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The Integrity of the Ball-and-Socket Joint Between V and C Domains Is Essential for Complete Activity of a Humanized Antibody

Nicholas F. Landolfi, 1 Archana B. Thakur, Helen Fu, Max Vásquez, Cary Queen, and Naoya Tsurushita

AF2 is a high affinity murine Ab possessing potent neutralizing activity against human IFN-γ. In carrying out the modifications to humanize this Ab, we discovered that an initial version displayed affinity for IFN-γ that was slightly less than that of AF2, but exhibited IFN-γ-neutralizing activity that was severely diminished. Characterization via site-directed mutagenesis revealed that the majority of this loss in IFN-γ-neutralizing activity was due to altering the V H framework residue at position 11. V H position 11 is distal to the binding surface of the Ab; however, it, along with residues 110 and 112, have been identified as forming the socket of a molecular ball-and-socket joint between the V and C domains of the Ig Fab, which influences the elbow angle between these domains. To determine whether disrupting the structure of this joint was the basis for reduced IFN-γ-neutralizing capacity, we altered residue 148 of C H1 , which with residue 149 comprises the corresponding ball portion of the joint. Changing this single C H1 domain residue diminished the ability of the Ab to neutralize IFN-γ to a level similar to that observed with the V H alteration. Thus, an intact ball-and-socket joint between the V and C domains in AF2 is required for potent neutralization of IFN-γ. These results suggest the importance of the elbow angle between Ig V and C domains in Ab activity, and support the hypothesis that this joint can be an important functional element of Ab structure. The Journal of Immunology, 2001, 166: 1748–1754.

Interferon-γ is a cytokine produced by activated T lymphocytes and NK cells that exhibits a variety of immunoregulatory activities (1–3). Possibly the most crucial of these is the role IFN-γ plays in the generation and maintenance of cells of the Th1 lineage (4, 5). As Th1 cells have been implicated in a variety of autoimmune diseases (6), neutralization of IFN-γ is an attractive approach for the potential treatment of such conditions.

AF2 is a high affinity murine anti-human IFN-γ mAb that displays potent IFN-γ-neutralizing activity (7). The determinant recognized by AF2 has been mapped and is proximal to, but distinct from, the surface of IFN-γ that interacts with its receptor, yet AF2 prevents IFN-γ from binding to IFN-γR 2 cells (7).

The neutralizing activity of AF2 makes it a suitable agent for interfering with IFN-γ activity in patients in whom IFN-γ may contribute to the disease state. However, as a murine Ab, AF2 would elicit a vigorous human anti-mouse Ab response (8). Humanization is a strategy proven to eliminate most, if not all, of the human anti-mouse Ab response (9). This technique involves replacing all residues of a murine Ab not essential for Ag binding with those that occur in a human Ig molecule. Thus, the murine C regions of both the H and L chains are replaced with human counterparts. The H and L chain V regions present more of a challenge, as retaining only complementarity-determining region (CDR) 2 residues of the murine Ab is usually insufficient to confer the level of Ag binding exhibited by the original murine Ab. Additional murine residues proximal to the CDRs in three-dimensional space are frequently required to retain the binding affinity and biological activity of the initial murine Ab (10). Frequently, humanized Abs have affinities and biologic activities indistinguishable from the murine counterpart, and in rare instances humanization can actually improve the affinity of a murine Ab (11).

Successful humanization of murine Abs has now become routine, and in a number of instances has allowed the therapeutic potential of murine mAbs to be realized. Four humanized Abs have been approved for use in humans (12–15) and several more are in late phase clinical trials. Thus, as a first step to clinical development of AF2 as an IFN-γ antagonist, we undertook humanization of the Ab.

The initial humanized version of AF2 displayed a relative binding affinity slightly lower than that of the original murine Ab; however, the capacity to neutralize IFN-γ was greatly diminished. Comprehensive mutational analysis implicated a V H framework residue distal to the Ag binding site as responsible for the majority of this loss. This framework residue maps to the interface between the V and C domains of Igs and has been identified as a component of a molecular ball-and-socket joint between these domains. It has been hypothesized that this general structural feature of Abs is one of functional importance, as it may modulate flexibility between these domains (16).

In this study, we present evidence that the integrity of the molecular ball-and-socket joint between V and C domains is essential for full biologic activity of AF2. Disruption of this joint by alterations in either the V or C domains profoundly interferes with the biological activity (neutralization of IFN-γ) of this Ab, with little
effect on Ag binding. These results support the contention that the ball-and-socket joint is a structural element of the Ig molecule that can possess functional importance.

Materials and Methods

Reagents

AF2 is a murine anti-human IFN-γ mAb previously described (7). Human rIFN-γ was purchased from R&D Systems (Minneapolis, MN). Hs294T, COS-7, and L243 cells were obtained from American Type Culture Collection (Manassas, VA). The HLA-DR-specific mAb L243 (17) was purified from exhausted culture medium of the hybridoma. Fluorescein-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP-conjugated avidin was obtained from Vector Laboratories (Burlingame, CA).

Computer analysis, modeling, and design of humanized AF2 versions

Three-dimensional models of the V domains of the AF2 Ab using the amino acid sequence determined (see below) were built with the help of the ABMOD and ENCAD programs (18, 19). Sequence analysis, homology searches, and structural analysis of three-dimensional models were conducted with the program Delta (20). For humanization, the approach of Queen et al. (21) was followed. First, sequences of human V regions most identical with AF2 were identified. Among the most similar VH sequences is Eu of Kabat’s subgroup I (22), with 58% identity in the framework region. This sequence was used to provide the VH framework of the humanized Ab. The Vκ from Eu was used to provide the humanized Ab Vk framework. This Vk sequence belongs to the Kabat subgroup I of κ-chains (22) and has 65% identity with AF2 in the respective framework region.

Based on their location relative to the CDRs in the three-dimensional model, the framework amino acids that could influence CDR conformation, and thus Ab-binding affinity and/or activity were identified. Amino acids from the murine AF2 sequence were used instead of the human Eu amino acids in those positions. Such substitutions were conducted at positions 48 from the murine AF2 sequence were used instead of the human Eu amino acids in those positions. Such substitutions were conducted at positions 48, 49, 50, 51, 52, 53, 54, and 55 of the VL domain, and at positions 28, 48, 66, 67, 69, 71, and 73 of the VH domain (the numbering scheme of Kabat et al. (22) is used unless otherwise noted). That these sets of framework amino acids contact the CDRs was confirmed using the structure of the chimeric AF2 Fab solved among the most similar VH sequences is Eu of Kabat’s subgroup I (22), with 58% identity in the framework region. This sequence was used to provide the VH framework of the humanized Ab. The Vκ from Eu was used to provide the humanized Ab Vk framework. This Vk sequence belongs to the Kabat subgroup I of κ-chains (22) and has 65% identity with AF2 in the respective framework region.

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Construction and expression of mutant and humanized versions of AF2

The cloning of the Vκ and VH domains of the AF2 Ab and their subcloning into mammalian expression vectors pVκ and pVγ1.d were as described (26). Genes encoding the humanized versions of the AF2 Vκ and VH were synthesized from a series of overlapping oligonucleotides (26). Site-directed mutagenesis of the murine and humanized AF2 genes was performed by the overlap-extension PCR method (27). The presence of the desired nucleotide substitution(s) was confirmed by DNA sequencing.

Ab expression

H and L chain expression vectors were transiently cotransfected into COS-7 cells using Lipofectamine (Life Technologies, Rockville, MD). After 72 h of incubation, the supernatants were collected and quantitated for Ab by ELISA. In most instances, Ab was purified from transfected supernatants using protein A-Sepharose chromatography (Bioprocessing, Princeton, NJ). All purified Ab preparations were examined by PAGE under both reducing and nonreducing conditions to verify the presence of only intact IgG. All experiments were conducted with intact whole Ab preparations.

IFN-γ neutralization assay

The IFN-γ-dependent induction of HLA-DR expression was conducted essentially as described (7), using the human melanoma cell line Hs294T (28). Cells were examined for the level of HLA-DR expression by indirect immunofluorescence. The level of inhibition of induction by the various Abs was calculated using the formula:

$$\% \text{ inhibition} = \left( \frac{\text{MCF}_{\text{max}} - \text{MCF}_{\text{min}}}{\text{MCF}_{\text{max}} - \text{MCF}_{\text{min}}} \right) \times 100,$$

where mean channel fluorescence (MCF)max is equal to the MCF of Hs294T cells incubated with IFN-γ and stained for HLA-DR expression, MCFmin is the MCF of Hs294T cells incubated with IFN-γ and 250 pM of the indicated Ab, and MCFmin is the MCF of Hs294T cells incubated in the absence of IFN-γ.

Competition ELISA

The competition ELISA using biotinylated AF2 (bAF2) to measure relative affinity differences was conducted as described (7).

Results

EuAF2 binds IFN-γ with affinity comparable with that of AF2

The murine Ab AF2 was humanized using the VH and VL frameworks of the human Eu Ab by the strategy outlined in Materials and Methods. The resulting humanized version of AF2, designated EuAF2, was expressed and purified from transfected supernatant, and compared with the original murine AF2 Ab for binding to human IFN-γ. The comparison was conducted using a competition ELISA; both AF2 and EuAF2 were assayed for their ability to competitively inhibit the binding of bAF2 to IFN-γ-coated ELISA plates. Fig. 2A demonstrates that EuAF2 competes with bAF2 slightly less efficiently than does AF2. The IC50 in this particular binding assay, which is representative of several, is 9.2 nM for AF2 and 20.8 nM for EuAF2. Thus, the humanized Ab exhibits a relative affinity ~2-fold lower than the murine Ab.

VH domain of EuAF2 is responsible for significant loss of IFN-γ-neutralizing activity

To further characterize EuAF2, its capacity to neutralize IFN-γ was compared with that of AF2. Induction of MHC class II expression on responsive cells is a sensitive assay for IFN-γ activity
Identification of AF2 V_H residues essential for IFN-γ neutralization

The H chain V region of EuAF2 contains 21-aa differences from AF2 exclusive of the CDR residues (Fig. 1). To establish whether the decrease in IFN-γ-neutralizing ability could be localized to individual residues on the humanized H chain, we constructed a series of mutants that introduced these 21 residues from the human V regions into the murine AF2 H chain individually or in groups into the EuAF2 H chain (Fig. 3). These mutant H chains were transfected along with the humanized EuAF2 L chain, and transfectant supernatants were collected after 72 h. The concentration of Ab was determined by ELISA for each variant, and each Ab was examined for the ability to neutralize IFN-γ, as expressed by the percentage of inhibition of MHC class II induction on Hs294T cells. Fig. 3 lists the mutants and depicts their relative efficiency in neutralizing IFN-γ. The majority of the mutants exhibited IFN-γ-neutralizing activity similar to that of AF2. However, two mutants exhibited decreased IFN-γ-neutralizing activity: a 4-aa change in framework region 1 (Asp10→Glu, Leu11→Val, Val12→Lys, and Met13→Lys) that exhibited a substantial decrease, and a change in framework region 2 (Lys38→Arg) that demonstrated a more subtle decrease (Fig. 3). The slight decrease in neutralizing capacity observed in the Ser75→Thr, Ser76→Asp mutant was not reproducible upon repeated analysis.

To determine the relative contribution of the four residues at positions 10–13 in V_H framework 1 to IFN-γ neutralization, each was introduced individually into the AF2 H chain, and the Abs were produced and assayed for IFN-γ-neutralizing activity. This experiment revealed one mutant (Leu11→Val) that lost an amount of IFN-γ-neutralizing activity roughly equivalent to that lost by the original 4-aa mutant; constructs containing the other three changes individually exhibited a level of IFN-γ-neutralization close to that of AF2 (Fig. 3). This analysis implicated the Leu at position 11 of the AF2 H chain as having a significant role in the IFN-γ-neutralizing activity of this Ab.

Modification of EuAF2 V_H to restore IFN-γ-neutralizing activity

The identification of the above two residues (11 and 38) as being capable of reducing IFN-γ-neutralizing activity of the murine AF2 V_H region led us to determine whether introducing the original murine amino acids at one or both of these positions would restore IFN-γ-neutralizing capacity to EuAF2. Thus, we introduced these 2 aa individually or together into the EuAF2 V_H, and transfected...
each along with the EuAF2 L chain into COS-7 cells, then determined the activity of the resulting Abs in the IFN-γ neutralization assay. Fig. 4 displays the neutralization curves for these versions and reveals that replacing residue 11 (Val) in the EuAF2 V\textsubscript{H} with a Leu significantly increased IFN-γ-neutralizing capacity, while replacing residue 38 (Arg) with a lysine had a smaller, but easily detectable effect. However, making both changes (Val-11→Leu, Arg-38→Lys) resulted in a humanized Ab version that most closely approximated the IFN-γ-neutralizing ability of AF2 (Fig. 4). This fully humanized Ab, designated HuZAF, differs from the initial EuAF2 version by only two residues (11 and 38) in the framework of the H chain V region.

We then compared HuZAF with AF2 in both the direct binding assay and the IFN-γ neutralization assay. HuZAF exhibits both IFN-γ-binding and IFN-γ-neutralizing capacity within 2- to 3-fold of that observed with AF2 (Fig. 5). Therefore, the 2-aa difference between EuAF2 and HuZAF, while having little or no effect on direct Ag binding, resulted in the latter exhibiting an IFN-γ-neutralizing capacity improved by ~10-fold.

**HuZAF C\textsubscript{H1} mutation can diminish IFN-neutralizing capacity**

Examination of the previously determined three-dimensional structure of the Fab of AF2 (23) indicates that Lys-38 is proximal to amino acids in the CDR portion of the Ab; thus, an effect an Ab activity can be reconciled, as this is the primary location of Ag binding (Fig. 6A). The location of Leu-11, in contrast, is distal to the Ag binding surface, making it unlikely that this residue affects the structure of the Ag binding surface. However, examination of this Fab structure indicates that Leu-11 is involved in the V-C interface, and it interacts closely with amino acids Phe-148 and Pro-149 of the C\textsubscript{H1} domain (Fig. 6B). To test the role of this interaction in neutralization capacity, the Phe-148 in HuZAF was replaced by Leu or Ala. Thus, these variants differ from HuZAF only by a single residue in the C region. As shown in Fig. 7, replacement of Phe-148 by either Leu or Ala substantially decreased the ability of the Ab to neutralize IFN-γ, and the magnitude of this loss was comparable with that observed for the change at V\textsubscript{H} position 11 (Fig. 2). Therefore, altering a single residue in C\textsubscript{H1} diminishes the ability of HuZAF to neutralize IFN-γ activity 5- to 10-fold, and indicates that this simple modification of the interface between V and C domains has ramifications for AF2 functional activity.

**Discussion**

In humanizing the murine anti-human IFN-γ mAb AF2, we noticed that while the IFN-γ-binding activity was minimally altered, the biological activity of the initial humanized version (EuAF2) was inordinately affected. Although EuAF2 exhibited relative Ag-binding activity within 2-fold of that observed with the original murine Ab, the capacity to neutralize IFN-γ was diminished by greater than 20-fold. As maintaining the potent IFN-γ-neutralizing activity of AF2 in any humanized version was essential, we investigated the basis of this discrepancy.

Our mutational analysis identified two V\textsubscript{H} framework residues in AF2 that had been changed to human residues in the initial humanization that were essential for full IFN-γ-neutralizing activity: a Leu at position 11 and a Lys at position 38. Reintroducing these two residues into the initial version of the humanized Ab recovered virtually all of the lost activity. The resulting humanized Ab, designated HuZAF, exhibits both IFN-γ binding and neutralization within 2- to 3-fold of the original murine AF2 Ab.

The participation of the V\textsubscript{H} framework residue 38 (Lys) in IFN-γ-neutralization is not surprising, even though the EuAF2 Ab has a very similar amino acid (Arg) at this position. Amino acid 38 is separated from the last residue of CDR1 by only 2 aa, and thus may play a structural role in the positioning of this CDR. Moreover, examination of the crystal structure of chimeric AF2 (23) reveals that the C\text{e} atom of Lys-38 (the first atom changed in a Lys to Arg mutation) is only 5 Å away from the side chain of a V\textsubscript{H} CDR2 residue (Phe-63), possibly influencing the configuration of this CDR also (Fig. 6A). In addition, several different Abs humanized by various groups retain the murine residue at this position (30–32), implying that involvement of V\textsubscript{H} Residue 38 in Ab activity is a characteristic not unique to AF2.

However, the importance of the Leu at V\textsubscript{H} position 11 to IFN-γ-neutralizing capacity was initially much more difficult to understand. This residue is located far away from the CDRs both in primary structure and three-dimensional space (Figs. 1 and 6A). Position 11 is separated by 19 aa from the beginning of CDR1, and the closest CDR residue in three-dimensional space to the C\text{e} of Leu-11 (the first atom changed in a Leu to Val mutation) is also the
Phe-63 in V\textsubscript{H} CDR2, although in this case the distance is more than 20 Å (23). Thus, it is unlikely that Leu-11 has a direct structural effect on the Ag binding site of the Ab.

However, V\textsubscript{H} framework residue 11 has been identified as a component of a ball-and-socket joint that exists between Ab V and C domains (16). Residues 11, 110, and 112 of V\textsubscript{H} form the socket, while residues 148 and 149 of C\textsubscript{H1} (numbered 149 and 150 in Ref. 16) form the corresponding ball of the joint (Fig. 6B). This structure has been hypothesized to modulate flexibility between the V and C domains of the Fab (16). It has been proposed that the orientation of this joint dictates the elbow angle, which is the angle formed by the approximate 2-fold axis of symmetry between the V\textsubscript{H}/V\textsubscript{L} domains with the equivalent axis formed by C\textsubscript{H1}/C\textsubscript{L}, and suggested that this structure has functional importance (16). Variations in this elbow angle define the second major form of Ab flexibility (the first being modulated by the hinge region connecting C\textsubscript{H1} with C\textsubscript{H2}) (33). Therefore, it seemed possible that the participation of the Leu-11 in IFN-\gamma neutralization was based upon the role it serves as a component of the ball-and-socket joint between the V and C domains. This hypothesis was tested by altering residue 148 of C\textsubscript{H1}, a residue that is part of the ball of the joint. Replacement of the Phe-148 with Ala or Leu significantly diminishes the ability of the Ab to neutralize IFN-\gamma. Phe-148 was chosen to test rather than Pro-149 (the residue that comprises the other half of the ball of the joint), as changing the latter could alter the backbone structure. The fact that a single mutation in C\textsubscript{H1} can so dramatically affect the biologic activity of this Ab with little consequence to Ag binding reveals that the ball-and-socket joint can have functional significance, and suggests that the appropriate elbow angle and/or V/C domain flexibility is essential for the IFN-\gamma neutralization activity of the AF2 Ab and its humanized versions.

It is interesting that 97% (152 of 157) of sequences available for members of the murine VH subgroup IIB have a Leu at position 11, making this a highly conserved residue in this subgroup (22).

The lack of strict correlation between binding affinity and Ab biological activity is an observation not confined to humanized versions of AF2. A humanized Fab variant of the murine anti-p185\textsuperscript{HER2} Ab 4D5 binds this Ag with affinity comparable with that observed with the murine Fab, but exhibits none of the antiproliferative effect of the latter (34). In addition, humanized versions of an anti-RSV single chain Ab fragment exhibit binding indistinguishable from the murine version; however, viral neutralization is severely impaired (35). Although neither of these cases appears to involve the ball-and-socket joint, it is clear that binding affinity alone is not a sufficient indication of a successful humanization, and the relevant Ab-mediated biological activity must be monitored.

It is unclear why IFN-\gamma neutralization is more susceptible to changes in the ball-and-socket joint than is direct Ag binding. It is possible that efficient IFN neutralization requires subtle molecular interactions, which are trivial to Ag binding, but not permitted by an altered ball-and-socket joint. AF2 inhibits the binding of IFN-\gamma.
to its receptor (7), and interrupting this interaction is presumably the mechanism of IFN-γ neutralization. Our results indicate that the Ab requires an intact ball-and-socket joint to exhibit optimal IFN-γ-neutralizing activity, and suggests that intramolecular flexibility of the Ab between V and C domains, and thus the elbow angle, is important. That IFN-γ-neutralizing activity relies upon flexibility between the V and C region domains may relate to the fact that the determinant on IFN-γ bound by AF2 is proximal to, but distinct from the surface of the molecule that interacts with the receptor (7). Possibly a flexible VIC interface permits the CDRs of the Ab to bind this determinant while allowing the remainder of the Ab molecule to interfere with IFN-γ binding to the receptor. This implies that simple binding to the epitope on IFN-γ is necessary, but not sufficient for IFN-γ neutralization, and suggests that the Ab must also possess the flexibility to block IFN-γ interaction with the receptor. A greater than 10-fold reduction in the ability of the Fab of HuZAF to neutralize IFN-γ (unpublished data) is consistent with this hypothesis. Therefore, alterations in the ball-and-socket joint might be expected to preferentially affect IFN-γ neutralization, while exhibiting little influence on binding. There is precedent for Ag-induced alteration in the elbow angle. A significant decrease in the elbow angle has been observed between the free Fab of NC6.8, an anti-guanidinium sweetener Ab, and the Fab-Ag complex (36), demonstrating Ag binding-dependent changes in the relative orientation of the V and C domains.

The successful humanization of AF2 provides a potent IFN-γ antagonist that may have therapeutic value. HuZAF exhibits an IC_{50} of <1 nM in the IFN-γ-mediated induction of MHC class II expression assay. The concentration of natural IFN-γ in this assay is 0.25 nM; thus, HuZAF can essentially abolish IFN-γ activity at a molar ratio of less than 4 HuZAF to 1 IFN-γ. This potent IFN-γ-neutralizing activity coupled with the long circulating t_{1/2} of a humanized Ig makes HuZAF a potentially ideal therapeutic agent for treatment of conditions in which IFN-γ may contribute to the pathology. The recent appreciation of the role that lymphocytes of the Th1 lineage play in a variety of autoimmune conditions, coupled with the integral involvement of IFN-γ in the generation and maintenance of this lineage, suggests HuZAF may possess a unique therapeutic potential. This potential will need to be tested in a clinical setting.

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