Isotype Choice for Chimeric Antibodies Affects Binding Properties*

Maurice M. Morelock‡, Robert Rothlein†, Susan M. Bright†, Martyn K. Robinson, Edward T. Graham**, John P. Sabo‡‡, Raymond Owens, David J. King, Stephen H. Norrist†, David S. Scher§§, James L. Wright¶¶, and John R. Adair†

From the Departments of †Biochemistry, ‡Management Informations Systems, ‡‡Drug Metabolism and Pharmacokinetics, and §§Pharmaceuticals, Research and Development Center, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut 06877-0368 and ¶¶Celltech Research, Celltech Limited, 216 Bath Road, Slough, Berkshire SLI 4BN, United Kingdom

Construction of a series of chimeric antibodies (mu-

rine variable region and human constant region) de-

rived from the murine antibody BIRR1, which recom-

nizes intercellular adhesion molecule 1 (ICAM-1), has

revealed differences in the relative binding abilities of

the chimeric antibody to antigen. The chimeric antibo-

dies show a ranking of their ability to compete with

BIRR1 for antigen on the surface of cells with the order

BIRR1 = cIgG1 (100%) > cIgG2 (90%) > cIgG3 (30%) > cIgG2 (10%) as de-

monstrated by solid-phase competitive enzyme-linked

immunosorbent assay. Papain digestion yielded Fab frag-

ments that were purified to homogeneity. Competitive

enzyme-linked immunosorbent assay showed that the

chimeric and murine Fab binding constants were equi-

valent. A solution-phase binding assay (analyzed by size

exclusion high performance liquid chromatography) be-

tween the intact mAbs and recombinant soluble ICAM-1

further established that the binding constants involving

the Fab arms of the two antibodies were equivalent.

In summary, the murine and chimeric anti-ICAM-1 anti-

bodies bind cellular ICAM-1 with equivalent affinities but

with differing avidities.

There is an increasing interest in the use of monoclonal an-

tibodies in the diagnosis and treatment of human diseases.

Many of these antibodies are of murine origin. As a conse-

quence, human anti-murine antibody responses have been ob-

served when these antibodies were used (LoBuglio et al.,

1986; Jonker and Den Brok, 1987) and have been shown to be

considerably less immunogenic than the original mouse antibody (reviewed by Adair (1992)). Human antibody genes have been shown to be compatible with human antibody genes. The resultant gene is a chimera with murine variable regions and human constant regions (Morrison et al., 1984). The aim of such a modification is to maintain the binding affinity of the murine mAb (since the murine variable domain is left intact and can fold independently) while minimizing the potential immunological problems. As an alternative, "CDR" grafting involves replacing the constant region and the framework residues that position the CDRs in the variable region of the murine antibody by their human equivalents (Jones et al., 1986). CDR grafting creates a molecule that contains the minimum amount of murine-encoded material while retaining antigen binding specificity. In some cases, however, this approach has the potential disadvantage of associated loss in antibody affinity (see, e.g., Riechmann et al. (1988); reviewed by Adair (1992)).

We have prepared chimeric derivatives of the murine anti-

intercellular adhesion molecule 1 (ICAM-1) mAb BIRR1 (Smith et al., 1988; Cosimi et al., 1990) using human IgG1, IgG2, and IgG3 constant regions. ICAM-1 is a member of the supergene family expressed on a variety of cell types (Dustin et al., 1986; Rothlein et al., 1986; Marlin and Springer, 1987; Staunton et al., 1988; 1990; Warwryk et al., 1989) and has been shown to be a ligand for the neutrophil-endothelial cell receptors LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (Rothlein et al., 1988; Dustin et al., 1986; Marlin and Springer, 1987; Smith et al., 1988; Diamond et al., 1990). BIRR1 has been shown to have beneficial effects in non human primates with renal allografts (Cosimi et al., 1990). In this study, the antigen binding ability of three different chimeric isotypes was examined. The chi-

result in a reduction in the immune response (Sears et al., 1984; Khazaeli et al., 1988); or co-administering interferon-γ with the antibody, which appears, at least in one study (Blotter et al., 1991), to delay the immune response to the murine mAb and to restrict the response, when it does arise, to the isotype.

One approach to reducing the human anti-murine antibody response involves replacing as much as possible of the non-human sequence with equivalent human sequence (Steplewski et al., 1988; LoBuglio et al., 1989). "Humanized" antibodies have been shown to be considerably less immunogenic than the original mouse antibody (reviewed by Adair (1992)). Humanization has been achieved in two ways. In the first instance, the constant regions of a particular mAb can be humanized by replacing the DNA sequences from the mouse constant heavy and light chain genes with suitable sequences from human antibody genes. The resultant gene is a chimera with mouse variable regions and human constant regions (Morrison et al., 1984). The aim of such a modification is to maintain the binding affinity of the murine mAb (since the murine variable domain is left intact and can fold independently) while minimizing the potential immunological problems. As an alternative, "CDR" grafting involves replacing the constant region and the framework residues that position the CDRs in the variable region of the murine antibody by their human equivalents (Jones et al., 1986). CDR grafting creates a molecule that contains the minimum amount of murine-encoded material while retaining antigen binding specificity. In some cases, however, this approach has the potential disadvantage of associated loss in antibody affinity (see, e.g., Riechmann et al. (1988); reviewed by Adair (1992)).

We have prepared chimeric derivatives of the murine anti-

intercellular adhesion molecule 1 (ICAM-1) mAb BIRR1 (Smith et al., 1988; Cosimi et al., 1990) using human IgG1, IgG2, and IgG3 constant regions. ICAM-1 is a member of the supergene family expressed on a variety of cell types (Dustin et al., 1986; Rothlein et al., 1986; Marlin and Springer, 1987; Staunton et al., 1988; 1990; Warwryk et al., 1989) and has been shown to be a ligand for the neutrophil-endothelial cell receptors LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (Rothlein et al., 1988; Dustin et al., 1986; Marlin and Springer, 1987; Smith et al., 1988; Diamond et al., 1990). BIRR1 has been shown to have beneficial effects in non human primates with renal allografts (Cosimi et al., 1990). In this study, the antigen binding ability of three different chimeric isotypes was examined. The chi-
meric cIgG₄ was able to compete equivalently with BIRR1 for binding to antigen on cells, confirming that the binding properties of the parental mAb was preserved in the formation of the chimeric antibody. Unexpectedly, however, the cIgG₄ and cIgG₃ antibodies showed decreased competitive abilities. We show in this paper that, while the binding site affinity of cIgG₄ is the same as BIRR1, the avidity of the antibody is markedly reduced. This observation may have wider consequences for the design of chimeric and humanized antibodies to cell surface antigens.

**Materials and Methods**

Reagents—Biotin-N-hydroxysuccinimide, streptavidin-β-galactosidase conjugate, and p-nitrophenyl-β-D-galactopyranoside were purchased from Pierce Chemical Co. A biotinylated amino acid protein determination kit (BCA-1) was purchased from Sigma. An ImmunoPure Fab preparation kit was purchased from Pierce Chemical Co. All other reagents were of the highest grade available and were obtained from Fisher or Sigma.

**Chimeric Gene Construction and Transfection**—Basic molecular biology procedures were as described by Sambrook et al. (1989). COS cell expression was as described by Whittle et al. (1987). Chinese hamster ovary transfections and cell culture were performed as described by Bebbington (1991).

Full-length cDNA was prepared by using oligo(dT) to prime first strand synthesis from mRNA prepared using the guanidinium/LiCl extraction procedure. After methylation and ligation to EcoRI linkers, the cDNA library was cloned into Escherichia coli (E. coli) in PSP64. E. coli colonies containing either heavy or light chain genes were identified by screening using oligonucleotide 5'-TCCAGATTTAATCCGTAC for the light chain, which is complementary to a sequence in the mouse κ constant (Ck) region, and by using a 980-base pair BamHI-EcoRI restriction fragment of a previously isolated mouse IgG₁ constant region clone for the heavy chain. DNA sequences for the 5'-untranslated regions, signals, sequences and variable regions of full-length cDNAs were obtained.

The chimeric light chain sequence was assembled from three fragments: a 397-base pair EcoRI-SfI-NI fragment coding for the 5'-untranslated region from the cDNA, the signal sequence, and the majority of the light chain variable region; an oligonucleotide adapter, which codes for the remainder of the 3' region of the variable region from the SfI-NI site and the 5' residues of the human constant region up to and including a unique NAR₁ site that had been previously engineered into the human Cκ gene at the third to fifth codons so as to not alter the coding potential², and the human Cκ gene as an NAR₁-EcoRI fragment. The ligated light chain gene was inserted into pEE6-hCMV-neo (Stephens and Coley, 1989) to give pAL7.

Chimeric IgG₄ and IgG₃ genes were assembled from three fragments: a 424-base pair EcoRI-BamI fragment coding for the 5'-untranslated region from the cDNA, the signal sequence and the majority of the heavy variable region sequence; an oligonucleotide adapter, which codes for the remainder of the 3' region of the variable region from the BamI site up to and including a unique HindIII site that had been previously engineered into the first two amino acids of the constant region (Whittle et al., 1987); and the IgG₄ or IgG₃ constant regions as HindIII-BamHI fragments. The ligated heavy chain genes were inserted into pEE6-hCMV-gpt to give pAL₈ and pAL₉, respectively. The chimeric IgG₄ heavy chain gene was assembled by excising the DNA coding for the heavy chain signal and variable region sequences along with the first 5 amino acids of the CH₁ domain from pAL₈ as a HindIII-Apal fragment and inserting the sequence 5' to the IgG₄ heavy chain constant region to the HindIII and Apal sites in pE1001, an E6 hCMV-gpt vector previously modified to contain the IgG₄ constant region 3' to the hCMV promoter, and to remove the Apal site in the gpt gene, to give pAL200. The first 5 amino acids of the CH₁ domains of IgG₄ and IgG₃ are identical, so this cloning procedure does not introduce any novel sequence motifs.

Cells were isolated after transformation into E. coli, and the linker and junction sequences for all of the constructions were confirmed by DNA sequencing. Stable cell lines were prepared by transfecting pAL₇ into CHO-K1 cells by the calcium phosphate precipitation procedure (reviewed by Bebbington (1991)). A neomycin-resistant, cell-line secreting light chain was selected and re-transfected with pAL₂₀₀, pAL₉, or pAL₈. Cell lines producing 3.8, 6.9, and 4.2 µg/m² cells/24 h of cIgG₄, cIgG₃, and cIgG₂ respectively, were used for antibody production.

**Antibody Purification**—Hybridoma cell line R6.5 D6 was generated as previously described (Smith et al., 1988). BIRR1 (IgG₂₄₅₆) mAb was produced by culturing the hybridoma in antibiotic-free Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum. Cell lines expressing chimeric antibodies were cultured in serum-free medium; in each case, the antibody in the culture supernatant was purified by affinity chromatography using protein A-Sephasor (Colcher et al., 1989) and concentrated by ultra-filtration. The pH was adjusted to 6.5 and the protein dialoged into phosphate-buffered saline (PBS). A small amount of dimethylaggregate was removed by size exclusion chromatography using a Superose-200 prep grade column (Pharmacia LKB Biotechnology Inc.) eluting with 64 mM sodium phosphate, 86 mM NaCl, pH 6, buffer. Purity and correct assembly of the antibody was tested by reducing and non-reducing SDS-polyacrylamide (SDS-PAGE) as described by Laemmli (1970), and high performance liquid chromatography (HPLC) gel filtration. Identity was confirmed by N-terminal amino acid sequencing for the light chain and amino acid composition analysis. Quantitative amino acid analysis was also used to determine accurate extinction coefficients at 280 nm for each of the chimeric antibodies, allowing accurate protein determinations to be made in pure chimeric antibody preparations.

**HPLC Analyses**—mAb and Fab fragments were evaluated for purity by size exclusion chromatography using a Waters 710B Wisp and 590 pump (Millipore, Milford, MA), a TSK G3000SW (Tosoh Corp., Tokyo, Japan) or DuPont GF250 column, and either a Spectraflow 773 absorbance detector (Kratos, Ramsey, NJ) or a 1044A and 390 detection system (Hewlett-Packard, Avondale, PA) as described by King et al. (1992).

**Preparation of BIRR1 and cIgG₃ Fab Fragments**—The fragments were generated according to the standard protocol accompanying the Pierce ImmunoPure Fab preparation kit with only slight modifications. This method involved overnight incubation at 38 °C with cysteine-activated immobilized papain for fragment cleavage, followed by protein A chromatography for purification. mAb and Fab buffer solutions were changed and protein concentrated using a Centricon-30 ultrafiltration device (Amicon, Beverly, MA). Final samples were exhaustively dialyzed against 64 mM sodium phosphate, 86 mM NaCl, pH 7.0 using Spectro/por tubing (M, 6,000–8000 cut-off) (Spectrum Medical Industries, Los Angeles, CA) and filtered using a 0.2-μm, low protein binding, sterile filter (Gelman Sciences, Ann Arbor, MI). Protein concentrations were determined using a biochiniconic acid protein assay (BCA-1, Sigma procedure TPI0-562).

**Competitive ELISA Binding Studies**—Wells of microtiter plates were coated with ICAM-1-bearing B lymphoblastoid cell line JY by the method of Norris et al. (1991). All additions to the wells, titration of samples, and determinations of optical density were done with a B-omek 1000 Automated Laboratory Workstation (Beckman, Palo Alto, CA), controlled by a Hewlett-Packard Vectra 9000 (Hewlett-Packard Co., Cupertino, CA). All plates were blocked prior to use with a 2% BSA, 0.1% sodium azide, PBS, pH 7.2 solution (BSA-BS). The microtiter plates were washed between steps with a 2% MgCl₂, 5 mM sodium phosphate, 3 mM potassium phosphate, 140 mM sodium azide, pH 7.0 solution using an UltraWash2 Microplate Washer (Dynatech Laboratories, Chantilly, VA). Biotinylated BIRR1 mAb (prepared by the method of Goding (1980)) was combined with native mAb in BSA-BS so that the final molar concentration ratios were (10⁻⁴/10⁻⁴), (10⁻⁴/10⁻⁵), (10⁻⁴/10⁻⁶), (10⁻⁴/10⁻⁷), (10⁻⁴/10⁻⁸), and (10⁻⁴/10⁻⁹), respectively; for Fab fragments, (5 × 10⁻⁶/5 × 10⁻⁶), (5 × 10⁻⁶/10⁻⁶), (5 × 10⁻⁷/2 × 10⁻⁷), (5 × 10⁻⁸/2 × 10⁻⁸), (5 × 10⁻⁹/2 × 10⁻⁹), and (5 × 10⁻¹⁰/2 × 10⁻¹⁰), respectively. Each sample was then titrated by a factor of 1.3 with BSA-BS so that 5 logs in concentration could be analyzed. Six 100-μl aliquots of the titrated samples were then transferred into the wells of the microtiter plates for each mAb concentration and incubated overnight at 4 °C. The following day (~20 h) the wells were washed with BSA-BS and incubated with 100 µl of a 1:500 dilution of streptavidin-β-galactosidase conjugate in BSA-BS. Plates were agitated for approximately 3 h and then washed with PBS, pH 7.2. 100 µl of a 0.5 mg/ml p-nitrophenyl-β-D-galactopyranoside, PBS solution, pH 7.2, was then added to each well and incubated with agitation for 1 h. The absorbance of the enzymatic product p-nitrophenol at 405 nm was then determined for each well.

**HPLC IA Binding Studies—ICAM-1 is available in soluble form (sICAM) (Marlin, 1990) and was provided by Dr. Steve Marlin and Dr. Richard Shansky (Boehringer Ingelheim Pharmaceuticals, Inc.). Binding of 1 ng of mAb and ICAM-1 was characterized by size exclusion chromatography (HPLC immunoassay, HPLC-IA), monitoring protein concentration at 220 nm. BIRR1 and cIgG₃ samples were prepared by combining...
Comparison between Murine Anti-ICAM-1 and Human Chimeras

siCAM-1 with the respective mAb in PBS, pH 7.0, at the same concentration (10^{-8} M). The association reaction was allowed to proceed at room temperature for 4 h. The samples were then titrated with PBS, pH 7.0, at a ratio of 2:3 down to a concentration of 1.7 \times 10^{-8} M. The dissociation reaction was allowed to proceed for 2 days at 4 °C. Concentrations of bound and unbound antibody were determined by peak area and peak height analyses of HPLC elution profiles.

Data Analysis—ELISA and HPLC-IA data files containing measurements of optical density were analyzed using the version 6.08 SAS statistical software system (SAS Institute Inc., Cary, NC) on an IBM 4381 mainframe computer running VM/CMS (IBM Corp., Armonk, NY). Data analysis was performed by applying nonlinear regression techniques to selected models using the Marquardt iterative method. Parameters were estimated with 95% confidence intervals and residual analysis conducted. SASGraph was used to display the data overlaid with the theoretical curve/surface.

RESULTS

Protein Characterizations—Reducing SDS-PAGE of all of the chimeric antibodies showed a single band of the expected heavy and light chains at approximately 55 and 28 kDa, respectively. Under non-reducing conditions, fully assembled antibody was seen in each case. The cIgG \textsubscript{1} mAb showed an additional minor band (10–15%) of monomeric cIgG \textsubscript{1} at about 80 kDa, similar to that seen with all other chimeric and natural IgG \textsubscript{1} antibodies (Angal et al., 1993) (Fig. 1). The biochemical explanation for the double band observed in the chimeric IgG \textsubscript{1} preparation is unknown (Fig. 1). The integrity of the cIgG \textsubscript{2} molecule was assessed by DNA sequence analysis of the assembled genes to confirm that the double banding seen in the non-reducing SDS-PAGE was not due to sequence heterogeneity of the cloned gene, by partial N-terminal amino acid sequencing of the light chain and by total amino acid composition analysis. Using size exclusion HPLC, all antibody preparations were observed to be a single species with less than 1% aggregate at 280 nm. Protein stability for all of the chimeric antibodies was measured by storage at 6 mg/ml at 4 °C or −70 °C for up to 1 month followed by SDS-PAGE and HPLC analysis and antigen binding assay. There was no evidence for alteration of structure or activity of the chimeric antibodies by such analyses (data not shown), suggesting that the material as produced by the cells was in a stable form.

BIRR1 and cIgG \textsubscript{1} Fab fragments prepared by papain digestion were observed to be a single species by size exclusion HPLC at 280 nm. Analysis by non-reducing SDS-PAGE showed a band at 50 kDa (>90%) with a minor band at 25 kDa. Reducing SDS-PAGE gels displayed one band at 25 kDa with slight microheterogeneity in each preparation. This heterogeneity is probably due to some digestion above the disulfide bonds yielding fragments (5–10%) that dissociate upon incubation with SDS as exhibited in the non-reducing analysis (not shown). The two Fab preparations were indistinguishable using these analytical techniques.

Characterization of mAb Binding by ELISA—Each of the chimeric antibodies reacted as strongly as the murine antibody with a panel of anti-idiotype monoclonal antibodies raised against BIRR1 (Rothlein et al., 1993), while nonspecific human immunoglobulins of the appropriate class did not react. These

---

**Fig. 1.** SDS-polyacrylamide gel electrophoresis of chimeric antibodies under non-reducing (panel A) or reducing (panel B) conditions. A, non-reducing 8% (w/v) acrylamide gel; B, reducing 12% (w/v) acrylamide gel. Samples were applied in the same order to each gel. Lane 1, 4 μg of chimeric B72.3 IgG, marker (King et al., 1991); lanes 2 and 10, molecular size marker mixture comprising phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa); lanes 3–9, chimeric IgG\textsubscript{1}, at loading of 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg, respectively; lanes 11–17, chimeric IgG\textsubscript{1} at loadings of 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg, respectively. Gels were run at 30 mA and stained with Coomassie Blue.

**Fig. 2.** JY cells expressing ICAM-1 absorbed to plastic were incubated with increasing concentrations of BIRR1 (●), cIgG\textsubscript{1} (○, panel A), cIgG\textsubscript{2} (□, panel B), and cIgG\textsubscript{3} (△, panel C). Biotinylated BIRR1 was then added at a fixed concentration and after further incubation binding of biotinylated antibody was revealed using streptavidin-β-galactosidase and p-nitrophenyl-β-D-galactopyranoside. In each panel inhibitory ability of the chimeric antibodies is compared to that of BIRR1 measured in the same experiment. The x axis shows concentration of these unlabeled antibodies.
data suggested that the antigen binding site from the murine antibody has been transferred to the chimeric antibodies. Competitive binding assays, however, indicated that while the cIgG1 antibody competed equally for antigen with the murine antibody (Fig. 2A), the cIgG2 and cIgG4 did not compete as well (Fig. 2, B and C), with the cIgG2 and cIgG4 showing approximately 40 and 10% relative potency, respectively.

In an attempt to further differentiate and quantitate the differences in antigen binding among the different antibodies, comprehensive data sets were generated using a JY cell ELISA assay (see “Appendix” for the development of mathematical models describing antibody/antigen binding). The absorbance curve for biotinylated BIRR1 at low mAb concentrations displays simple binding behavior. This segment of the data is fit nicely by the one-site model with \( A_{\max} \) and \( A_{\min} \) being well defined (Fig. 3, curve a). Since the curve breaks in the \( 10^{-9} \) M range \( (K_R \sim 10^9 \text{ M}^{-1}) \), the antibody is presumed to be binding in a cooperative bivalent manner and this site is defined as being minimally composed of two ICAM-1s. At higher mAb concentrations, however, absorption increases and the one-site model gives a poor fit due primarily to the uncertainty in \( A_{\max} \) (Fig. 3, curve b). Consequently, a two-site model was developed to allow for tight binding at low concentrations (site 1: cooperative bivalent, and monovalent at high mAb concentrations) and for weaker binding at higher concentrations (site 2: monovalent only). The two-site model results in a good fit for the data at low and high concentrations (Fig. 3, curve c). It should be noted that although \( K_{R1} \) is determined with excellent confidence limits, data at very high antibody concentrations are required for obtaining a good fit for \( K_{R2} \). Since the BIRR1 and chimeric mAbs are expected to display both monovalent and bivalent binding with fixed JY cells, this two-site model was adopted for analysis of the mAb competitive ELISA data.

The competitive binding data taken for BIRR1 are shown in Fig. 4. This experimental design results in statistically significant estimates for \( A_{\min} \) (low concentration data), \( \mu \) (intermediate concentrations wrapping around the face of the surface), and \( A_{\max} \) (high concentration data). In addition, the reporter mAb is evaluated in each experiment and serves as an indicator for the degree of competition with which the sample mAbs were challenged. The binding behavior along the reporter mAb axis was similar to the data observed for biotinylated BIRR1 in Fig. 3. The simulated fit projected a surface that conformed closely with the data. The fitted values for BIRR1 and cIgG4 given in Table I indicated that the bivalent binding displayed by the chimeric IgG4 was about 30% that of the murine antibody, while the monovalent binding was statistically equivalent. \( K_{R1} \) for the biotinylated BIRR1 reporter was \( (8.6 \pm 0.6) \times 10^9 \text{ M}^{-1} \) and \( (1.05 \pm 0.08) \times 10^9 \text{ M}^{-1} \) in the BIRR1 and cIgG4 experiments, respectively, and indicated that the binding constants for BIRR1 and cIgG4 were determined under comparable competitive conditions.

Characterization of Fab Binding by ELISA—To address whether the loss of binding could be attributed to affinity differences, Fab fragments were prepared for the BIRR1 and cIgG antibodies. Competitive JY cell ELISA experiments were carried out with the Fab fragments to measure each antibody’s affinity. Biotinylated BIRR1 intact mAb was used in each experiment as the reporter. The data were fit to response surfaces (not shown) defined by a two-site model (see “Appendix”) and resulted in comparable \( K_{S12} \) values for each fragment (see Table I). These data suggest that the binding site affinity has not been perturbed for the cIgG, but that the avidity of the antibody has been altered as disclosed by the binding of the whole antibody to cell surface antigen. The binding constant \( K_{R1} \) for the biotinylated BIRR1 was \( (2.4 \pm 1.1) \times 10^9 \text{ M}^{-1}(2.7 \pm 0.7) \times 10^9 \text{ M}^{-1} \) for the BIRR1 and cIgG experiments, respectively, indicating that \( K_{S12} \) values for the Fab fragments were determined under equivalent competitive conditions. (Note that the biotinylated mAb used in the Fab experiments displayed lower \( K_{R1} \) values than the preparation used in the mAb ELISA experiments (see above) and reflects the variation in batch to batch antibody biotinylations.)

Characterization of mAb Affinity—In the JY cell ELISA, cooperative bivalent binding was observed. In solution, however, the cooperative effect is expected to be minimized because two ICAM-1 molecules may bind a single antibody independently. Thus, a study of solution binding was performed to yield information concerning the affinity of the intact antibody. The
HPLC immunoassay experiment was carried out by combining mAb and sICAM at a ratio of 1:1 and titrating the reactions from $\frac{10^{-8}}{M}$ to as low as $2 \times 10^{-6} M$. The model used to fit the data (not shown) allowed for mAb-antigen complex ratios of 1:1 and 1:2. The parameter estimates (murine: (2.7 ± 0.7) x 10^{11}; cIgG: (2.4 ± 0.6) x 10^{11}) indicated that the affinities of the intact BIRR1 and cIgG mAbs were equivalent.

**DISCUSSION**

This paper describes the production of IgG_{1}, IgG_{2}, and IgG_{4} human-mouse chimeric forms of the mouse monoclonal anti-ICAM-1 antibody BIRR1, some of the physical properties of these chimeric molecules, and a comparison of their antigen binding ability with that of the native mouse antibody.

With regard to the physical nature of the chimeric antibodies, the cIgG_{4} has, in common with normal human IgG, and all other reported chimeric mouse-human and fully humanized IgG antibodies analyzed to date, a proportion of the molecules in which it is believed the hinge disulfide bonds do not form, leading to a tetrmeric (bivalent) antibody that is non-covalently linked between the heavy chains (Angal et al., 1993), and references therein). This feature of human IgG_{4} has not been associated with marked difference in binding avidity in other situations when chimeric or humanized isotypes have been compared (Shaw et al., 1988; Colcher et al., 1989; Hardman et al., 1989; Hutzel et al., 1981; Shearman et al., 1991). The significance of the double-banded appearance of the cIgG_{4} is not clear. The SDS-PAGE data suggest that there are two variants of the molecule which appear to be stable forms of the antibody. Natural IgG, and other mouse-human chimeric and fully humanized IgG antibodies have not previously shown this phenomenon (Bruggemann et al., 1988), and in the case of the cIgG_{4} we cannot exclude the possibility that this feature of the antibody contributes to the effect on binding avidity. Integrity of the mouse variable region in the chimeric molecules has been confirmed by DNA sequence analysis of the assembled genes, by DNA sequence of the constant regions for the IgG_{4} molecule to confirm the double banding seen in the non-reducing SDS-PAGE was not due to sequence heterogeneity of the cloned gene, by partial N-terminal amino acid sequencing of the light chain, and by total amino acid composition analysis for all of the antibody fragment proteins. Furthermore, these chimeric molecules require with a set of monoclonal anti-idiotypic antibodies that were raised against the murine anti-ICAM-1. The data suggest that the conformation of the mouse variable region in the chimeric molecules remains intact.

Preliminary competitive binding studies (Fig. 2) revealed that the order of binding was BIRR1 $\sim$ cIgG_{4} $<$ IgG_{2} $<$ IgG_{1}. Since time-resolved fluorescence depolarization studies of antigen-antibody complexes have shown that the flexibility of the hinge region for these human constant regions also decreases in the order IgG_{1} $>$ IgG_{2} $>$ IgG_{4} (Oi et al., 1984; Dangl et al., 1988; Schneider et al., 1988; Tan et al., 1990), it was postulated that the decreased ability to bind cellular ICAM-1 was reflective of restricted Fab arm movement resulting from decreased hinge region flexibility. In order to test this hypothesis, we have compared the binding abilities of BIRR1 and cIgG_{4} with cell-bound, as well as solubilized, ICAM-1 in an attempt to differentiate affinity associated with the mouse variable region from avidity, which additionally depends upon a mouse versus a human hinge region.

ICAM-1 is an $\alpha$-regulated cell surface marker. On live cells the density of antigen will increase from a situation where monovalent binding predominates to one where there will be a combination of monovalent and bivalent occupancy of the antigen binding sites by the antibody, depending on dosage and pharmacokinetics. Since regulation of ICAM-1 is a dynamic phenomenon, it would be difficult to analyze the type and degree of binding with living cells in vivo or in vitro. With fixed JY cells, however, the constitutively expressed ICAM-1 density and distribution are fixed such that monovalent and bivalent interactions can be observed separately as a function of antibody concentration. Cooperative bivalent binding, for example, is a strong antigen/antibody interaction usually observed at low antibody concentrations, i.e. $10^{-8}$ to $10^{-11} M$. Since this type of interaction depends upon the ability of the Fab arms to assume a particular spatial arrangement dictated by proximal ICAM-1 molecules, bivalent binding is descriptive of antibody avidity. Monovalent binding, on the other hand, is a much weaker interaction since only one of the Fab arms is involved with the binding site at any given time. Thus, mAb monovalent interactions are descriptive of binding affinity and should be observed in a concentration range expected for Fab fragments, i.e. $10^{-6}$ to $10^{-9} M$. In contrast to bivalent binding, monovalent interactions are for the most part independent of hinge region flexibility. Since BIRR1 and cIgG_{4} have the same mouse variable regions, a comparison of their respective binding curves should reveal equivalent binding affinities at the higher mAb concentrations even though their binding avidities at the lower mAb concentrations are very different.

Binding response surfaces were generated in all of the competitive ELISA experiments (e.g. Fig. 4). A comparison of the binding constants indicates that while the $K_{s}$ value for cIgG_{4} is only 30% that for the murine IgG_{2}, the $K_{v}$ values descriptive of monovalent binding are statistically equivalent. In addition, the constants obtained for the Fab ELISA data also argue for equivalent monovalent binding behavior. For a visual comparison of mAb and Fab binding, representative slices of the raw data from their respective response surfaces at a fixed reporter concentration are shown in Fig. 5. While the cIgG_{4} mAb displays a decrease in cooperative bivalent binding ability relative to the murine analog, the Fab isotherms are nearly identical (see also Table 1). These data suggest that although the affinities of the two mAbs are equivalent, their avidities are markedly different.

In summary, the constants obtained in the competitive ELISA experiments are consistent with monovalent and cooperative bivalent binding for the mAbs and monovalent binding for the Fab fragments (Mason and Williams, 1986). Since a reporter is not required for the mAb solution assay, the monovalent and non-cooperative bivalent binding observed in the HPLC immunoassay is a direct measurement of the mAbs's affinity. Thus, the mAb and Fab competitive ELISA (indirect) and the mAb HPLC-1A (direct) independently demonstrate equal affinities between the two antibodies. These results establish that BIRR1 and cIgG_{4} bind ICAM-1 with equivalent affinities but with inequivalent avidities.

A recent publication suggests that the subtype of the IgG

---

*4 M. M. Morelock, R. Rothlein, S. M. Bright, M. K. Robinson, E. T. Graham, J. P. Sabo, R. Owens, D. J. King, S. H. Norris, D. S. Scher, J. L. Wright, and J. R. Adair, our unpublished observations.
contributes the avidity of an antibody to its multimeric antigen (Horgan, 1993). This finding is consistent with what we report here. There are two possible explanations for the differential binding results for the chimeric antibodies presented. First, the constant regions of the molecules could be affecting assay results by interacting with Fc receptor on the JY cell. We think that this is unlikely, since we have been unable to detect any differences in the binding via the Fc of human IgG1, IgG2, or IgG3 to JY cells and FcR, the only Fc receptor to bind monomeric antibody and not normally found in human B-cell-derived lines (reviewed by Burton and Woof (1992)). The second explanation, and the one that we favor, is that the antigen binding results reflect differences in avidity imposed on the molecules by the different constant region structures. For bi- variant binding to occur, flexibility is required in the hinge and switch regions for rotation and elbow bending of the Fab arms (Valentine and Green, 1967; Romans et al., 1977; Jackson et al., 1983; Nezlin, 1990; Schumaker et al., 1991; Borrebaeck, 1992). Since constant regions have been shown to differ in the segmental flexibility of their hinge regions, the potential exists for antibodies with the same binding site but different constant regions to have differing abilities for spatially binding the antibody to the antigen site. In particular, the reported hinge flexibility of the human IgGs correlates with the retention of binding activity noted here for the chimeric antibodies, in that the IgG1, with the most flexible hinge has the best binding avidity, while the IgG2 with the least flexibility has the lowest binding avidity.

In conclusion, the engineering of chimeric antibodies by exchanging human constant regions for mouse constant regions not only alters the effector functions of the antibody, but can also alter its antigen binding properties. Thus, while the ability or inability of an IgG isotype to mediate biological events may be paramount in determining therapeutic efficacy, the ability to bind cell surface antigens must also be considered in the design and characterization of chimeric antibodies.

Acknowledgments—We thank Rowena Reedman and Alan Lyons for providing expression vectors containing the chimeric antibodies, Graham Roberts and Carol Gofton for construction of cell lines, and Karen Proudfoot and Janet Deystung for protein purification. We also thank Tobin Cammett for the biotinylation procedure of BIRR1, Rod Deleon for the JY cell culture, Karen Laszlo Harbasak for the preparation of JY cell ELISA microriter plates, and Paul McGoff for assistance with the chimeric purification and HPLC experiments. We also thank Tapon Roy for advice concerning the statistical treatment of the data.

APPENDIX

Since a cell-based ELISA involves solid/liquid phase reactions, the binding kinetics cannot be strictly characterized using equilibrium relationships for solutions. For example, on-rates will be slower as a result of diffusion-limited mass transport of the antibody in solution to the antigen on the cell surface; in addition, off-rates will also be slower due to the relatively high concentrations of the antigen at the cell surface (Nygren and Stenberg, 1989), and references therein). Nevertheless, it is precisely this cell surface antibody/antigen interaction that is of interest in considering the in vivo therapeutic aspects of these antibodies. Consequently, we have developed binding models based on equilibrium expressions (Pincus and Rendell, 1981; Mason and Williams, 1980) and we note that $K$ is an apparent binding constant. The models used herein postulate that antigen sites are uniformly distributed on the cell surface and that the binding of one antibody does not affect the binding of another antibody.

**Reporter mAb/Direct Binding**

**One-site Model**—In the simplest case, binding is between an antibody and a population of identical binding sites. The equilibrium equation for a one-site model is

$$K_p = \frac{R + G}{\theta_R G}$$  \hspace{1cm} (Eq. 1)

where $R$ is the reporter antibody, $G$ is a free antigen, $RG$ is the antibody-antigen complex, and $K_p$ is the apparent binding constant. An association equilibrium expression may be written for the complex formation as

$$K_p = \frac{[RG]}{[R][G]} = \frac{[RG]/[G]}{[R]/(G/G)} + \frac{\theta_{RG}}{\theta_G}$$  \hspace{1cm} (Eq. 2)

where $[G]_R$ is the total concentration of antigen binding sites, $\theta_{RG}$ is the fraction of sites complexed with an antibody and $\theta_G$ is the fraction of sites free. Noting that $\theta_{RG} = 1$ and solving for $\theta_G$, we obtain Equation 3.

$$\theta_G = \frac{K_p [R]}{1 + \theta_{RG}}$$  \hspace{1cm} (Eq. 3)

Although $[R]$ is not determined within the experiment, the initial antibody concentration $[R]_i$ may be used in Equation 3 as a first approximation, since $[R]_i > [G]_i$, except at very low antibody concentrations. In the ELISA technique used, the experimentally measured absorbance $A$ is a function of the bound fraction of total antigen sites, i.e.,

$$A = \theta_{RG} \cdot (A_{max} - A_{min}) + A_{min}$$  \hspace{1cm} (Eq. 4)

When $\theta_{RG} = 1$, $A = A_{max}$; for $\theta_{RG} = 0$, $A = A_{min}$. Thus, Equation 4 may be rewritten to relate absorbance to the fraction of bound antigen sites as defined by equilibrium relationships. Substituting for $\theta_{RG}$, we obtain Equation 5.

$$A = \frac{K_p [R]_i}{1 + \theta_{RG}} \cdot (A_{max} - A_{min}) + A_{min}$$  \hspace{1cm} (Eq. 5)

In this paper, $R$ is biotinylated BIRR1, $A$ is the experimentally determined absorbance of the enzymatic product p-nitrophenol at 405 nm, $A_{max}$ is the maximum absorbance determined under...
saturation conditions, and $A_{\text{min}}$ is the minimum or background absorbance due to nonspecific binding of streptavidin-$\beta$-galactosidase with the plate wells. Equation 5 is sigmoidal in nature with $A_{\text{max}}$ and $A_{\text{min}}$ being the fitted upper and lower asymptotes, respectively.

**Two-site Model**—The high concentration data in Fig. 3 can be fit to a model that is characterized by two sites with different binding affinities. Site 1 is considered to be made up of at least two ICAM-1 antigens and is capable of both cooperative bivalent binding (one mAb/site; $K_{R1}$) and monovalent (one or two mAbs/site; $K_{S2}$). Site 2 consists of a single ICAM-1 molecule and displays only monovalent binding (one mAb/site; $K_{S2}$). The equilibrium binding isotherm for a two-site model is described by Equation 6, where the fractions of total binding determinants for site 1 and site 2 are given by $\mu$ and $(1 - \mu)$, respectively.

$$\theta_{\text{total}} = \frac{\mu (K_{R1} + K_{R1} \cdot K_{S2} \cdot [R]_2) + (K_{S2} + K_{S2} \cdot [R]_2)}{1 + K_{R1} + K_{R1} \cdot K_{S2} \cdot [R]_2} \cdot [S]_2$$

Substitution of Equation 7 into Equation 8 gives the expression used for fitting the mAb direct binding ELISA.

**Reporter + Sample mAb/Competitive Binding: Two-site Model**

The equations developed for competitive binding are derived in a manner similar to direct binding. The competition of reporter and sample antibody are:

$$\mu = \theta_{\text{total}} - \theta_{\text{free}} = \frac{(1 - \mu) \cdot [R]_2 \cdot K_{R1} \cdot [S]_2}{1 + K_{R1} \cdot [R]_2 \cdot K_{S2} \cdot [S]_2}$$

where $K_{R1}$ and $K_{S2}$ are the apparent binding constants for the biotinylated mAb and sample Fab, respectively. Substitution of Equation 11 into Equation 4 gives the expression used for fitting the competitive Fab ELISA data. As in the other ELISAs discussed above, the affinity constants for the Fab fragments derived from this competition assay should be viewed as apparent binding constants.

**REFERENCES**

Adair, J. R. (1992) *Immunol. Res.* 19, 5-40

Angel, S., King, D. J., Bodmer, M. W., Turner, A., Lawton, A. D. G., Roberts, G., Pedley, B., and Adair, J. R. (1993) *Mol. Immunol.* 30, 105-108

Bebbington, C. R. (1991) Methods 5, 136-145

Benjamin, R. J., Cobbold, S. P., Clark, M. R., and Waldmann, H. (1986) *J. Exp. Med.* 165, 1589-1552

Blattiere, H. M., Stapelewski, Z., Herlyn, D., and Douillard, J. Y. (1991) *Hum. Antib.* Hybrid., 2, 16-25

Borrello, C. A. (1992) *Antibody Engineering*, pp. 69-75, W. H. Freeman and Co., New York

Bruggemann, M., Williams, G. T., Bindon, C. I., Clark, M. R., Walker, M. R., Jeffers, R., Waldmann, H., and Neuberger, M. S. (1987) *J. Exp. Med.* 165, 422-443

Burton, D. R., and Woof, J. M. (1992) *Advs. Immunol.* 51, 1-84

Chatenoud, L. (1996) *Immunol. Today* 7, 367-368

Chatenoud, L., Badraudriat, M. P., Chikoff, N., Kreis, H., Goldstein, G., and Bach, J. F. (1988) *J. Immunol.* 137, 830-838

Colcher, D., Melinec, D., Rosselli, M., Raubitshek, A., Yarranton, G., King, D., Adair, J., Whittle, N., Bodmer, M., and Schlom, J. (1999) *Cancer Res.* 59, 1378-1385

Cosimi, A. B., Coit, D., Delmonico, F. L., Preffer, P. I., Wei, S.-L., Rothlein, R., Faanes, R., and Colvin, R. B. (1990) *J. Immunol.* 144, 4604-4612

Dangl, J. L., Wessel, T. G., Morrison, S. L., Stryer, L., Herzenberg, L. A., and Otten, S. P. (1987) *EMBO J.* 7, 1989-1994

Diamond, M. S., Staunton, D. E., Peplow, R., Stacker, S. A., Garcia-Aguilar, J., Hibbs, M. L., and Springer, T. A. (1990) *J. Cell Biol.* 111, 3129-3139

Dillman, R. G. (1999) *Annu. Rev. Immunol.* 17, 1-15

Dustin, M. L., 13054 Comparison between Murine Anti-ICAM-1 and Human Chimeras

Evans, R. J., Dear, P. H., Foote, J. Neuberger, M. (1991) *Nature* 137, 830-838

Fujita, T., Bridges, S. T., Adair, J. R., Youle, R. J., Johnson, V., Kim, J., Muto, M., and Bach, J. F. (1986) *Science* 234, 927-942

Garland, C. L., Wessel, T. G., Morrison, S. L., Stryer, L., Herzenberg, L. A., and Otten, S. P. (1988) *J. Immunol.* 137, 830-838

Horgan, C., Brown, K., and Pincus, S. H. (1993) *J. Immunol.* 150, 640-6407

Huttell, P., Kashmiri, S., Colcher, D., Primus, F. J., Horan Hand, P., Roselli, M., Fisch, M., Yarranton, G., Bodmer, M., Whittle, N., King, D., Leoluis, C. C., McCoy, D. W., Callahan, R., and Schom, J. (1999) *Cancer Res.* 59, 181-1819

Jackson, A. P., Siddle, K., and Thompson, G. P. (1985) *Biochem.* 23, 1-5

Jonker, M., and Den Brek, J. H. A. M. (1993) *Eur. J. Immunol.* 23, 1547-1553

Khazaeli, M. B., Saleh, M. N., Wheeler, R. H., Huster, W. J., Holden, H., Carrano, V., and LoBuglio, A. F. (1986) *J. Natl. Cancer. Inst.* 80, 927-942

King, D. J., Adair, J. R., Angi, S., Low, D. C., Proudfoot, K. A., Loy, J. C., Bodmer, M., and Yarranton, G. T. (1991) *Biochem.* 231, 317-323

Lampert, U. K. (1970) *Nature* 227, 680-685

LoBuglio, A. F., Wheeler, R. H., Trang, J., Haynea, A., Rogers, K., Harvey, E. B., Sun, L., Ghrayeb, J., and Khazaeli, M. B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 4229-4234

Marlin, S. D., and Springer, T. A. (1977) *Cell* 131, 813-839

Marlin, S. D., Staunton, D. E., Springer, T. A., Stratowa, G., Sonnergerwuber, G., and Merluzzi, V. J. (1990) *Nature* 344, 70-72

Mason, D. W., and Williams, A. F. (1980) *Biosci. Rep.* 1351-1361

Mason, D. W., and Williams, A. F. (1986) *Biochem.* 187, 1-7

Mason, D. W., and Williams, A. F. (1986) *Handbook of Experimental Immunology*, Vol. 1, Blackwell Scientific Publications, Oxford

Mathews, P. W., Cobbold, S. D., Hale, G., Clark, M. R., Olivera, D. B., Lockwood, C. M., and Waldmann, H. (1990) *N. Engl. J. Med.* 323, 250-253

Morrison, S. L., Johnson, M. J., Herzenberg, L. A., and Otten, S. P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 686-687

Neelin, R. (1990) *Advs. Immunol.* 46, 1-40
Comparison between Murine Anti-ICAM-1 and Human Chimeras

Norris, S. H., Johnstone, J. N., DeLeon, R., and Rothlein, R. (1991) J. Pharm. Biomed. Anal. 9, 211–217

Nygren, H., and Stenberg, M. (1989) Immunology 66, 321–327

Oi, V. T., Vuong, T. M., Hardy, R., Beidler, J., Dangi, J., Herzenberg, L. A., and Stryer, L. (1984) Nature 307, 136–140

Pinches, M. R., and Rendell, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5924–5927

Reynolds, J. C., Del Vecchio, S., Sakahara, H., Lera, M. E., Carrasquillo, J. A., Neumann, R. D., and Larson, S. M. (1989) Nucl. Med. Biol. 16, 121–125

Riechmann, L., Clark, M., Waldmann, H., and Winter, G. (1988) Nature 332, 323–327

Romans, D. G., Tilley, C. A., Crookston, M. C., Falk, R. E., and Dorrington, K. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 2531–2535

Rothlein, R., Dustin, M. L., Marlin, S. D., and Springer, T. A. (1986) J. Immunol. 137, 1270–1274

Rothlein, R., Kennedy, C., Czajkowski, M., and Barton, R. W. (1993) Int. Arch. Allergy Immunol. 100, 121–127

Schneider, W. P., Wensel, T. G., Stryer, L., and Oi, V. T. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3509–3513

Shaw, D. R., Khazaeli, M. B., and LoBuglio, A. F. (1988) J. Natl. Cancer Inst. 80, 1553–1558

Shearman, C. W., Karuzy, E. J., Lawrie, D. K., Li, Y.-W., THANMAN, P., Moore, G. P., and Kurrie, R. (1991) J. Immunol. 146, 925–935

Shumaker, V. N., Phillips, M. L., and Hanson, D. C. (1991) Mol. Immunol. 28, 1347–1350

Smith, C., Rothlein, R., Hughes, B., Mariscalco, M., Rudloff, M. E., Schmalstieg, F. C., and Andersson, D. (1988) J. Clin. Invest. 82, 1746–1756

Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L., and Springer, T. A. (1988) Cell 52, 925–933

Staunton, D. E., Dustin, M., Erickson, H. P., and Springer, T. A. (1990) Cell 61, 243–254

Stephens, P. E., and Cockett, M. I. (1988) Nucleic Acids Res. 17, 7110

Steplewski, Z., Sun, L. K., Shearman, C. W., Ghrayeb, J., Daddona, P., and Koprowski, H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4852–4856

Tan, L. K., Shopes, R. J., Oi, V. T., and Morrison, S. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 162–166

Valentine, R. C., and Green, N. M. (1987) J. Mol. Biol. 207, 615–617

Whittle, N., Adair, J., Lloyd, C., Jenkins, L., Devine, J., Schlom, J., Rauh, R., Velich, K., Fecondo, J., and Boyd, A. W. (1989) Immunol. Rev. 108, 135–161

Welch, E., Forbes, Z., and Boyce, Z. H. (1987) Protein Eng. 1, 499–505