If the main association of cross-idiotypes is with antigen specificity of the Ab as first described (30, 31), then conservation of the WA XId suggests an antigen selection process for WA XId-positive cells. The recent finding of chronic hepatitis C virus (HCV) infection in patients with mixed cryoglobulinemia (32) suggests that HCV may be the pathogen that stimulates proliferation of the WA XId-positive RF-negative cells.

The mRFs encoded by germline genes have been found to cross-react with non-IgG antigens (15, 33). The finding that random pairing of 17.109 L chains and G6 H chain did not produce IgM with RF activity and that certain somatic mutations of CDR3 are required for generation of reactivity with IgG and other self-antigens (6) is consistent with the possibility that the germline genes that encode the 17.109-positive G6-positive IgM are preserved by selection for reactivity with antigens other than IgG. Hence, the RF activity of WA mRFs may be incidental and unrelated to the physiologic role of these Abs but may play a role in disease as a result of untoward proliferation and involvement in immune complex-mediated inflammation.

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