Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5322–5326, July 1990
Immunology

Expression and characterization of an antibody binding specificity joined to insulin-like growth factor 1: Potential applications for cellular targeting

(chimeric antibody/immunotherapy/growth factor receptors/fusion protein)

SEUNG-UON SHIN AND SHERIE L. MORRISON*

Department of Microbiology, University of California at Los Angeles, and The Molecular Biology Institute, 405 Hilgard Avenue, Los Angeles, CA 90024

Communicated by Stanley G. Nathenson, April 26, 1990

ABSTRACT **To create antibody molecules with improved functional properties, a growth factor (insulin-like growth factor 1, IGF1) was used to replace the constant region of a chimeric mouse–human IgG3 anti-dansyl antibody. The chimeric heavy chain was expressed with an anti-dansyl-specific chimeric κ light chain. The IgG3–IGF1 chimeric protein retained its specificity for the antigen dansyl. The chimeric proteins bound to the IGF1 receptors of the human lymphoblast IM-9, albeit with reduced affinity, and elicited some of the same biologic effects (increased glucose and amino acid uptake) in human KB cells as did human IGF1, but with reduced specific activity. The reduced affinity and biologic activity may result from several things: the presence of the unprocessed IGF1 moiety, the large size of the IgG3–IGF1 chimeric protein (160 kDa) compared with IGF1 (7 kDa), and three amino acid substitutions in rat IGF1 compared with human IGF1, which may lead to decreased affinity for the human IGF1 receptor. The chimeric proteins show that it is feasible to produce a new family of immunotherapeutic molecules targeted to growth factor receptors.**

Monoclonal antibodies, because of their inherent specificity, seem ideal agents for recognizing and destroying malignant cells. However, a major challenge has been to produce antibodies that are truly tumor specific and that are effectively targeted to malignant cells while leaving normal cells untouched.

Transfected cells (transfectomas) provide an approach to improving monoclonal antibodies. Genetically engineered antibodies can be expressed following gene transfection into lymphoid cells (1–5). One of the major advantages of expressing genetically engineered antibodies is that one is not limited to using antibodies as they occur in nature. In particular, nonimmunoglobulin sequences can be joined to antibody sequences, creating multifunctional molecules. We have exploited this characteristic by joining growth factors to an antibody combining specificity; we anticipate that these molecules will be more effective in targeting tumor cells possessing growth factor receptors, since both the growth factor and an anti-tumor specificity can be contained in a single molecule and function synergistically. Growth factor receptors have also been reported to be on the blood–brain barrier; the chimeric molecules may be able to utilize growth factor receptors for transcytosis into the brain (6–8).

For these initial studies, we have joined insulin-like growth factor 1 (IGF1) to a murine anti-dansyl (Dns) combining specificity and the heavy (H) chain constant (C) region C_H1 domain, hinge, and a portion of the C_H2 domain from human IgG3. When this chimeric H chain was transfected into a myeloma cell along with the dansyl-specific light (L) chain, the expected molecule was produced, assembled, and secreted. The resulting chimeric proteins bound the hapten Dns. They also were bound by the growth factor receptor, but with reduced efficiency, and exhibited some of the functions of IGF1 such as increasing uptake of α-aminoisobutyric acid and 2-deoxy-D-glucose (2-dGlc) (9). Therefore, these molecules have great potential as immunotherapeutic reagents.

MATERIALS AND METHODS

Cell Lines. Human lymphoblasts, IM-9, which express IGF1 receptors on their surface (10), were obtained from American Type Culture Collection and were grown in RPMI 1640 medium with 10% (vol/vol) fetal calf serum (HyClone). Human epidermoid carcinoma, KB, which expresses growth factor receptors on its surface (11), was a gift from H. Ginsberg (Columbia University, New York). KB cells and P3X63Ag8.653 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; GIBCO) with 10% calf serum (HyClone).

Construction and Characterization of the IgG3–IGF1 Chimeric Protein. A unique restriction enzyme site (*Pvu* II) was generated at the 5′ end of the C_H2 domain of the mouse–human IgG3 H chain gene specific for the hapten Dns by site-directed mutagenesis (12) and at the 3′ end of the leader sequence of rat IGF1 cDNA, a gift from A. Efstratiadis (Columbia University). IGF1 was joined to IgG3 by using the *Pvu* II sites. The chimeric IgG3–IGF1 H chain and κ L chain genes were transfected into P3X63Ag8.653 simultaneously by protoplast fusion as described (13, 14). Transfected cells were selected with G418 (GIBCO) at 1.0 mg/ml and screened by enzyme-linked immunosorbent assay (ELISA) for transfectomas producing the chimeric protein (14). The IgG3–IGF1 chimeric proteins biosynthetically labeled with [^{35}S]methionine (Amersham) were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO$_4$/PAGE) with or without 2-mercaptoethanol (Kodak). The IgG3–IGF1 chimeric proteins were purified by affinity column as described (15). The purity of the IgG3–IGF1 chimeric proteins was determined by silver-staining gel (16) and fast protein liquid chromatography (FPLC; Pharmacia).

Receptor Binding Assays. IM-9 cells were washed twice with 100 mM Hepes buffer containing 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO$_4$, 15 mM HOAc, 10 mM glucose, and 1% bovine serum albumin (BSA; pH 7.5) (Hepes/BSA buffer), resuspended in Hepes/BSA buffer, and incubated for 1 hr at

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IGF1, insulin-like growth factor 1; FPLC, fast protein liquid chromatography; 2-dGlc, 2-deoxy-D-glucose; Dns, dansyl; H, heavy; C, constant; V, variable; L, light.

*To whom reprint requests should be addressed.

room temperature (17) at a concentration of 4–6 × 10^7 cells per ml. A 50-μl aliquot of this cell suspension (2–3 × 10^6 cells) was removed and incubated with 50 μl of human IGF1 (Amgen Biologicals), IgG3–IGF1, IgG3, or buffer, and with 100 μl of buffer containing 1 μl of ^{125}I-labeled IGF1 [^{125}I-IGF1 (Amersham); 2.5 μCi (1 μCi = 37 kBq) in 2 ml of phosphate-buffered saline (PBS)] at 15°C for 2 hr. The cells were pelleted by 10-sec centrifugation in a microcentrifuge, 100 μl of the supernatant (S; total, 200 μl) was saved for determination of unbound radioactivity, and the pellets (P) were washed with 0.5 ml of ice-cold PBS and assayed for radioactivity in a gamma counter (Beckman Gamma 5500). The percent inhibition was calculated as {1 − [P/(2S + P)]/C} × 100%, where C is the cpm bound in the absence of a competitor.

Determination of α-[1-^{14}C]Aminoisobutyric Acid and 2-[1-^{14}C]dGlc Uptake. KB cells (5 × 10^5 cells per ml) grown to confluence in a 24-well plate were washed three times with IMDM. After incubation with IMDM without serum overnight at 37°C, cells were washed with Hepes/BSA buffer, and 100 μl of buffer was added. Hepes/BSA buffer without glucose was used for 2-dGlc uptake. A 100-μl aliquot of various concentrations of the test samples (IGF1, IgG3–IGF1, or IgG3) was added into each well and incubated at 37°C for 6 hr. After incubation, 24 μM α-[^{14}C]aminoisobutyric acid (DuPont) or 15 μM 2-[^{14}C]dGlc (Amersham) was added into each well (9). The plates were rapidly washed with ice-cold PBS after a 15-min incubation at 37°C, and cells were lysed with 300 μl of 1 M NaOH. Aliquots were assayed for ^{14}C and normalized to the amount of total protein determined by the bicinchoninic acid protein assay (Pierce).

RESULTS

Construction and Transfection of a Hybrid Human IgG3–IGF1 Rat Gene. Experiments were undertaken to produce an antibody combining site joined to a growth factor so that the resulting molecule would retain its ability to bind to both antigen and the growth factor receptor. For the initial construction, rat IGF1 was chosen as the growth factor. Mature IGF1 (a 70-amino acid protein that exists as a monomer) is formed from the IGF1 precursor (a 130-amino acid protein) through proteolytic processing of both its leader peptide and its carboxyl terminus; the amino acid at which the leader peptide is cleaved is a convenient site for joining to the immunoglobulin molecule. Human IgG3, which has an extended hinge region of 62 amino acids, was chosen for use as the immunoglobulin; placing the IGF1 distal to the hinge region results in a spacing and flexibility that should facilitate simultaneous antigen and receptor binding.

To facilitate construction of the fused genes, a unique restriction site (*Pvu* II) was generated by site-directed mutagenesis at the 5′ end of the C_H2 exon in human IgG3 cDNA and at the 3′ end of the leader sequence of the rat IGF1 cDNA (Fig. 1*A*). These mutations were confirmed by sequencing. The human IgG3 gene [which contains the C_H1 domain, hinge, and 4 base pairs (bp) of the C_H2 exon] was joined to the rat IGF1 gene (which contains 2 bp of leader sequence, exon 2, exon 3, and exon 5). The IgG3–IGF1 chimeric C region gene was joined to a mouse anti-Dns variable (V) region gene in the vector pSV2ΔH-gpt (Fig. 1*B*). The chimeric mouse–human κ L chain specific for Dns (pSV184ΔH-neo) is available in our laboratory.

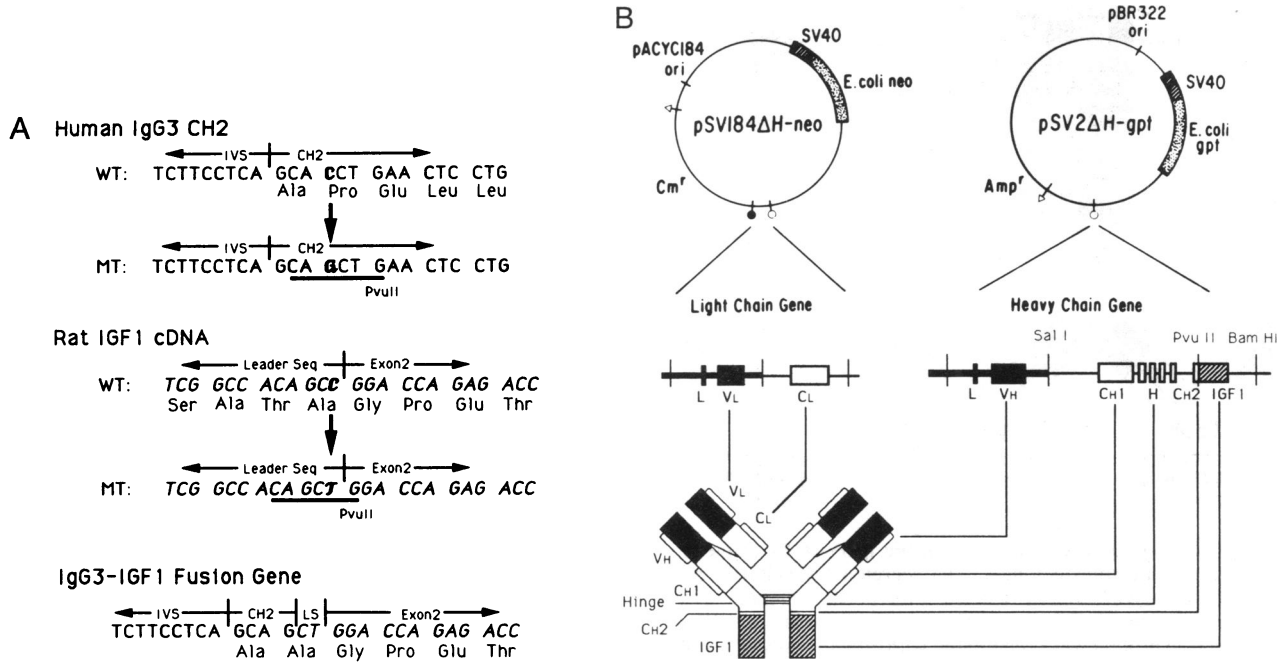

FIG. 1. (*A*) Strategy for the construction of the IgG3–IGF1 fusion gene. The fourth base (C) of the gene for the C_H2 domain in human IgG3 was mutated to G by site-directed mutagenesis, resulting in the introduction of a unique *Pvu* II restriction enzyme site. In addition, a unique *Pvu* II site was introduced into the IGF1 gene by changing the last base (C) of the leader sequence (Leader Seq. or LS) of rat IGF1 cDNA (italic letters) to T. Human IgG3 and rat IGF1 cDNA digested with *Pvu* II were ligated, resulting in an in-frame IgG3–IGF1 fusion gene without any significant amino acid substitutions. (*B*) Schematic representation of the transfection vectors, the genetically engineered IgG3–IGF1 fusion gene, and a proposed chimeric antibody. The mouse–human κ L chain gene was cloned into pSV184ΔH-neo, which is derived from pACYC184 and contains the pACYC origin of replication, a chloramphenicol-resistant gene (Cm^r) for selection in *Escherichia coli*, and the *neo* gene (the dotted box) with the simian virus 40 (SV40) early region promoter (the shaded box) for selection in eukaryotic cells. The mouse–human IgG3–rat IGF1 H chain gene is cloned into pSV2ΔH-gpt gene (the stippled box). Boxes in the chimeric genes represent exons. The thick black solid line and boxes represent DNA of mouse origin, while the thin solid line and open boxes represent human DNA segments. The shaded box in the H chain genes represents the rat IGF1 cDNA. The sites of cleavage by restriction endonucleases *Eco*RI (△), *Bam*HI (○), and *Hin*dIII (●) are shown. The mouse–human IgG3–rat IGF1 chimeric protein produced by expression of both transfection vectors is shown at the bottom of *B*. The black region of the chimeric molecule represents the mouse V region domains specific for the hapten Dns, the open regions represent the C domains of human κ L chain and IgG3, and the hatched region represents the rat IGF1.

The bacteria containing both vectors were used to transfect a nonproducing myeloma cell line (P3X63Ag8.653) by protoplast fusion. Stable transfectomas were selected by using G418, and the supernatants of stable transfectomas were tested for the presence of an antibody protein by an ELISA with anti-human κ chain antibody. Seventy-seven stable transfectomas secreting complete antibody molecules were identified and recovered for further characterization. The frequency of the desired transfectomas was 1.54×10^{-6} recipient myeloma cells. These transfectomas secreted 0.5–30 μg of chimeric molecule per 10^6 cells per 24 hr. The production level of the immunoglobulin–nonimmunoglobulin chimeric molecules is not different from that of wild-type chimeric antibodies.

Characterization of the IgG3–IGF1 Chimeric Protein. The secreted IgG3–IGF1 chimeric proteins were biosynthetically labeled with [^{35}S]methionine, and the labeled proteins were purified by immunoprecipitation with Dns-BSA-conjugated Sepharose beads or a rabbit anti-human IgG Fab antiserum and IgGsorb. Reactivity of the fusion proteins with Dns-BSA showed that they retained their ability to react with their specific antigen. NaDodSO$_4$/PAGE analysis (Fig. 2) showed that the stable transfectomas produce chimeric molecules. As expected, the size of these chimeric molecules is smaller than

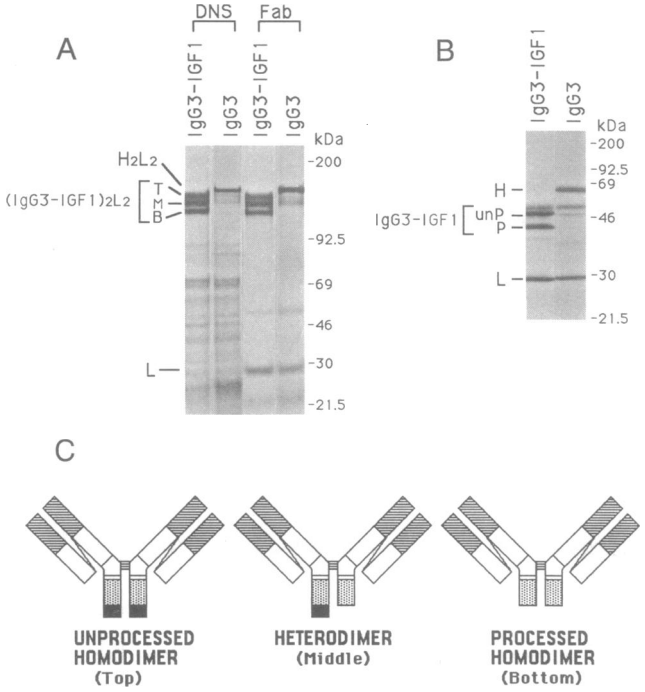

FIG. 2. NaDodSO$_4$/PAGE analysis of the IgG3–IGF1 chimeric protein secreted by transfectomas. The secreted IgG3–IGF1 chimeric protein biosynthetically labeled with [^{35}S]methionine was analyzed under nonreducing (*A*) and reducing (*B*) conditions. The labeled chimeric protein was precipitated with either Dns-Sepharose (lanes DNS in *A*) or anti-human IgG Fab antiserum/*Staphylococcus* protein A/IgGsorb (lanes Fab in *A*). Anti-Fab precipitates free L chains as well as L chains covalently attached to H chains. The secreted IgG3 chimeric antibody consisting of mouse V region–human IgG3 C region has the same basic structure as the IgG3–IGF1 chimeric protein and is used as a control. Under nonreducing conditions (*A*), the three bands represent the heterogeneous assembly of the processed and unprocessed chimeric protein; under reducing conditions, the processed (P) and unprocessed (unP) chimeric protein are seen (*B*). The schematic diagrams of heterogeneous assembly patterns are shown in *C* in which the hatched regions represent the mouse V regions, the open regions represent human C regions, the stippled regions represent the mature IGF1, and the black regions represent the carboxyl terminus of the unprocessed IGF1.

that of a normal IgG3 antibody. However, the secreted chimeric heavy chain appeared to be heterogeneous in size because the recipient cells only partially process the IGF1 precursor at its carboxyl terminus to mature IGF1. The incomplete proteolytic processing at the carboxyl terminus of IgG3–IGF1 H chain results in two distinctive H chains: a processed and an unprocessed H chain (Fig. 2*B*). When the chimeric molecules assemble to form the H_2L_2 antibody molecule, assembly of two unprocessed H chains results in the highest molecular weight chimeric molecule [unprocessed homodimer:top band (T) in Fig. 2 *A* and *C*], and assembly of two processed H chains results in the lowest molecular weight chimeric molecule [processed homodimer: bottom band (B) in Fig. 2 *A* and *C*]. Assembly between a processed and an unprocessed H chain results in an intermediate-size chimeric molecule [heterodimer:middle band (M) in Fig. 2 *A* and *C*]. Unprocessed IGF1 contains two possible N-linked glycosylation sites that appear to be used (data not shown). The IgG3–IGF1 chimeric proteins were recognized by anti-IGF1 antisera, demonstrating that the IGF1 in the fusion protein assumes a native configuration. The band migrating at about 50 kDa in Fig. 2*B* appears to be a nonimmunoglobulin protein precipitated by the antiserum.

IgG3–IGF1 chimeric proteins were purified from culture supernatants by using an affinity column with 2-dimethylamino naphthalene-5-sulfonyl chloride (M_r 269; dansyl isomer) coupled to AH-Sepharose 4B. Bound protein was eluted with *N*-5-carboxypentyl-2-dimethylamino naphthalene-5-sulfonamide (M_r 364; dansyl isomer), and free hapten was removed by extensive dialysis. Purified IgG3–IGF1 chimeric proteins were tetrameric and heterogeneous in size, as had been the case with the biosynthetically labeled proteins. No other protein was observed in the silver-stained gel (data not shown). Because of its size heterogeneity, the purified chimeric IgG3–IGF1 protein was eluted in a broad peak on Superose-12. No evidence of aggregation was seen. The approximate molecular mass of the $(IgG3–IGF1)_2L_2$ chimeric protein is 160 kDa (data not shown).

Binding of the IgG3–IGF1 Chimeric Protein to the IGF1 Receptor. A critical attribute of the fusion protein is whether it retains its ability to bind to the IGF1 receptor. To assess this, unlabeled recombinant human IGF1, wild-type chimeric IgG3, and the IgG3–IGF1 chimeric protein were used to inhibit the binding of ^{125}I-IGF1 to human IM-9 cells (17). Both IGF1 and IgG3–IGF1 inhibited the binding of ^{125}I-IGF1 in a dose-dependent manner (Fig. 3). The 50% inhibition of ^{125}I-IGF1 binding occurred at a recombinant IGF1 concentration

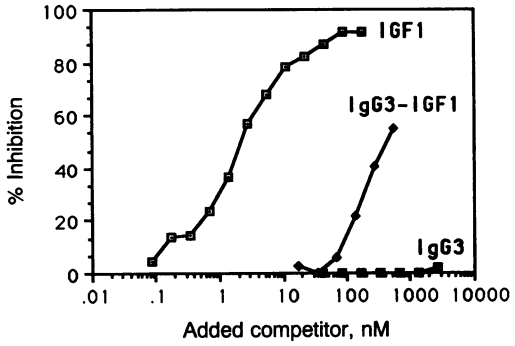

FIG. 3. Competitive inhibition of binding of ^{125}I-IGF1 to IM-9 lymphocytes. Approximately 3×10^6 IM-9 cells were incubated at 15°C with a constant amount of ^{125}I-IGF1 and the indicated concentration of unlabeled competitors (recombinant IGF1, IgG3–IGF1 chimeric protein and IgG3 chimeric antibody). After 2 hr of incubation, the amount of receptor-bound radioactivity was determined. Values are expressed as the relative inhibition of binding compared to use of only labeled tracer ^{125}I-IGF1. Results shown for each curve are the means of duplicate experiments.

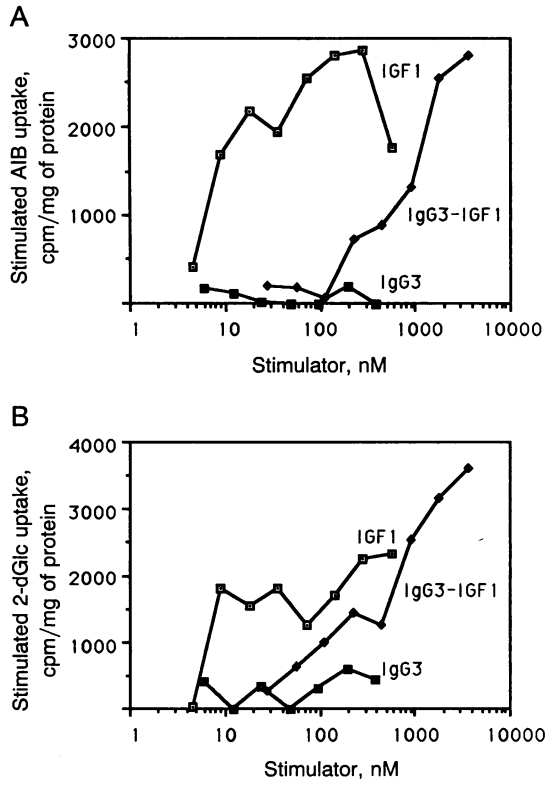

FIG. 4. Relationship between IGF1 and IgG3–IGF1 stimulatory effects on α-aminoisobutyric acid (AIB) (*A*) and dGlc (*B*) uptakes in KB cells. Uptakes were determined in the presence of the indicated concentrations of IGF1, IgG3–IGF1, and IgG3 as control.

of 2.25 nM and at an IgG3–IGF1 chimeric protein concentration of 0.315 μM. Therefore, the IgG3–IGF1 chimeric protein was 0.7% as effective as recombinant human IGF1 in inhibiting ^{125}I-IGF1 binding. However, the wild-type chimeric IgG3 did not show any inhibition of ^{125}I-IGF1 binding, even at a concentration as high as 2.7 μM. Therefore, the competition by the chimeric protein was a consequence of the presence of the IGF1 moiety.

In human epidermoid carcinoma KB cells, fluid-phase endocytosis and exocytosis are stimulated by growth hormones (insulin, IGF1, and epidermal growth factor); these cells possess 7.5×10^4 IGF1 receptors per cell (11). The ability of the IgG3–IGF1 chimeric protein to stimulate 2-dGlc and α-aminoisobutyric acid uptake (9) was investigated and compared with IGF1 and IgG3. The dose–response relationships of IgG3–IGF1 chimeric protein stimulation of 2-dGlc and α-aminoisobutyric acid uptake in KB cells are shown in Fig. 4. Based on half-maximal effective concentration, the relative potencies of IGF1 and IgG3–IGF1 were 200:1 for α-aminobutyric acid uptake and 25–100:1 for 2-dGlc uptake. IgG3 alone did not affect 2-dGlc and α-aminobutyric acid uptake by KB cells. Therefore, the chimeric proteins exert the expected biological effects but are less potent than the human IGF1 standard.

DISCUSSION

Among the problems encountered when investigators have attempted to use monoclonal antibodies as immunotherapeutic agents is efficiently targeting the antibodies to tumor cells while leaving normal cells untouched. As a first step towards producing antibodies with greater specificity, we have produced a chimeric antibody in which an anti-Dns murine VH region joined to the C_H1 and hinge regions of human IgG3 was fused with rat IGF1 and expressed with a Dns-specific chimeric L chain. In the future we will change the combining specificity to be directed against tumor-associated antigens. We anticipate that simultaneous binding by the antibody combining site to the antigen on the surface of the tumor cell and by the hormone to the hormone receptor will increase the specificity of targeting. We found that the chimeric protein was efficiently produced and secreted (up to 30 μg per 10^6 cells per 24 hr) in a recipient nonproducing murine myeloma. Thus, high-level expression of these recombinant molecules is feasible.

The chimeric proteins assemble and are secreted as H_2L_2 tetramers. This result is somewhat surprising because these molecules are missing a normal C region. Murine IgG molecules lacking a segment of the C region fail to covalently assemble H_2L_2 molecules and instead are secreted as HL half-molecules (18). Similarly IgA molecules with deletions in the C region do not form inter-H-chain bonds (19). It would appear that the presence of IGF1 at the carboxyl terminus facilitates the formation of the inter-H-chain disulfide, permitting the formation of H_2L_2 molecules. Extensive cis interactions occur between the C_H3 domains; the ability to assemble H_2L_2 molecules may suggest extensive cis interactions between the IGF1 moieties and may in part explain the low specific activity of the molecules.

The IgG3–IGF1 chimeric proteins retain their specificity for the antigen Dns; although we have not determined the actual affinity of the chimeric antibody for Dns, we would expect no change. The chimeric protein was able to bind to the IGF1 receptor with reduced affinity and to elicit some of the biologic effects of receptor binding. The reduced affinity for receptor and biologic activity may result from several things: first, the presence of the unprocessed IGF1 moiety results in a heterogenous population of the IgG3–IGF1 chimeric protein, which may explain the low potency. Second, the large size of the IgG3–IGF1 chimeric protein (160 kDa) compared with IGF1 (7 kDa) may lead to decreased accessibility to the IGF1 receptor and decreased binding affinity. To address this issue we can produce an Fab–IGF1 chimeric protein that would be reduced in size. Finally, three amino acid substitutions in rat IGF1 compared with human IGF1 may lead to lower affinity for the human receptor. If we decide that this is the case, we can alter these amino acids by site-directed mutagenesis.

The flexibility and accessibility of IGF1 may also play an important role in the chimeric protein. In the initial construct, we positioned the IGF1 moiety carboxyl-terminal to the extended hinge of human IgG3. IgG3 is the most flexible human IgG (20), and we reasoned that this flexibility would optimize the ability to simultaneously bind the IGF1 receptor and antigen. However, as discussed above, the position of IGF1 immediately carboxyl-terminal to the hinge may bring the IGF1 molecules into contact with each other and interfere with their binding. A possible improvement in the molecules may be to place the IGF1 on a β-strand in C_H2 more distal to the hinge. Since the C_H2 domains do not normally contact each other, this should place the two IGF1 moieties at some distance from each other and thereby improve their binding efficiency.

The blood–brain barrier in a normal brain effectively restricts transport between blood and the central nervous system of certain molecules, especially those that are water soluble, charged, and larger than several hundred daltons (21). However, recent data suggest specific receptors facilitate the transport of certain growth factors across the blood–brain barrier, including insulin, transferrin, IGF1, and IGF2 (6–8). The IgG3–IGF1 chimeric proteins bind specifically to the IGF1 receptor and to human brain endothelial cells. Therefore, by including the proper growth factor, we may be able to target molecules to the brain. Just as it may be possible to use the growth factor to deliver an antibody combining site

to a novel locale, it may be possible to use the antibody combining specificity to deliver hormones that have an intracellular function to cells that either lack the receptor or have a defective receptor.

The question remains as to the antigenicity of the chimeric antibodies. Even though each component may not be antigenic by itself, the novel combination of components may produce neoantigenic determinants that will elicit an immune response.

Even though the chimeric molecules as currently constituted have limitations, they indicate that the general approach is feasible. Expanded studies could lead to a new family of immunotherapeutic molecules with increased specificity of binding to tumors and access to locales such as the brain.

This work is supported in part by Grants CA 16858 from the National Institutes of Health and ACS IM-550 from the American Cancer Society. S.-U.S. is supported by a Cancer Research Institute/J. M. Foundation Fellowship.

1. Morrison, S. L., Johnson, M. J., Herzenberg, L. A. & Oi, V. T. (1984) *Proc. Natl. Acad. Sci. USA* **84**, 6851–6855.
2. Neuberger, M. J., Williams, G. J., Mitchell, E. B., Jonhal, S. S., Flanagan, J. G. & Rabbitts, T. H. (1985) *Nature (London)* **314**, 268–270.
3. Boulianne, G. L., Hozumi, N. & Shulman, M. J. (1984) *Nature (London)* **312**, 643–646.
4. LoBuglio, A. F., Wheeler, R. H., Trang, J., Haynes, A., Rogers, K., Harvey, E. B., Sun, L., Ghrayeb, J. & Khazaeli, M. B. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4220–4224.
5. Liu, A. Y., Robinson, R. R., Hellstrom, K. E., Murray, E. D., Jr., Chang, C. P. & Hellstrom, F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3439–3443.
6. Frank, H. J. L., Pardridge, W. M., Morris, W. L., Rosenfeld, R. G. & Choi, T. B. (1986) *Diabetes* **35**, 654–661.
7. Pardridge, W. M., Eisenberg, J. & Yang, J. (1985) *J. Neurochem.* **44**, 1771–1778.
8. Fishman, J. B., Rubin, J. B., Handrahan, J. V., Connor, J. R. & Fine, R. E. (1987) *J. Neurosci. Res.* **18**, 299-304.
9. Bequinot, F., Kahn, C. R., Moses, A. C. & Smith, R. J. (1985) *J. Biol. Chem.* **260**, 15892–15898.
10. Rosenfeld, R. G. & Hintz, R. L. (1980) *Endocrinology* **107**, 1841–1848.
11. Miyata, Y., Hoshi, M., Koyasu, S., Kadowaki, T., Kasuga, M., Yahara, I., Nishida, E. & Sakai, H. (1988) *Exp. Cell Res.* **178**, 73–83.
12. Zoller, M. J. & Smith, M. (1982) *Nucleic Acids Res.* **10**, 6487–6500.
13. Oi, V. T. & Morrison, S. L. (1986) *BioTechniques* **4**, 214–221.
14. Shin, S.-U. & Morrison, S. L. (1989) *Methods Enzymol.* **178**, 459–476.
15. Morrison, S. L., Canfield, S., Porter, S., Tan, L. K., Tao, M.-H. & Wims, L. A. (1988) *Clin. Chem. (Winston-Salem, N.C.)* **34**, 1668–1675.
16. Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310.
17. Misra, P., Hintz, R. L. & Rosenfeld, R. G. (1986) *J. Clin. Endocrinol. Metab.* **63**, 1400–1405.
18. Yelton, D. E. & Scharff, M. D. (1982) *J. Exp. Med.* **156**, 1131–1148.
19. Zack, D. J., Morrison, S. L., Cook, W. D., Dackowski, W. & Scharff, M. D. (1981) *J. Exp. Med.* **154**, 1554–1569.
20. Dangl, J. L., Wensel, T. G., Morrison, S. L., Stryer, L., Herzenberg, L. A. & Oi, V. T. (1988) *EMBO J.* **7**, 1989–1994.
21. Shapiro, W. R. & Shapiro, J. R. (1986) *Semin. Oncol.* **13**, 56–69.