

HUMAN RHEUMATOID FACTOR CROSSIDIOTYPES
I. WA and BLA Are Heat-labile Conformational Antigens Requiring
Both Heavy and Light Chains

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Three human monoclonal rheumatoid factor (mRF) crossidiotypic (XId) groups, WA, PO, and BLA, were defined using polyclonal antisera (1, 2). Both the WA and the BLA XIds appeared to be associated with the antigen-combining site (2), and serologic studies (3, 4) indicated that the antisera defining the WA XId appeared to recognize a determinant requiring both H and L chains. Structural characterization of the XIds was not done. Extensive studies (5-7) have recently been performed, however, to determine the structural basis of the WA XId. Investigators using antisera to primary structures on the H and L chains postulated that the WA XId resides on the L chain. Our study, in which we used the original antisera that defined the WA and BLA XIds and additional polyclonal antisera, presents evidence that the WA and BLA XIds are conformational antigens requiring both L and H chains, and that with denaturation, the antigens that define the XId and the antigen-binding activity are lost in parallel.

Materials and Methods

Preparation of Reagents and Antisera. IgM mRFs were obtained and isolated as previously described (2). Antisera used to type the WA XId (anti-McD) and BLA XId (anti-Bla) were previously described (2). Anti-PSL2, a synthetic peptide corresponding to the second complementarity-determining region (CDR) and adjacent residues on the L chain of the IgM RF Sie (8), was provided by Drs. P. P. Chen and D. A. Carson (Scripps Clinic, La Jolla, CA).

Preparation of H and L Chains. H and L chains of mRFs were prepared by routine partial reduction with 10 mM DTT, alkylated and isolated in 1 M propionic acid on a G100 Sephadex column. Purity of H and L chains was established, and recombination of H and L chains was performed by mixing H and L chain pools in a 1:2 M ratio, respectively, and by dialyzing them for 72 h at 4°C against a 0.5 M Tris, 0.15 M NaCl buffer (pH 7.2). Recombined H and L chains were isolated with G200 Sephadex column chromatography. The recombinant pools were tested for free κ or μ chains by immunodiffusion in agarose gel using nonspecific antisera to κ and μ chains.

Immunodotting and Blotting. 100 μ l of each mRF diluted to 10 μ g/ml in PBS was "dotted" onto 0.45- μ m nitrocellulose paper using a hybrid dot filtration manifold (Schleicher & Schuell, Inc., Keene, NH). Parallel sample sets were stained with Coomassie blue or blocked with 1% BSA/PBS and probed with WA or BLA XId-typing reagents. The filter

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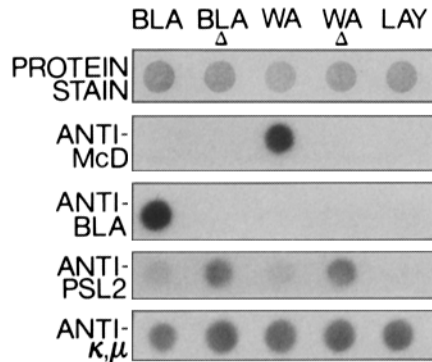


FIGURE 1. Replica nitrocellulose strips dotted with 1 μ g of Bla mRF, Bla mRF heated (Δ), Wa mRF, Wa mRF heated (Δ), and Lay mRF (PO XId group). Top strip is stained for protein with Coomassie blue. Second strip is probed with anti-McD, the WA XId typing reagent. Third strip is probed with anti-Bla, the BLA XId typing reagent. Fourth strip is probed with anti-PSL2, and the fifth strip is probed with a mixture of anti- κ and anti- μ .

strips were washed with PBS plus 0.02% BSA and 0.05% Tween 20 (PBT) and were incubated with 125 I-labeled F(ab')₂ rabbit anti-goat IgG (5.2 μ Ci/mg at 10^6 cpm/ml) for 1 h at 37°C, were washed several times with PBT, and exposed to XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY). The reactivity of anti-McD and anti-Bla with denatured H and L chains isolated by SDS-PAGE was tested by probing Western blots of the gel in the same method noted previously for dots.

Direct WA XId Assays. Wa mRF was coated on microtiter plates (NUNC/Immulon II; USA Scientific Plastics Inc., Ocala, FL) at 0.1 μ g/ml in 0.05 M Tris (pH 9.5) overnight at room temperature. Equal volumes of F(ab')₂ goat anti-McD at 1:500 and PBS or inhibitor were incubated for 1 h at 37°C, and 100 μ l of the inhibitor or control mixture was added per well, incubated 1 h at 37°C, and assayed with alkaline phosphatase-labeled F(ab')₂ rabbit anti-goat IgG H- and L-chain serum (Pel-Freeze Biologicals, Rogers, AR). The BLA Id assay was performed in a similar way using a Bla mRF coat (0.1 μ g/ml) and F(ab')₂ goat anti-Bla diluted 1:10,000. The same format was used for the IgG-binding assay, except that human IgG was coated at 10 μ g/ml, mRFs were incubated with the coat 1 h at 37°C and overnight at 4°C, and peroxidase-labeled F(ab')₂ goat anti-human IgM (1:500) was used as the second antibody. Direct optical density readings were taken as measure of binding.

Results

The reactivity of anti-McD, the WA XId typing antiserum, and anti-Bla, the BLA XId-typing antiserum, as determined by immunoblot assay, is shown in Fig. 1. The WA XId reagent reacted only with Wa mRF. When heated at 90°C for 20 min, Wa mRF showed no reactivity. Similarly, the BLA XId reagent reacted only with native Bla. Four other tested mRFs of the WA group, McD, Dri, Blo, and Gar, lost reactivity with the WA XId reagent when heated at 90°C for 20 min. No other mRFs of the BLA group were available for testing. We also tested the reactivity of the WA and BLA XId reagents with completely denatured H and L chains of Bla and Wa mRFs separated on SDS-PAGE. Probes of Western blots of SDS-PAGE separation of mRFs at 20 times the concentration used in Fig. 1 showed no positive reactions with κ chains and only occasional weak reactivity of the WA XId reagent with Wa H chains. In contrast to the anti-XId sera, which did not react with heat-denatured mRF, anti-PSL2 raised against the

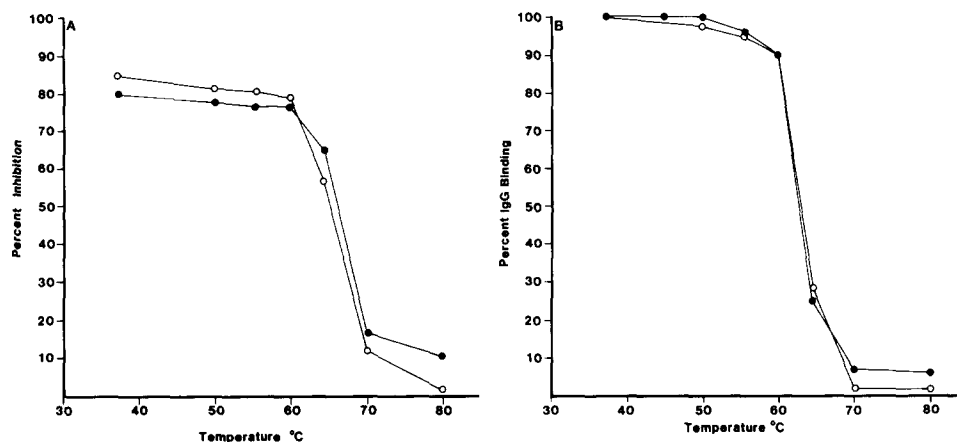


FIGURE 2. (A) Inhibition in their respective Xid assays of samples of Bla mRF (solid circles) and Wa mRF (open circles) at $1 \mu\text{g/ml}$ heated at various temperatures for 20 min. (B) Duplicates of each sample run in experiments shown in A tested for binding to IgG Sepharose.

TABLE I
Effect of Acid Conditions on WA Xid

Acid condition	Decrease WA Xid (%) after incubation for:			
	4°C			25°C
	24 h	48 h	72 h	72 h
pH 2.8, glycine-HCl	4	6	17	36
pH 4.0, 0.1 N acetic acid	9	11	21	54
pH 2.3, 1.0 N acetic acid	28	34	42	100

L chain second hypervariable region (L-CDR2) of Sie, a Wa mRF, and shown to react with mRFs of the WA Xid group but not the PO Xid group (8), reacted more strongly with both heat-denatured Wa and Bla mRFs. Routine anti- κ and anti- μ typing sera reacted equally well with both native and denatured mRFs.

Results of further studies of the effect of heat on the WA and BLA Xid antigens are shown in Fig. 2 A. There was a sharp loss of Wa and Bla reactivity in their respective Xid assays beginning at $\sim 60^\circ\text{C}$ with almost total loss of reactivity at 70°C . Fig. 2 B shows that antigen-binding activity by Wa and Bla mRFs appears to be lost over an almost identical temperature range.

Sucrose density gradient ultracentrifugation was performed on the heated mRFs. With increasing temperature between 60°C and 70°C there was progressive aggregation of the 19S IgM mRFs so that at 70°C no monomeric IgM mRF was detectable.

The effect of three acid conditions commonly used in the preparation of RFs on the Xid antigens of the Wa and Bla mRFs was tested at 4°C and at room temperature (25°C) for varying times. The results obtained with Wa mRF are shown in Table I; results with Bla mRF were similar. The greatest decrease in Wa Xid was caused by 1 N acetic acid. Treatment with pH 2.8 glycine-HCl caused the smallest loss of Xid antigens.

The requirement of both H and L chains for expression of both WA and BLA Xids was shown by chain recombination experiments (Fig. 3). The reactivity of

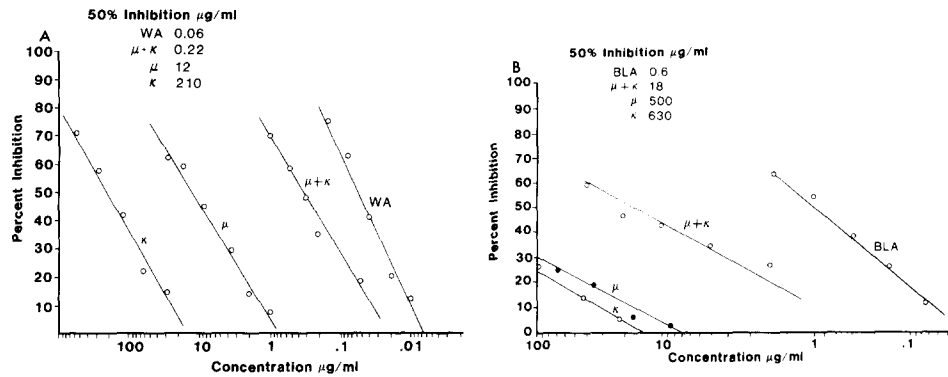


FIGURE 3. (A) Inhibition in WA XId assay of whole Wa mRF (WA), Wa μ chains (μ), Wa κ chain (κ), and isolated recombinant Wa μ and Wa κ chain ($\mu + \kappa$). Concentration of each preparation giving 50% inhibition is shown at top left corner. (B) Same experiment as shown in A with use of Bla preparations in BLA XId assay.

the partially reduced μ chain as compared with that of the partially reduced κ chain cannot be assessed accurately because antibodies specific for either chain cannot be excluded. In both the WA and BLA experiments, however, the μ chains showed greater inhibition than the κ chains.

Discussion

This study provides evidence that the WA and BLA XIds as originally defined (1, 2) are present on the native antibody molecules, require both H and L chains, and are lost with denaturation. These findings also apply to the Bla private Id since the BLA XId was tested on Bla and not on another rheumatoid factor of the BLA group. Heating of Bla and Wa mRFs resulted in aggregation. It is unlikely, however, that loss of reactivity of the XId antisera was due to random masking of XId determinants because reactivity with other antisera to H and L chains was unaffected. Anti-PSL2 has been shown to react ~ 10 times greater with denatured isolated L chains than with the intact mRF (8). The increased reactivity of anti-PSL2 with heated Wa and Bla mRFs indicates loss of native structure and exposure of hidden primary structures with heating, thus supporting the possibility that heating destroys the conformational determinants of the XId. These findings also indicate that heating can differentiate between WA and BLA XIds determined by conformational antigens and the primary sequence-dependent XId, PSL2-CRI (5). These experiments also show that PSL2-CRI is not unique to Wa mRFs since it is present on Bla, the prototype mRF for the BLA group.

The possibility that the parallel loss of XIds and antigen-binding activities is coincidental cannot be excluded. Nevertheless, in view of the previous evidence (2) that antigens determining the BLA and WA XIds are associated with the antigen-combining sites, it is likely that the XIds are destroyed with heating along with the antigen-binding activity of the antibodies.

The relatively narrow temperature range over which denaturation of XIds was seen in our study has not been noted previously. Whether this is a feature of other Ids remains undetermined. Preliminary studies with polyclonal RFs

indicate that binding activity diminishes over a similar temperature range (9). Therefore, heat lability may be a common feature of combining-site Ids of RFs. Another observation in our study has not been noted previously: denaturation of RFs under the acid conditions commonly used for their preparation can cause marked loss of Id antigens.

In view of our data, it is untenable to postulate that PSL2 is the structural correlate of the WA XId (7). Use of antisera to primary structures of the CDR to delineate the structural basis of the WA XId is unlikely to be a productive approach since this Id is a conformational antigen requiring both H and L chains. A more promising approach would be assorted chain recombination studies, such as those used to delineate Ids of antiarsonate antibodies (10). We did not perform assorted chain recombination studies, and therefore the relative roles of the κ and μ chains in the formation of the WA XId were undetermined. Nevertheless, results of the limited studies performed suggest that the H rather than the L chains may play the major role. The role of the κ IIb L chains in the WA XId may be nonspecific, as previously postulated (4). The XId defined by anti-PSL2 may be totally unrelated to the WA XId or antibody specificity.

Summary

Evidence was obtained that both the WA and BLA crossidiotypic (XId) groups are conformational antigens requiring both L and H chains and that with heat denaturation the antigens that define the XIds and antigen-binding activity are lost in parallel. In contrast, the primary structure-dependent crossreactive idiotype (CRI), PSL2, which is only weakly detected on native Wa and Bla monoclonal rheumatoid factors (mRFs), became prominently detected on the heated Wa and Bla mRFs. Heat denaturation may provide a simple method for distinguishing Ids determined by conformational antigen from primary structure-dependent Ids. In addition to heat denaturation, some acid conditions commonly used for preparation of RFs were also found to cause marked loss of Id antigen. The finding of PSL2-CRI on Bla mRF indicates that this Id is not unique to the WA XId.

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