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Humanization of immunotoxins

(chimeric antibody/ribonuclease/angiogenin)

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ABSTRACT **The construction and expression of a chimeric gene encoding a mouse/human antibody to the human transferrin receptor fused to the gene for angiogenin, a human homolog of pancreatic RNase, are described. F(ab')$_2$-like antibody–enzyme fusions were prepared by linking the gene for human angiogenin to a chimeric anti-transferrin receptor heavy chain gene. The antibody–enzyme fusion gene was introduced into a transfectoma that secretes the chimeric light chain of the same antibody, and cell lines were cloned that synthesize and secrete the antibody–enzyme fusion protein of the expected size at a concentration of 1–5 ng/ml. Culture supernatants from clones secreting the fusion protein caused inhibition of growth and protein synthesis of K562 cells that express the human transferrin receptor but not toward a non-human-derived cell line that lacks this receptor. Whereas excess antibody to the same receptor did not itself inhibit protein synthesis, it was able to completely prevent the protein synthesis inhibition caused by the fusion protein. These results indicate that the cytotoxicity is due to a transferrin receptor-mediated mechanism involving the angiogenin portion of the fusion protein and demonstrate the feasibility of constructing recombinant antibody–RNase molecules capable of killing tumor cells bearing the transferrin receptor. The significance of the acquired cytotoxicity of a mouse/human chimeric antibody linked to a human protein may bear importantly in human therapeutic strategies that use mouse antibodies linked to toxins from plants or bacteria to target tumor cells. It is expected that the humanization of immunotoxins will lead to less toxicity and immunogenicity than currently available reagents.**

Recently results from our laboratory demonstrated that bovine pancreatic RNase linked to transferrin (1) or antibodies to the transferrin receptor (D. Newton, S.M.R., and R.J.Y., unpublished) displayed receptor-mediated cytotoxicities characteristic of antibody–toxin conjugates. Those results provide evidence that mammalian RNase can possibly be used in lieu of bacterial or plant toxins in the construction of immunotoxins. The reduction in the immunogenicity of these reagents would be furthered by the use of human RNase in these conjugates. Angiogenin (Ang) is a human protein with homology to pancreatic RNase (2) and RNase activity albeit different from that of the pancreatic enzyme (3, 4). Ang is also a potent inhibitor of protein synthesis in cell-free extracts (5) and upon injection into *Xenopus* oocytes (6). Extracellular Ang is not cytotoxic toward a wide variety of cultured cells (S.M.R., unpublished) and is normally present in human plasma (7). Thus it was important to determine whether Ang could acquire cytotoxic properties when fused to an antibody to the transferrin receptor that could allow it to enter the cell.

The ability of myeloma cells to express and secrete antibody–cytokine (8, 9) and antibody–enzyme (10–12) hybrid molecules has previously been described. The results presented herein demonstrate the feasibility of producing recombinant antibody–human RNase fusion proteins with cell-type-specific cytotoxic activity. These results may have important implications in the design and construction of a new generation of immunotoxins.

MATERIALS AND METHODS

Materials. The materials and their sources used in the construction and expression of the chimeric antibodies are described by Hoogenboom *et al.* (13). Ang was cloned from the gene for human angiogenin (14) and expressed in *Escherichia coli* at Biogen (Cambridge, MA) (unpublished). Since it is a recombinant form of the human plasma protein and contains an additional N-terminal methionine, it is designated as Ang in these studies. Rabbit antibodies to human Ang were a generous gift from Karen Olson and James Fett (Center for Biochemical and Biophysical Research in the Sciences and Medicine of Harvard Medical School). E12B5 is a chimeric cell line that produces and secretes the chimeric mouse/human light chain for the E6 anti-transferrin receptor antibody (13), and the cell culture of the other cell lines used in this study were reported recently (1).

Construction and Expression of the Chimeric Antibody–Ang Gene. The methods used in cloning and expressing the chimeric antibody–Ang fusion protein were the same as described in detail elsewhere (13). The ELISAs used to detect human IgG or κ chain production measured antibody concentrations ranging from 1 to 100 ng/ml and were specific for the detection of human γ or κ chain. Detection of Ang in transfectoma supernatants was accomplished by using a method previously published (7) and constructing a standard by coating the wells with known amounts of Ang in cell culture supernatant.

Biosynthetic labeling of secreted proteins with [^{35}S]methionine, immunoprecipitation, and immunoblotting were accomplished as described (13). Detection of Ang by immunoblotting was performed with modifications of a protocol devised by Karen Olson. After the transfer the blot was incubated in phosphate-buffered saline (PBS)/0.05% Tween overnight at 4°C. The blot was then incubated with rabbit anti-Ang for 2 hr at room temperature, washed with PBS/0.05% Tween, and incubated with biotin-labeled anti-rabbit IgG for 2 hr at room temperature, followed by a 90-min incubation with streptavidin/horseradish peroxidase. Detection of human Ang was accomplished using 4-chloro-1-

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Abbreviations: Ang, angiogenin; PRI, placental RNase inhibitor; gpt, xanthine–guanine phosphoribosyltransferase; TNF, tumor necrosis factor.

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naphthol or the same procedure as that used to detect human IgG or human κ chain (13).

Fractionation of Culture Supernatant and *in Vitro* Assays. Cell culture supernatant (500 ml) collected from CH2.5 Ang-secreting cells (grown in T150 flasks and stored frozen at −20°C) was applied to an *O*-carboxymethylcellulose (CM-cellulose) column (3 × 10 cm) equilibrated with 10 mM Tris (pH 7.5). All column operations were conducted at room temperature. The column was washed with 2 column volumes of equilibration buffer and eluted with stepwise increases in NaCl concentrations. At each salt concentration 10-ml fractions were collected until the A_{280} was <0.02 absorbance unit. Peak fractions were pooled and dialyzed against PBS. An aliquot of each pool was concentrated using a Centricon microconcentrator and analyzed by SDS gel electrophoresis. The proteins were detected using Coomassie blue stain and silver stain. Immunoblot analysis was performed to detect human γ and κ chains as well as human Ang.

The *in vitro* translation assay (5) and the growth and protein synthesis assays (1) were performed as described.

RESULTS

Construction of the Anti-Transferrin Receptor–Ang Chimeric Gene. Construction of the antibody–Ang fusion required modification of the Ang gene. PCR was used to introduce *Eco*RI sites 5′ to the first codon of the mature protein (14) and past the *Eco*RV site in the 3′ untranslated region of the gene to include the stop and poly(A) signals of the native gene. This Ang gene bordered by two *Eco*RI sites was cloned into pBluescript and completely sequenced, and a clone without mutations was selected for the fusion to the mouse/human chimeric anti-transferrin receptor antibody gene (Fig. 1). The antibody gene (13) was previously fused to the gene for TNF at the 5′ region of the CH2 domain of the antibody, thus leaving the hinge region unaffected and dimerization of the heavy chain possible (9). The pUC-CH2-TNF chimeric gene was used to generate a *Hin*dIII–*Eco*RI fragment that encompassed the antibody gene free of the TNF gene. The *Eco*RI Ang fragment was ligated to the antibody gene, and a clone with the Ang gene in the correct orientation was obtained in pBluescript. The chimeric heavy chain Ang gene was cloned into the *Hin*dIII–*Bam*HI sites of pSV2gptMOV$_H$NP (pSVgpt in Fig. 1). In this vector the expression of the chimeric gene is regulated by an immunoglobulin transcription enhancer element and promoter, both situated upstream of the gene (15). Secretion of the gene product is directed by an immunoglobulin-derived signal peptide sequence.

Isolation and Analysis of Transfectomas. An anti-transferrin receptor chimeric light-chain-producing cell line, E12B5 (13), was transfected with the pSV2-derived vector containing the

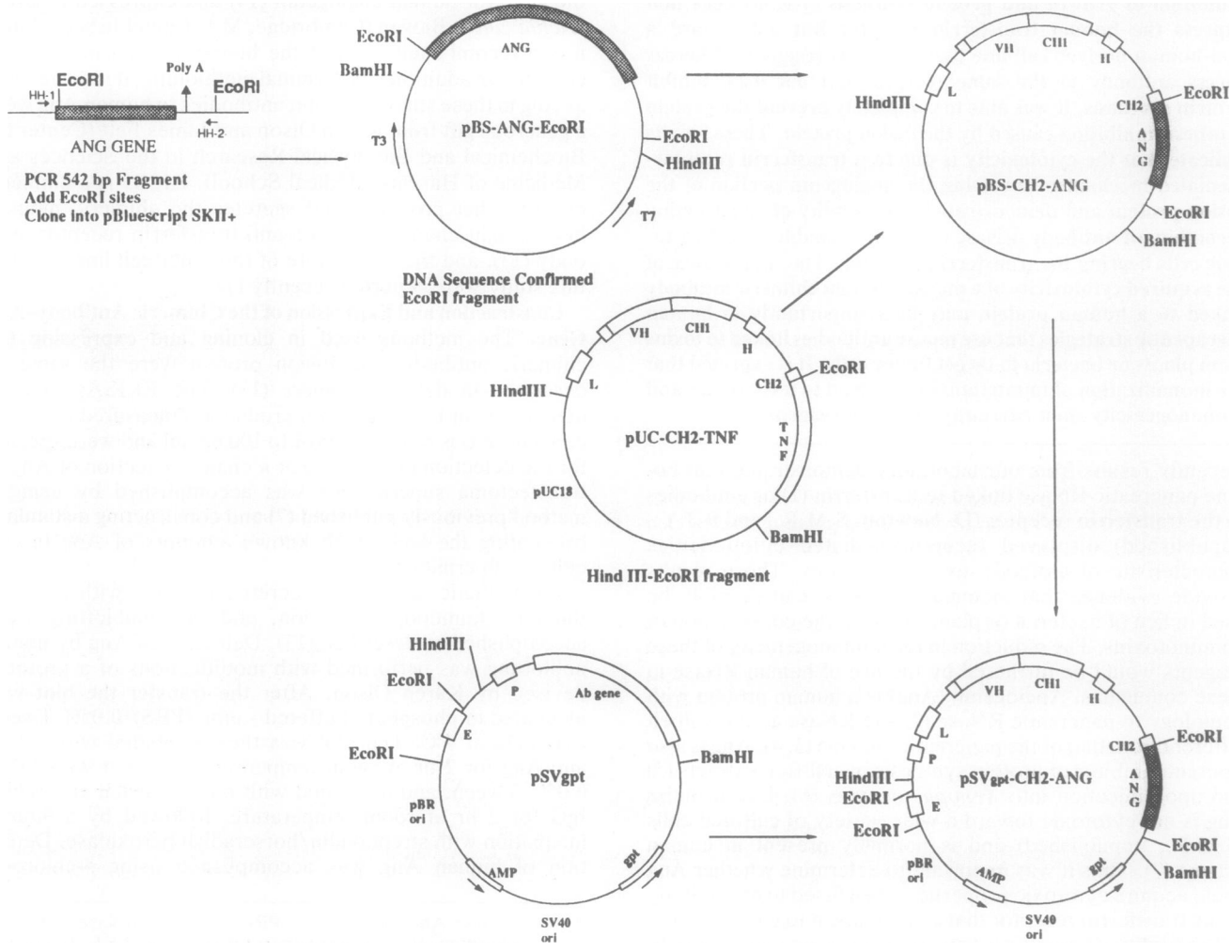

FIG. 1. Outline of the strategy used to construct expression plasmid pSVgpt-CH2-ANG. Only the restriction sites used in the construction are indicated. Detailed structure and construction of pUC-CH2-TNF and expression vector pSVgpt are described in refs. 13 and 15, respectively. E, enhancer; P, promoter; L, immunoglobulin leader sequence; VH, mouse variable regions of E6 antibody gene; CH1 and CH2, constant regions 1 and 2, respectively, of the heavy chain gene; H, hinge region of heavy chain gene; Ang, DNA coding for human Ang (shown in a shaded box); *gpt*, xanthine–guanine phosphoribosyltransferase; AMP, ampicillin-resistance gene; TNF, tumor necrosis factor; SV40, simian virus 40.

CH2–Ang gene. After selection for the presence of the *gpt* gene, culture supernatants of clones testing positive for human IgG were followed for reproducible human IgG activity. The eight highest producing clones were subcloned by limiting dilution. Of these, clone CH2.5 Ang was selected for further characterization. The amount of secreted CH2.5 Ang ranged from 1 to 5 ng/ml, as determined in a human IgG-detecting ELISA, and from 1 to 2 ng/ml when Ang was detected by ELISA. The presence of the heavy chain chimera of the appropriate molecular size as well as the light chain was also observed by immunoprecipitation and immunoblot analysis (not shown).

CM-Cellulose Fractionation of CH2.5 Ang Culture Medium and Characterization of Ang-Specific *in Vitro* Activity. To purify the fusion protein, CH2.5 Ang culture medium was applied to a CM-cellulose column, washed, and then treated with increasing salt in a stepwise manner (Fig. 2 *Top*). At neutral pH Ang is cationic and binds to CM-cellulose (2), whereas most of the components of the growth medium used for these experiments do not adsorb to the column (Fig. 2 *Middle*). Human IgG also fails to adsorb to the CM-cellulose column at neutral pH (not shown). In contrast to growth medium (Fig. 2 *Middle*), most of the IgG-immunoreactive material in the CH2.5 Ang culture medium eluted with 0.5 M salt and the specific activity of the IgG-reactive material in this fraction was increased relative to the starting material. Since only the Ang portion of the fusion protein binds to CM-cellulose, the anti-human reactive material eluting with 0.5 M salt must be linked to Ang. These data indicate that a fusion protein consisting of the chimeric antibody and Ang exists in the medium.

The human serum RNase inhibits the translational capacity of rabbit reticulocyte lysates in a distinctive manner and the inhibition is prevented by a placental RNase inhibitor (PRI; ref. 5). Two concentrations of partially purified 0.5 M CM-cellulose material containing CH2.5 Ang at 200 or 400 ng/ml were added to a standard rabbit reticulocyte lysate *in vitro* translation system and incorporation of [^{35}S]methionine into newly synthesized proteins was measured (Fig. 2 *Bottom*). A concentration-dependent inhibition of protein synthesis was observed that was completely reversed by PRI and partially reversed by an excess of tRNA, an *in vitro* substrate for Ang (16). These results demonstrate that chimeric Ang expresses characteristic *in vitro* activities of the native protein.

Attempts to isolate CH2.5 Ang from transfectoma medium were hampered by the very low concentration of the fusion protein in the culture supernatant. For this reason further biological characterization of the fusion protein was carried out using the cell culture supernatant without further purification.

Characterization of the Effect of CH2.5 Ang Culture Medium on Growth and Protein Synthesis in Human Leukemia Cells. K562 human erythroleukemia cells were plated into CH2.5 Ang culture medium and the growth of the cells was compared to K562 cells plated into the same growth medium that had not been incubated with CH2.5 Ang-secreting cells. The growth of the cells in the CH2.5 Ang medium was inhibited and by 2 weeks the wells contained mostly cell debris (Fig. 3 *Upper*). This effect cannot be ascribed to effects of spent medium since growth medium from transfectomas engineered to secrete a Fab-like fragment supported the growth of K562 cells (Fig. 3 *Upper*). In another experiment, a 10-fold higher initial density of K562 cells was plated and inhibition of cell growth with the CH2.5 Ang medium was also observed (Fig. 3 *Lower*).

The effect of CH2.5 Ang medium on protein synthesis in K562 cells was examined and compared to the effects of purified recombinant Ang protein and the mouse monoclonal anti-transferrin receptor antibody (E6) that contributes the variable domains for the chimeric antibody gene used for

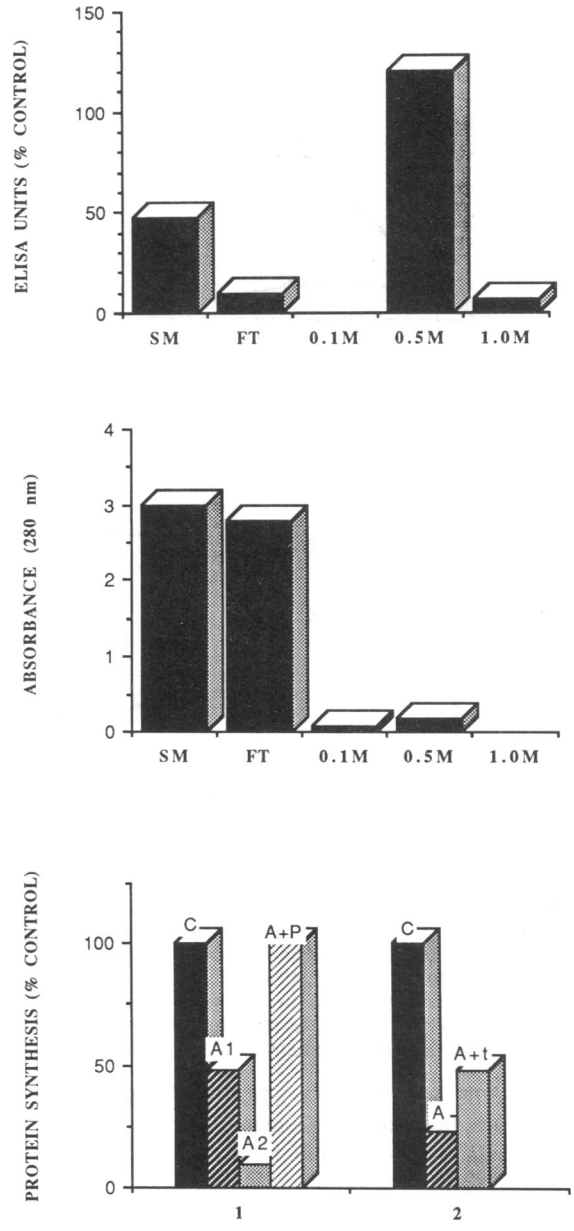

FIG. 2. CM-cellulose fractionation of CH2.5 Ang cell culture supernatant. Transfectoma medium was fractionated as described in the text. The distribution of protein was followed by the reactivity of the samples with antibody to human IgG (*Top*) or by following absorbance of the samples at A_{280} (*Middle*). SM, starting material; FT, flow through; 0.1, 0.5, and 1.0 M NaCl in the elution buffer. (*Bottom*) *In vitro* protein synthesis assay of material eluting with 0.5 M NaCl. The CM-cellulose pool eluting with 0.5 M NaCl contained the highest amount of IgG-reactive material and was assayed for effects on *in vitro* protein synthesis using the rabbit reticulocyte lysate system. Experiment 1: C, control medium; A1, 0.5 M CM-cellulose pool with Ang added to the assay at a final concentration of 40 ng/ml; A2, 0.5 M CM-cellulose pool with Ang at 80 ng/ml in the assay; A+P, 0.5 M CM-cellulose pool (Ang, 80 ng/ml final assay concentration) plus PRI, 80 units. Experiment 2: C, control medium; A, 0.5 M CM-cellulose pool (Ang, 80 ng/ml final assay concentration); A+t, 0.5 M CM-cellulose pool (80 ng/ml) plus calf tRNA (20 μg/ml).

these studies. This chimeric E6 antibody retains the specificity of the mouse E6 antibody (13), and neither the chimeric E6 antibody nor mouse monoclonal E6 antibody competes for transferrin and thus does not block cell growth. Although neither Ang, E6, nor a mixture of the two proteins inhibits protein synthesis in K562 cells, CH2.5 Ang medium inhibits

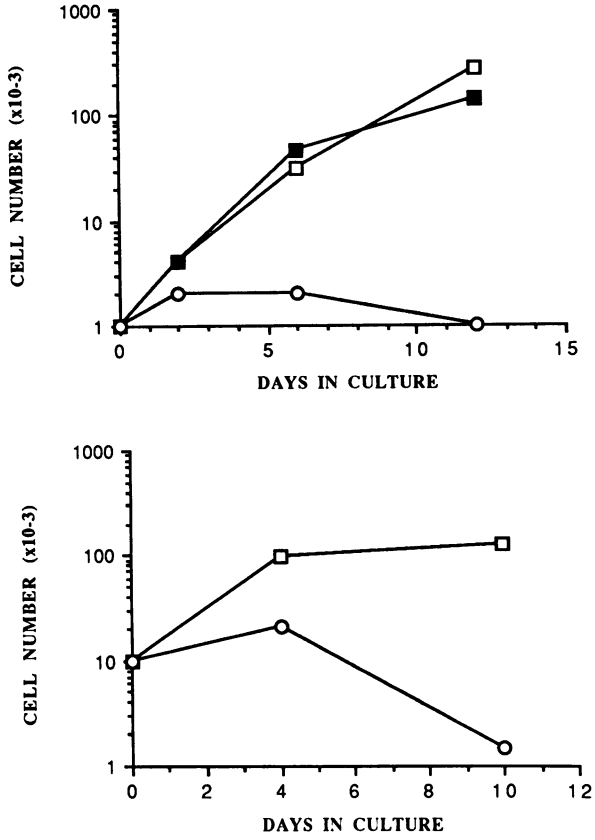

FIG. 3. (*Upper*) Growth of K562 cells in transfectoma culture supernatant. K562 cells (10^3) were plated into 24-well plates in 1 ml of growth medium (□), 1 ml of growth medium that contained 1–2 ng of CH2.5 Ang per ml (○), or 1 ml of growth medium from transfectomas engineered to produce a Fab-like antibody–Ang conjugate (■). Duplicate cell counts were determined and are representative of several experiments. (*Bottom*) In another experiment K562 cells (10^4) were plated and counted as described for *Upper*. □, Growth medium; ○, growth medium containing 1–2 ng of CH2.5 Ang per ml.

protein synthesis by 50% after a 24-hr incubation (Fig. 4 *Top*). Furthermore, the addition of excess E6 antibody immediately after plating K562 cells into CH2.5 Ang medium blocks the toxicity when protein synthesis is measured at 24 hr (Fig. 4 *Middle*). Since neither E6, Ang, nor the chimeric antibody used to make the fusion protein inhibits protein synthesis or cell growth, these results imply that inhibition of protein synthesis of the CH2.5 Ang medium is due to the combined functions of the secreted fusion protein. In other experiments protein synthesis was measured after a 48-hr and a 72-hr exposure to the Ang fusion protein and it continued to decline (not shown).

The antibody part of the fusion protein recognizes the human transferrin receptor and not the transferrin receptor on monkey cell lines (P. J. Nicholls, personal communication). Incubation of Vero cells, a monkey cell-derived line, does not result in inhibition of protein synthesis (Fig. 4 *Bottom*) or Vero cell growth (not shown). Assuming that Vero cells express the same sensitivity to Ang as the K562 cells, this experiment shows cell-type specificity implying transferrin receptor-mediated toxicity of the antibody–Ang fusion protein.

DISCUSSION

The purpose of the construction of a fusion protein consisting of a chimeric antibody to the human transferrin receptor linked to a human serum protein with ribonucleolytic activity was to evaluate the potential of the construct to target and kill

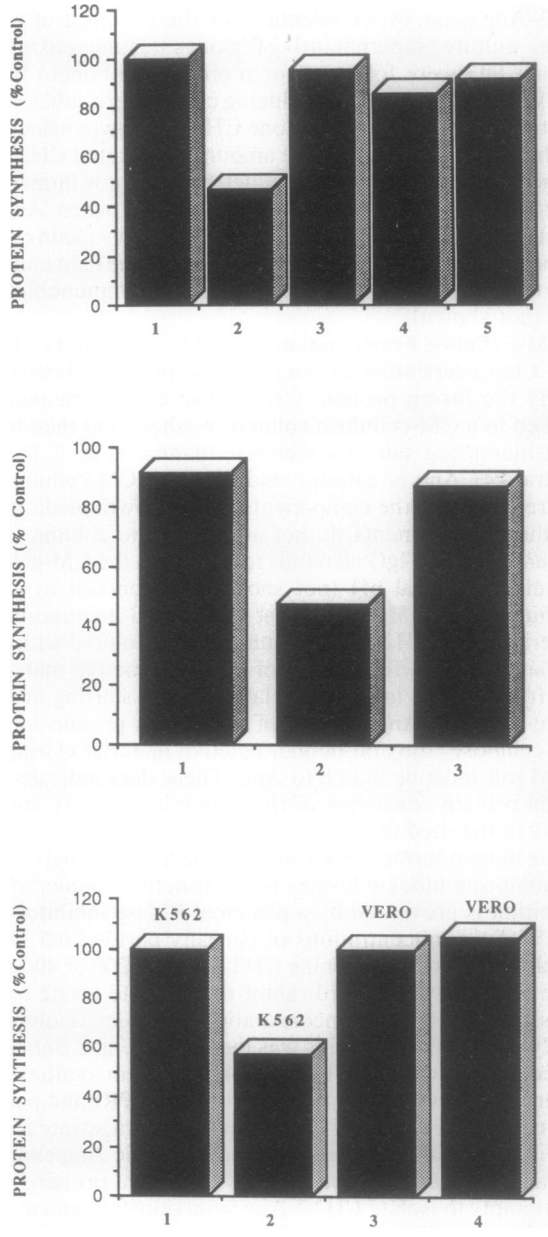

FIG. 4. Protein synthesis in K562 cells. (*Top*) K562 cells were plated with growth medium alone (1), medium containing 1–2 ng of CH2.5 Ang per ml (2), growth medium containing 1 μg of Ang protein per ml (3), E6 anti-transferrin receptor antibody, 4 μg/ml (4), or the same amounts of a mixture of Ang and E6 (5). Protein synthesis was measured after 24 hr. (*Middle*) Inhibition of protein synthesis by CH2.5 Ang medium is blocked by E6. Protein synthesis was measured in K562 cells as described above after 24 hr with additions: E6 anti-transferrin receptor antibodies added to growth medium, 4 μg/ml (1); CH2.5 Ang medium, 1–2 ng/ml (2); CH2.5 Ang medium plus E6, 4 μg/ml (3). (*Bottom*) CH2.5 Ang medium does not inhibit protein synthesis in non-target cells. Cells were plated into medium and protein synthesis was measured as described above. Control growth medium (1 and 3); CH2.5 Ang medium (2 and 4). Data from each of the panels were derived from separate experiments.

tumor cells. This follows from the recent observation that bovine pancreatic RNase chemically linked to transferrin or antibodies to the transferrin receptor acquired cytotoxicity toward cells that expressed the target antigen (ref. 1 and D. Newton, S.M.R., and R.J.Y., unpublished). In addition, a chimeric toxin consisting of bacterial RNase and *Pseudomonas* exotoxin A has been published (17). Yet the logic of using mammalian RNase is based on many studies that demonstrate the cytotoxic properties of this family of proteins (ref.

1 and references therein). Furthermore, human serum contains several RNases (18, 19) that are expressed in a tissue-specific manner. The function of these extracellular RNases is unknown, but the discovery that proteins involved in the host defense activity of the eosinophil are homologous to RNases and express RNase activity (20, 21) suggests the intriguing possibility that human serum RNases also may have host defense activities. Thus targeting human RNase with antibodies may in effect be enhancing a physiological defense system.

Ang, an interesting member of the RNase superfamily, was discovered by fractionating tumor cell-conditioned medium and following the specific activity to elicit angiogenesis in the chick chorioallentoic membrane assay (2). The purified material was shown to have potent activity as an angiogenic factor. Yet, the mRNA for this gene is expressed in tumor and normal human cells (22) and is expressed in a wide variety of tissues in the rat, with rat liver expressing the highest levels (23). In addition, Ang is present in normal human plasma (7). This could imply other functions for Ang as well, since neovascularization is a rare event in the normal adult. The structure of Ang contains about 65% homology to pancreatic RNase (14, 24) and the active site residues are conserved, yet it has very little activity toward standard substrates for the pancreatic enzyme (4). Studies on *in vitro* protein synthesis demonstrated that Ang inhibited the translational capacity of the rabbit reticulocyte lysate (5) by a mechanism markedly different from pancreatic RNase A. Although it was shown that a ribonucleolytic activity of Ang was responsible for this inhibition, no cleavage of rRNAs could be demonstrated at concentrations of the enzyme that completely inhibited protein synthesis. Since the base cleavage specificities of Ang and pancreatic RNase were the same toward 5S rRNAs (3), the substrate for Ang may be a unique RNA molecule.

The functional experiments that demonstrate growth and protein synthesis inhibitory activity of CH2.5 Ang culture supernatants toward target cells provide evidence that recombinant antibody–RNase fusion proteins may be used to target tumor cells. The inhibitory effects of the supernatant are blocked by E6, the parent murine monoclonal antibody. This antibody does not compete for transferrin and does not have inhibitory effects on K562 or other cultured cells (13). Furthermore, the cloned chimeric antibody derived from E6 has been shown to retain the same functional characteristics as E6 and also does not inhibit growth of cultured cells until it is fused to TNF (8, 9). These results argue that Ang acquires cytotoxicity when linked to antibody directed to the human transferrin receptor. The transferrin receptor is expressed on malignant cells and is a suitable target for immunotoxin models and also for therapeutic strategies under certain circumstances (ref. 25 and references therein). Although the possibility exists that immunotoxins directed to the transferrin receptor could be cytotoxic to normal cells that express this receptor, recent results demonstrate that anti-transferrin receptor immunotoxins can be administered to the central nervous system for the treatment of intrathecal neoplasia without a direct effect of the drug to cause neurotoxicity (unpublished results).

The results presented here significantly extend the results reported previously on the action of chemically linked RNase hybrid proteins (1). The construction of a unique linkage between RNase and a cell-binding ligand eliminates the heterogeneity of chemically linked antibody–RNase conjugates. This may contribute to the increased potency of the recombinant antibody–RNase fusion. Estimates of the amount of CH2.5 Ang in the cell culture supernatants range from 1 to 5 ng/ml, based on the reactivity to anti-human IgG antibodies, and 1 to 2 ng/ml, based on Ang immunoreactivity. Thus the recombinant fusion protein is ≈1000 times more potent than chemically linked RNase hybrids. Interestingly, these results demonstrate that RNase does not have to be linked through a reducible disulfide linkage to cause toxicity. It is possible that other mechanisms for separating Ang from the ligand exist—i.e., hydrolytic cleavage—or that the entire conjugate can enter the cytosol.

In conclusion, this paper demonstrates the acquired cytotoxic potential of a human RNase when it is linked to a chimeric antibody. This represents a significant step toward the humanization of immunotoxins. The use of humanized antibodies with human proteins should contribute to alleviating some of the problems of immunogenicity that hamper the success of immunotoxins constructed with bacterial or plant toxins. Although the novel construction of these chimeras might form new antigenic determinants, it is expected that the recombinant molecules will be far less antigenic.

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