

COMPARISON OF THE EFFECTOR FUNCTIONS OF HUMAN IMMUNOGLOBULINS USING A MATCHED SET OF CHIMERIC ANTIBODIES

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The five Ig classes have distinct biological roles. The IgG subclasses also show marked differences in their ability to mediate a variety of effector functions. A detailed comparison of the properties of the human Ig classes and subclasses is not only of interest for relating the functions of antibodies to their structures but is also of great importance for the implementation of therapy based upon immunological intervention. Indeed, this second aspect has become particularly significant as the development of techniques for the production of chimeric antibodies (1–3) should ensure that immunological intervention is now likely to make use of mAbs that have human effector functions; several cell lines have already been established that secrete chimeric antibodies directed against human cancer cells (4–6).

Much of our knowledge of the properties of human Igs has been obtained from the study of myeloma proteins (reviewed in references 7–9). However, generally myeloma proteins do not bind identified antigens and, moreover, different myeloma proteins differ not only in their heavy chain class/subclass but also in their light chains and variable regions. As the initiation of antibody effector activity is usually a consequence of antigen binding and is indeed influenced by the quality of that binding, previous studies on myeloma proteins, although valuable, may not provide a sufficient picture of antibody effector function for therapeutic purposes. To carry out a detailed and controlled comparison of the effector functions of the different human C_H regions, we have established a panel of cell lines that secrete a matched set of human chimeric antibodies. These antibodies are directed against the hapten 4-hydroxy-3-nitrophenacetyl (NP).¹ This specificity for a known hapten has allowed us to compare the effector functions of the IgG subclasses not only when interacting with soluble antigen but also when interacting with cell-bound antigen. This has enabled us to determine the efficacy with which different subclasses lyse their

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¹Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytotoxicity, NP, nitrophenacetyl; NIP, 5-iodo-4-hydroxy-3-nitrophenyl.

target cells by means of both cell-mediated and complement-dependent mechanisms.

Materials and Methods

Plasmids. Plasmid pSV-V_{NP} (Fig. 1) contains a single Bam HI site for the insertion of restriction fragments containing C_H exons; this plasmid, as well as pSV-V_{NP}H_ε, has been previously described (3). Derivatives of pSV-V_{NP} with Xba I or Hind III sites in place of the Bam HI site were created by inserting octameric linkers into the Bam HI site that had been blunted using *Escherichia coli* DNA polymerase I Klenow fragment. Construction of the Hind III vector required prior destruction of two other Hind III sites in pSV-V_{NP} by Klenow treatment. The origin and manipulation of the DNA inserts specifying the different C_H genes is described in the legend to Fig. 1.

Cell Lines² and Transfection. J558L plasmacytoma cells (10) were obtained from Dr. S. L. Morrison (Dept. of Microbiology, College of Physicians and Surgeons, Columbia Univ., New York) and were grown in DME containing 10% FCS. Transfection was performed by spheroplast fusion (11) in the case of the IgG2-, IgG4-, and IgE-secreting cell lines, and by electroporation for the other constructs. For electroporation (12), 2 × 10⁷ cells that had been washed and resuspended in 0.2 ml cold PBS were mixed with linearized plasmid DNA (15 μg in 20 μl H₂O) and placed in a 1 cm × 0.4 cm × 4.5 cm plastic microcuvette that had been equipped with aluminum electrodes. The sample was given 15 2-kV pulses at 1-s intervals from an Apelex power supply, left on ice for 30 min, and then resuspended in 50 ml DME/10% FCS/gentamycin before transferring to 24-well plates. Selective medium containing mycophenolic acid was applied 24 h later (11). Anti-NP antibody in the culture medium was assayed by RIA (13) and cells were cloned by limiting dilution.

Protein Purification and Analysis. For antibody purification, cells were allowed to grow to saturation in 2 liters of DME/10% FCS. The culture medium was filtered, supplemented to 0.05% in NaN₃, and passed over a 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP)-caproate Sepharose affinity column. This column was prepared according to a procedure provided by M. Cramer (University of Cologne, Cologne, Federal Republic of Germany). 80 ml of Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) that had been washed and resuspended in an equal volume of ice-cold 2 M Na₂CO₃, was activated by mixing with 4 ml of 0.5 g/ml CNBr in acetonitrile. After extensive washing, the activated Sepharose was mixed with 40 ml of bis(3-aminopropyl)amine that had been brought to pH 10 by the slow addition of 12 M HCl at 4°C. After gentle mixing overnight at 4°C, the derivatized Sepharose was washed in H₂O, resuspended in cold 3% NaHCO₃ and mixed with 60 mg of NIP-caproate-*O*-succinimide (Cambridge Research Biochemicals, Harston, Cambridge) that had been dissolved in 1 ml dioxane. After mixing overnight at 4°C, the NIPcap-Sepharose was washed with 0.1 M glycine/HCl, pH 3, followed by PBS and then stored in PBS/0.05% NaN₃. After samples had been passed through the column and extensive washing, antibody was eluted from the sorbent with 0.5 mM NIP-caproate or NP-caproate in PBS and dialyzed extensively. Antibody samples were centrifuged extensively to remove aggregates.

Protein samples were analyzed on SDS/PAGE gels (14). Samples were also purified on NIP-caproate Sepharose from cells labeled with [¹⁴C]-L-lysine in the presence or absence of 10 μg/ml tunicamycin as previously described (15).

Serological Assays. Serologic analysis was performed in an ELISA assay as follows: wells of flat-bottomed microtiter plates were coated overnight at 4°C with 100 μl purified anti-NP antibody diluted to 1 μg/ml in 0.2 M NaHCO₃, pH 9.6, or with purified human paraproteins of serologically defined class, subclass, and allotype. After washing with PBS/0.05% Tween 20 (PBS/Tween), 100 μl of a 1:100 dilution of mAb ascitic fluid in

² The cell lines will be deposited in the European Centre of Animal Cell Cultures, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts.SP4 OJG, United Kingdom, where they may be obtained by scientists affiliated with nonprofit research in pursuance of an academic research program.

PBS/Tween was added to each well and incubated at 37°C for 2 h. After extensive washing with PBS/Tween, 100 μ l of a 1:4,000 dilution in PBS/Tween of polyclonal sheep anti-mouse Ig antibody conjugated to horseradish peroxidase (BDS Biologicals Ltd., Birmingham, United Kingdom) was added and incubated for a further 2 h at 37°C before repeated washing in PBS/Tween. Bound sheep antibody was revealed by addition of the substrate (2.2 mM *o*-phenylene diamine). The reaction was quenched after 15 min by the addition of 50 μ l 12.5% H₂SO₄ and the OD₂₉₅ was measured using a Titertec Miniscan (Flow Laboratories, Irvine, Scotland). Positive reactions were scored where the OD₂₉₅ was 0.5 OD units above control values.

Protein A Binding. Purified antibodies (100 μ g/ml in PBS) were coated onto the wells of microtiter plates (Cooke; Dynatech Laboratories, Inc., Alexandria, VA) before blocking with PBS/3% BSA. The wells were then incubated with [¹²⁵I]protein A (50,000 cpm per well; Amersham International, Amersham, United Kingdom), which had been diluted in citrate/phosphate buffer of the appropriate pH. The wells were washed at the same pH. Parallel incubations were also performed in which the plates were washed at the various pHs and the immobilized antibody developed with radio-iodinated monoclonal anti- λ_1 antibody Ls136 (13) in PBS/1% BSA. This confirmed that the pH dependence observed in the protein A binding assays was indeed a result of the pH dependence of antibody/protein A interactions rather than artefact due to washing the chimeric antibody off the microtiter plate.

C1q Binding and Hemolysis Assays. Human C1q that had been radioiodinated by the lactoperoxidase method was a gift from Dr. N. Hughes-Jones and B. Gorick (MRC Centre, Cambridge, United Kingdom). Human erythrocytes were coupled with NIP-kephalin (gift of Dr. U. Weltzien, Max Planck Institute For Immunology, Freiburg, Federal Republic of Germany) as previously described (16), and, if required, were labeled with sodium [⁵¹Cr]chromate (Amersham International) as previously described (17). For C1q binding assays, washed NIP-red cells (20 μ l at 10⁹ cells/ml) in PBS/1% BSA were coated with saturating amounts of antibody (10 μ l at 200 μ g/ml) and then supplemented with [¹²⁵I]C1q (10 μ l at 10–60 μ g/ml). After rotation at 37°C for 1 h, samples were centrifuged in microfuge tubes through 150 μ l of oil of density 1.028 (made by mixing four parts di-*n*-butyl phthalate with one part dinonyl phthalate). The cell pellets were separated by clipping off the bottom of the tube and the radioactivity in the bound and free fraction was determined. Controls were performed without added antibody; nonspecific binding was always <1%.

For hemolytic complement assays, NIP-human red cells were labeled with [⁵¹Cr]-chromate, washed, and 50 μ l of cells (10⁹/ml) were mixed in microtiter wells with 50 μ l of appropriate dilutions of the chimeric antibodies. After 10 min at room temperature, 100 μ l of diluted human complement was added to give a final concentration of 20%. After a 30-min incubation at 37°C, the cells and supernatant were separated by centrifugation (100 g, 2 min). Samples incubated with no antibody were used to calculate the spontaneous ⁵¹Cr release. The percentage specific ⁵¹Cr release is calculated as: Percent release = 100 \times [(Test release – spontaneous release)]/[(Total radioactivity) – (spontaneous release)].

Antibody-dependent Cell-mediated Cytotoxicity. This was performed essentially as described (18) but with modifications. Cells (2 \times 10⁶) of the human T cell line HPB-ALL were labeled in 100 μ l of medium with 50 μ Ci of sodium [⁵¹Cr]chromate for 30 min at 37°C, and 2 μ l of NIP-kephalin (100 μ g/ml) was then added and the incubation was continued for another 15 min. These target cells were then washed with HEPES-buffered Iscove's modified DME containing 1% BSA. The effector cells were obtained from healthy donors by venipuncture and, after defibrination with glass beads, mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (19) and cultured overnight in Iscove's MDM/5% FCS. Antibody-dependent cell-mediated cytotoxicity (ADCC) was measured by mixing labeled target cells (50 μ l at 4 \times 10⁵/ml) with dilutions (100 μ l) of the chimeric antibodies and then supplementing with the effector cells (50 μ l at 1.2 \times 10⁷/ml). The cells were pelleted (200 g, 10 min) and incubated at 37°C for 4 h. The radioactivity in the supernatant was then measured. Assays were performed in triplicate and controls

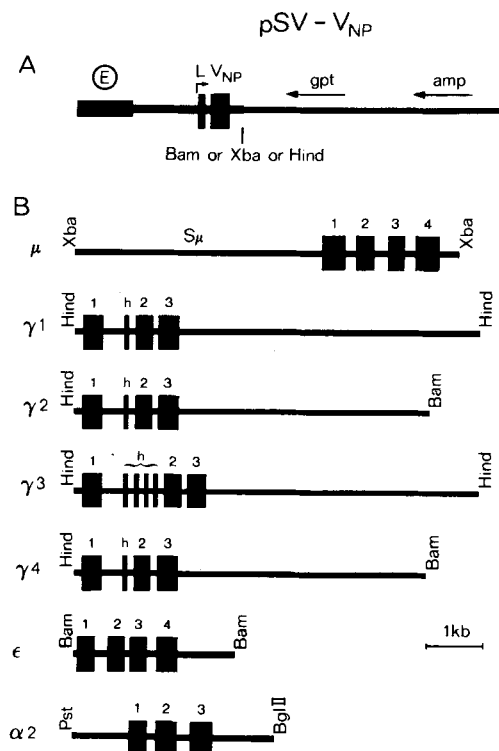


FIGURE 1. Structure of plasmids. (A) The pSV- V_{NP} vector. The mouse IgH enhancer (E) is located upstream of the leader (L) and V exons of the V_{NP} variable region. The family of vectors have a Bam HI, Hind III, or Xba I site located downstream of V_{NP} for the insertion of germline C_H regions. The transcriptional orientations of the L/ V_{NP} , *gpt*, and ampicillin resistance (*amp*) genes are marked with arrows. (B) The C_H region inserts. The exons for the 3' end of the mRNA of the membrane forms of the heavy chains are not depicted, although they are probably present in their entirety in the γ but not in the μ , ϵ , or α constructs. Only restriction sites at the ends of the C_H gene inserts are depicted. The C_H inserts originate: μ , Xba I fragment of phage λ C75R (20); $\gamma 1$, Hind III fragment of phage Charon 4A HI γ 10 (21); $\gamma 2$ and $\gamma 4$, Hind III-Bam HI fragments of cosIg8 (22), which were obtained as Bam HI-Bam HI fragments after cloning of the genomic Hind III fragments in pUC19; $\gamma 3$ was obtained as a 7.4-kb Hind III fragment from either cosIg1 (22) yielding an IgG.G3m(g) antibody or from phage λ EZZ $\gamma 3$ (23) yielding an IgG.G3m(b) antibody. The $\alpha 2$ insert originated from cosIg10 (22) and was obtained as a Hind III fragment from a plasmid subclone whose insert extended from the Pst I site 5' of $C_H 1$ to the Bgl II site 3' of $C_H 3$. Plasmid pSV- V_{NP} He has been described previously (3).

performed without effector cells (no lysis was then observed). The specific ^{51}Cr release was calculated as described above.

Results

Cell Lines Expressing Human Chimeric Antibodies. The basic plasmid that was used for the construction of the chimeric heavy chain genes (pSV- V_{NP}) is depicted in Fig. 1. The plasmid contains the V_H gene of a mouse anti-NP antibody. Upstream of the V_H promoter is the mouse IgH enhancer element; downstream of V_H is a unique Bam HI restriction site. Different human C_H genes were inserted either into this Bam HI site or into derivatives of pSV- V_{NP} in which the Bam HI site was converted to a Hind III or Xba I site by insertion of linkers. The exact C_H fragments inserted are described in Fig. 1. In the case of $\gamma 3$, two constructs were assembled with different $\gamma 3$ genes that originated from different sources, having been cloned from different individuals.

The *gpt* marker present in plasmid pSV- V_{NP} allows stably transfected cells to be selected by virtue of their resistance to the drug mycophenolic acid. The plasmids were introduced into the mouse plasmacytoma J558L as described in Materials and Methods. This plasmacytoma secretes a λ_1 light chain but expresses no heavy chain of its own. The V_λ of the endogenous light chain complements the V_{NP} of the transfected heavy chain to yield an NP-specific antibody; such antibodies display a slightly greater affinity for the iodinated derivative, NIP, than for NP itself.

Antibodies were purified to homogeneity from the culture medium of cloned

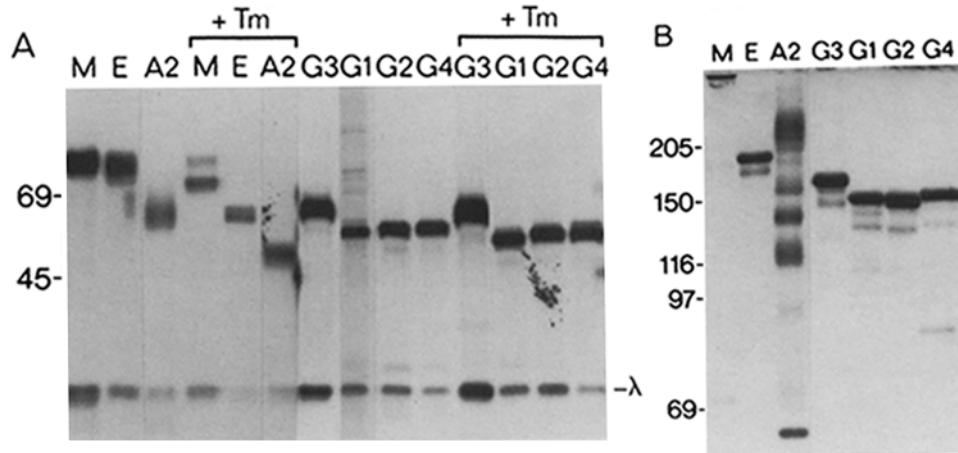


FIGURE 2. Analysis of chimeric antibodies by SDS-PAGE. (A) Antibodies from cells biosynthetically labeled in the presence or absence of tunicamycin (*Tm*) were analyzed on 10% SDS-PAGE gels after reduction. (B) Antibodies purified from culture supernatants were analyzed without reduction on a 7% SDS-PAGE gel. The positions of molecular mass (kD) markers are indicated.

transfectants; yields were typically in the range 2 to 10 mg/liter although sometimes up to 30 mg/liter was achieved.

Analysis of Antibodies on SDS/PAGE. To characterize the chimeric antibodies, purified samples were reduced and analyzed by SDS-PAGE. The mobilities of the heavy chains were much as predicted on the basis of their DNA sequences except for μ , ϵ , and α_2 . We have previously shown (3) that the chimeric ϵ heavy chains become heavily glycosylated. We therefore resorted to biosynthetic labeling to compare the antibody normally secreted by the transfectants with that made in the presence of the glycosylation inhibitor tunicamycin. The results (Fig. 2A) demonstrate the IgM, IgE, and IgA2 secreted by the J558L transfectants are indeed heavily glycosylated on their heavy chains. A much smaller amount of glycosylation is apparent on the γ heavy chains. The sizes of the heavy chains of the unglycosylated antibodies are exactly as predicted from the DNA sequences (the γ_3 heavy chains standing out from the other γ chains because of the long hinge).

To examine whether the secreted antibodies were correctly assembled, unreduced samples were analyzed on 7% SDS-PAGE gels (Fig. 2B). The IgGs and IgE exhibit mobilities consistent with their having the expected H_2L_2 structures whereas the IgM is clearly of very large molecular weight and, as expected for the pentameric form, scarcely enters the gel. IgA2 gives the most complex pattern and appears to contain H, HL, H_2L_2 , and $(H_2L_2)_2$ forms; this may reflect the secretion of some noncovalently linked molecules.

Serological Characterization. For potential therapeutic applications, it is clearly important to establish whether chimeric human antibodies secreted by a mouse plasmacytoma are indeed homologous to human mAbs. While the analysis on SDS-PAGE indicated that the chimeric antibodies resembled their human counterparts as regards the molecular weights of both the native and the unglycosylated heavy chains, we decided to extend this characterization to include a wide

TABLE I
Serological Typing of Chimeric Antibodies

Specificity	Number used	Chimeric antibody						
		IgG1	IgG2	IgG3*	IgG3 [†]	IgG4	IgA2	IgM
IgG	7	+	+	+	+	+	ND	ND
IgG1	1	+	-	-	-	-	ND	ND
G1m(f)	1	-	-	-	-	-	ND	ND
IgG2	1	-	+	-	-	-	ND	ND
IgG2,3,4	1	-	+	+	+	+	ND	ND
IgG1,2,4	5	+	+	-	-	+	ND	ND
IgG3	1	-	-	+	+	-	ND	ND
G3m(u)	1	-	-	+	+	-	ND	ND
nG3m(g)	4	+	+	-	+	+	ND	ND
IgG4	1	-	-	-	-	+	ND	ND
IgG1,2,3	2	+	+	+	+	-	ND	ND
IgA	1	ND	ND	ND	ND	ND	+	ND
IgA1	3	ND	ND	ND	ND	ND	-	ND
IgA2	1	ND	ND	ND	ND	ND	+	ND
nA2m(2)	1	ND	ND	ND	ND	ND	+	ND
IgM	6	ND	ND	ND	ND	ND	ND	+

* The $\gamma 3$ C_H region derives from clone cosIg1 (22).

[†] The $\gamma 3$ C_H region derives from phage λ EZZ $\gamma 3$ (23).

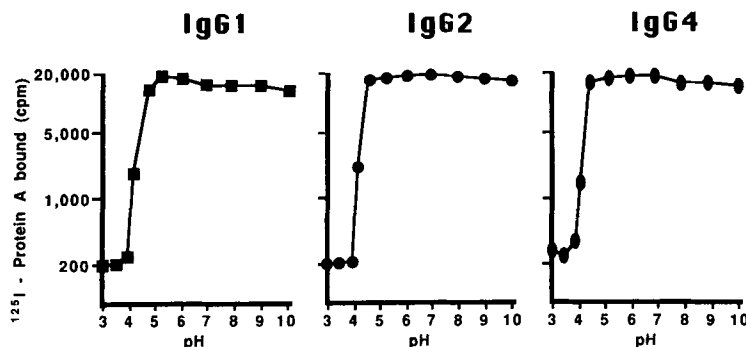


FIGURE 4. Binding of protein A to chimeric antibodies. The amount of radio-iodinated protein A bound to immobilized antibodies is plotted as a function of pH. No binding was detected to the IgM, IgE, IgA2, or either of the IgG3s over the entire pH range tested.

assayed using various concentrations of radiolabeled C1q and hapten-derivatized red cells that were coated with amounts of antibody that were shown to be saturating. The results (Fig. 5, *left*) show that C1q binding is detected with the IgM, IgG1, and both IgG3 antibodies but not with IgG2, IgG4, or IgE. The binding to IgM is weaker than to IgG1 and IgG3 and, of these two IgG subclasses, the IgG3s bound more C1q than did the IgG1.

Complement-mediated Hemolysis. Antibodies were tested over a wide concentration range for their ability to lyse hapten-coupled human red cells in the presence of human complement. The results (Fig. 5, *right*) show that IgG2, IgG4, and IgE did not mediate hemolysis, whereas IgM, IgG1, and both IgG3s were effective. Indeed, it is notable that the IgG1 was considerably more effective in this hemolytic assay than the IgG3s. We have previously demonstrated (17) that

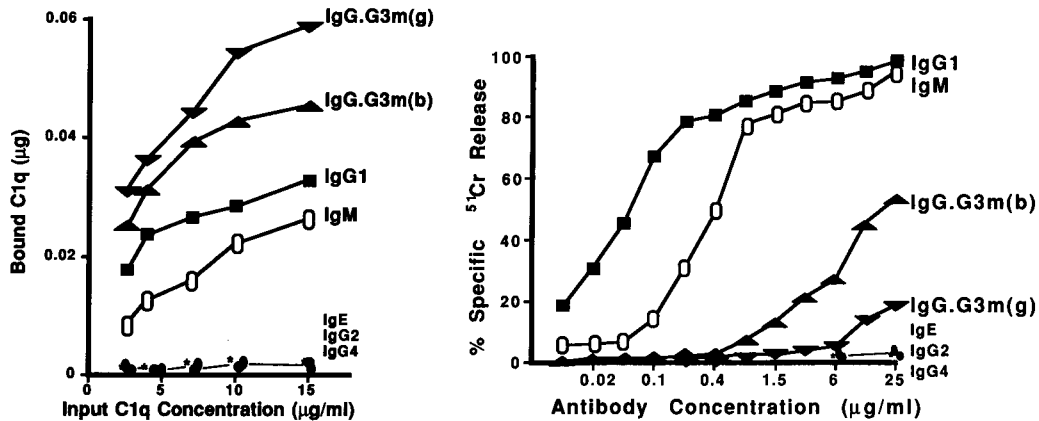


FIGURE 5. Binding of Clq and complement-dependent hemolysis by chimeric antibodies. (Right) Binding of various concentrations of radiolabeled Clq to NIP-human red cells that have been coated with saturating amounts of chimeric antibody. (Left) Lysis of [⁵¹Cr]NIP-human red cells by various concentrations of chimeric antibody in the presence of human complement. The allotypes of the two IgG3 antibodies [IgG.G3m(b) and IgG.G3m(g)] are indicated.

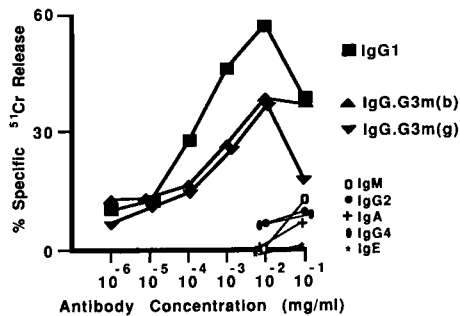


FIGURE 6. ADCC by chimeric antibodies. The lysis of [⁵¹Cr]NIP-conjugated human target cells (HPB-ALL T cell line) by human PBMC was measured as a function of the concentration of anti-NP chimeric antibody.

the efficacy of anti-NP antibodies in this hemolytic assay is critically dependent on their affinity for the hapten. However, this cannot explain the results obtained here. Binding-inhibition assays (not shown) indicate that the IgG1 and IgG3 antibodies have indistinguishable affinities for NIP-caproate. Furthermore, the IgG1 and IgG3 samples gave very similar titers in hemagglutination assays using hapten-derivatized red cells. Thus, although IgG3 is the best subclass as regards C1q binding it is considerably less potent than IgG1 in hemolytic assays. Interestingly, a similar but less marked reversal of hemolytic efficiency as compared with C1q binding is observed with the two IgG3 allotypes. Whereas the IgG.G3m(g) binds Clq slightly better, the IgG.G3m(b) is somewhat more effective in hemolysis.

Antibody-dependent Cell-mediated Cytotoxicity. The efficacy of the antibodies in mediating ADCC was tested using a hapten-derivatized human T cell line (HPB-ALL) as target and mononuclear cells from human volunteers as effector cells. It was found (Fig. 6) that only IgG1 and IgG3 were effective in mediating ADCC with the IgG1 showing greater potency than either of the IgG3s.

Discussion

The cell lines established during the course of this work provide a source of chimeric antibodies that can be easily purified to homogeneity in a one-step purification on hapten sorbents. To confirm that these chimeric human antibodies made in mouse plasmacytoma cells exhibit the features expected of antibodies possessing human C_H regions, we have characterized the antibodies as regards their serological properties, the mobilities in SDS-PAGE of both the native and the unglycosylated forms as well as their binding to protein A. In all these respects, the chimeric antibodies behave exactly as expected from their authentic human counterparts.

The known antigen specificity of these chimeric antibodies has allowed us to assay their efficacy in complement-mediated lysis and in ADCC. This has given rise to some novel and unexpected findings. The hierarchy of the binding of the aggregated chimeric IgG subclasses to human C1q described here agrees well with the previous results on the hierarchy in C1q binding of monomer IgG myeloma subclasses (28); however, aggregated IgG will bind with a higher avidity than monomeric IgG. Nevertheless, when we measured the efficacy of the antibodies in complement-mediated hemolysis, a very different result was obtained. It is evident that the IgG1 is very much more effective than the IgG3 in this assay. At present it is not clear why there is this lack of correlation between C1q binding and hemolytic efficacy; it will clearly be of interest to compare the IgG1 and IgG3 chimeric antibodies in intermediate stages of the pathway such as C1 activation and C4 and C3 binding. It will also be of interest to determine how the comparative hemolytic efficacy of IgG1 and IgG3 depends upon the density and nature of the antigen on the target cell.

The results presented here also indicate that the IgG1 and IgG3 are the only antibodies that are really effective in ADCC. The greater effectiveness of IgG1 as compared with IgG3 is a novel and interesting finding. The relative inactivity of the IgG2 and IgG4 antibodies was, however, predictable. Although the exact nature of the effector cell responsible for mediating ADCC has not been unambiguously identified, the Fc receptors found on the possible effector cell types (lymphocytes and monocytes) bind IgG1 and IgG3 much better than IgG2 and IgG4 (reviewed in reference 9).

Finally, it is worth noting that the results obtained in this work suggest that for many therapeutic purposes an IgG1 antibody might be greatly preferred to the other IgG subclasses as it appears to be considerably more effective in mediating both complement-dependent lysis and ADCC.

Summary

Cell lines have been established that secrete a matched set of human chimeric IgM, IgG1, IgG2, IgG3, IgG4, IgE, and IgA2 antibodies that are directed against the hapten 4-hydroxy-3-nitrophenacetyl. These chimeric antibodies secreted from mouse plasmacytoma cells behave exactly like their authentic human counterparts in SDS-PAGE analysis, binding to protein A and in a wide range of serological assays. The antibodies have been compared in their ability to bind human C1q as well as in their efficacy in mediating lysis of human erythrocytes in the presence of human complement. A major conclusion to emerge is that

whereas IgG3 bound C1q better than did IgG1, the chimeric IgG1 was much more effective than all the other IgG subclasses in complement-dependent hemolysis. The IgG1 antibody was also the most effective in mediating antibody-dependent cell-mediated cytotoxicity using both human effector and human target cells. These results suggest that IgG1 might be the favoured IgG subclass for therapeutic applications.

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