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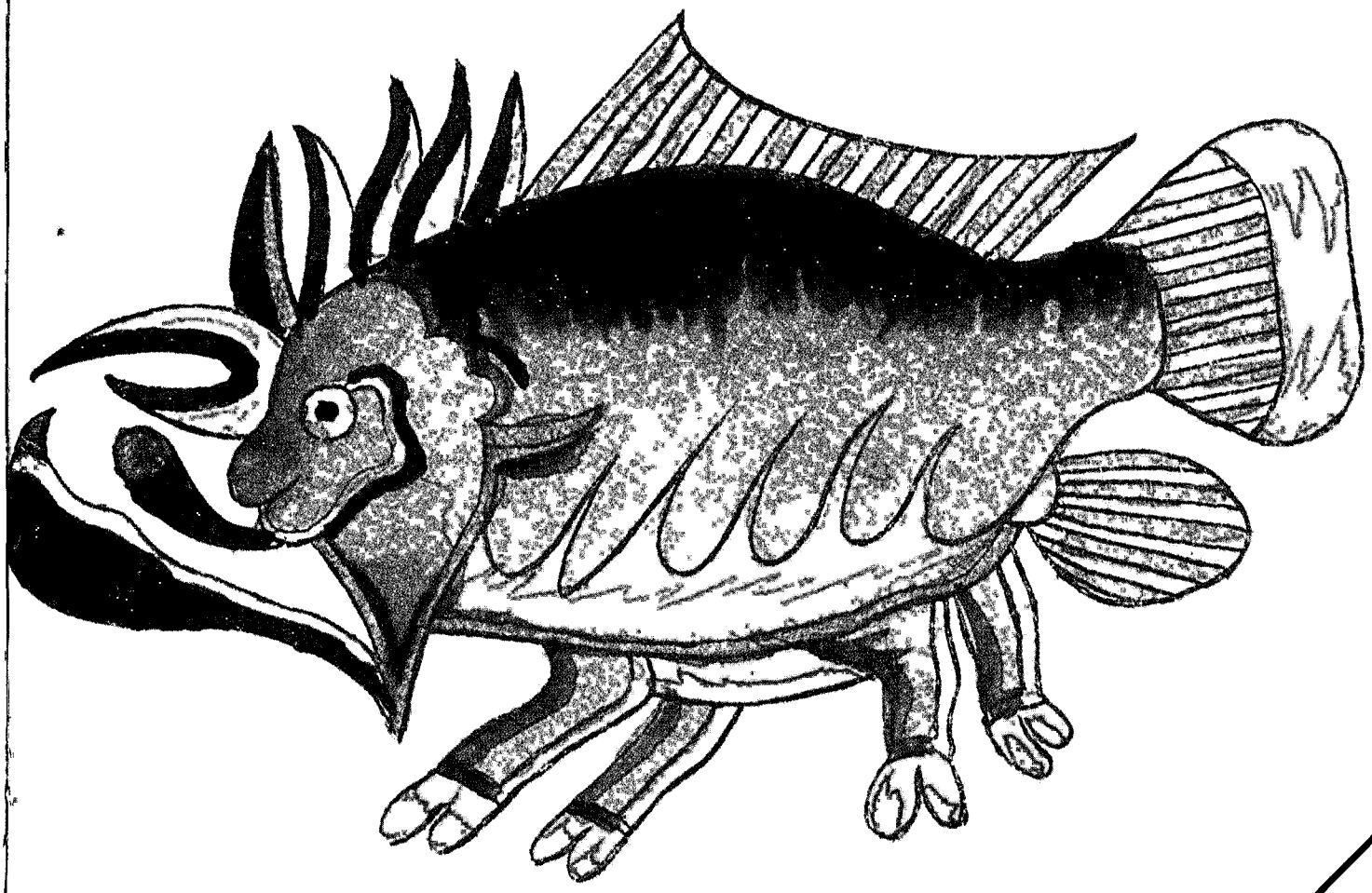

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7 January 1988; accepted 18 March 1988

Escherichia coli Secretion of an Active Chimeric Antibody Fragment

MARC BETTER, C. PAUL CHANG, RANDY R. ROBINSON, ARNOLD H. HORWITZ

A chimeric mouse-human Fab protein that binds specifically to the human carcinoma cell line C3347 has been expressed and secreted from *Escherichia coli*. This molecule, which contains functionally assembled kappa and Fd proteins, binds as effectively to sites on the surface of C3347 cells as Fab fragments prepared proteolytically from whole chimeric or mouse antibody. The production in *Escherichia coli* of foreign heterodimeric protein reagents, such as Fab, should prove useful in the management of human disease.

THE BINDING-SELECTIVITY OF ANTIbody molecules makes them suited for applications as diverse as affinity chromatography, diagnostic reagents, and therapeutics in the detection and treatment of human diseases. Monoclonal antibodies are especially useful for these purposes because they can be prepared with homogeneous recognition specificities targeted at virtually an unlimited number of antigenic determinants. The protein domains that confer these antigen recognition determinants can be proteolytically separated from the remainder of the molecule and still retain their antigen-binding ability. This portion of an antibody (Fab) is roughly one-third the size of an intact immunoglobulin G (IgG) (about 48 kD) and exhibits monovalent antigen binding. The similar $F(ab')_2$ portion retains divalent antigen-binding capacity and contains both recognition domains linked by two interchain disulfide bridges. Antibodies differ, however, in their susceptibility to proteolytic cleavage, and preparations can be heterogeneous. The relatively simple structure of a Fab (5 disulfide bonds) compared to an intact antibody (16 disulfide bonds) and the therapeutic usefulness of Fab molecules make them attractive targets for production by microbial fermentation after appropriate protein engineering. Here we discuss the expression of a mouse-human chimeric Fab in *Escherichia coli*, that is, a molecule that contains the variable regions (antigen recognition domains) from a mouse monoclonal antibody and the C_H1 and C_κ constant regions from a human IgG1 antibody.

Each protein chain of a Fab has two intrachain disulfide bonds that stabilize functional domains, and a single cysteine involved in interchain disulfide linkage. *Escherichia coli* has been used to produce individual immunoglobulin chains internally that are not properly folded (*1*, *2*), or individually secreted chains (*3*); however, for *E. coli* to assemble the truncated heavy chain (Fd) and κ into the correct heterodimeric molecule, both chains must be translated simultaneously and secreted. This operation would then mimic the cognate immunoglobulin assembly process.

The chimeric L6 antibody (*4*), directed toward a ganglioside antigen expressed on (*5*), has been described. This antibody, prepared from the culture supernatant of an Sp2/0 transfectoma cell line, is expressed from the cDNA copies of the chimeric L6 IgG γ and κ chain genes. The cDNA clones for these two chimeric genes were used as the starting point for expression of a Fab molecule in mammalian cells and bacteria.

A termination codon was introduced into the chimeric heavy chain gene at amino acid 228 by site-directed mutagenesis (*6*) (Fig. 1a) in a manner that introduced a Bcl I restriction site. A similar step introduced a restriction site, Sst I, into the coding region at the processing cleavage site of the native heavy chain leader peptide and the mature heavy chain. Site-directed mutagenesis was

Table 1. Binding activity of bacterial Fab to human carcinoma cells. Target cells were incubated for 30 min at 4°C with each antibody or Fab, followed by incubation with fluorescein isothiocyanate (FITC)–labeled goat antibody against human κ for the bacterial Fab, FITC-labeled goat antibody against mouse IgG for the L6 mouse antibody, FITC-labeled goat antibody against mouse κ for L6 mouse Fab, or goat antibody against human IgG for the chimeric L6 antibody. We determined antibody binding to the cell surface by using a Coulter model EPIC-C cell sorter. FITC-labeled antibodies were obtained from TAGO.

Antibody	Binding ratio*	
	C3347 cells L6+	T51 cells L6−
Mouse L6	95	1
Sp2/0 chimeric L6	116	1
Bacterial L6 Fab	54	1
Mouse L6 Fab†	16	1

*The binding ratio is the number of times brighter a test sample is than a control sample treated with FITC-conjugated second antibody. Quantitative differences in binding to C3347 cells probably reflect the relative activity of individual FITC-conjugates. Data shown are from one of two similar binding assays. †Prepared by

International Genetic Engineering, Inc. (INGENE),

also used to insert an Aat II restriction site into the chimeric κ chain gene at the junction of the leader peptide and the mature processed κ chain. Each coding sequence, Fd and κ, was fused to the leader peptide segment of the bacterial *pel*B gene (pectate lyase) from *Erwinia carotovora* (*7*) generating a gene fusion between the leader peptide segment of the pectate lyase gene and the mature coding sequence of the Fd and κ genes. This bacterial leader sequence was chosen to direct membrane translocation in *E. coli* since pectate lyase can be secreted to high levels under the control of a regulated promoter. To ensure that both Fd and κ were also translated in close physical proximity, we assembled a plasmid that codes both genes in a single dicistronic message (Fig. 1b). This operon was placed under the control of the inducible ara*B* promoter from *Salmonella typhimurium* (*8*) and expressed in *E. coli*.

Examination of culture supernatants or extracts of the periplasmic space (*9*) of *E. coli* by enzyme-linked immunosorbent assay (ELISA) for chimeric κ with antibody against human κ (Cappel), or Fab production with antibody against human Fd (Calbiochem) and antibody against human κ, revealed that about 90% of the secreted κ chain accumulated in the culture medium. This was a surprising observation that allowed simple purification of this material from induced bacterial cultures. Approximately 2 mg/liter of material reactive as Fab in an ELISA is secreted into culture supernatants of MC1061 (pIT106). Immunoblot analysis with antibody against human κ revealed that under nonreducing gel conditions, the predominant reactive species had a molecular size of about 48 kD. Under gel conditions where disulfide linkages were reduced, the predominant species had a molecular size of about 23 kD. These observations are consistent with the predicted molecular sizes of the processed chimeric Fab (κ, 23.3 kD; Fd, 24.7 kD), and suggest that the material is properly assembled.

For purification of Fab, bacterial supernatants were concentrated, filtered, and loaded on an SP disk equilibrated with 10 m*M* phosphate buffer, *p*H 7.5. Fab was eluted with 0.2*M* NaCl and purified by S Sepharose column chromatography, where it was eluted as a single peak with a linear 0 to 0.12*M* NaCl gradient. The immunologically reactive material was more than 90% pure as determined by SDS–polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining (Fig. 2). Purified material has a molecular size of about 48 kD under nonreducing gel conditions and about 24.5 and 23 kD under reducing gel conditions. The 23-kD band is immunologically distinguishable by using antibody against human κ.

Purified bacterial Fab was tested for binding to L6 antigen–containing cells (Table 1). Bacterial Fab bound specifically to the human colon carcinoma cell line C3347; cells from the T cell line T51 served as a negative control. Bacterially produced Fab also exhibited characteristic binding inhibition of FITC-labeled mouse L6 antibody to the surface of antigen-positive C3347 colon carcinoma cells (Fig. 3). We tested bacterially produced chimeric Fab, proteolytically prepared Fab from L6 mouse antibody, L6 chimeric antibody, and Fab prepared from Sp2/0 cells transfected with the truncated chimeric Fd and the chimeric κ gene. All Fab preparations have essentially identical binding inhibition profiles. The proteolytically produced Fab contains a significant proportion of degraded, low molecular size peptides, whereas chimeric Fab from bacteria or Sp2/0 cells is homogeneous (Fig. 2).

Protein engineering allowed the expression in *E. coli* of a functional chimeric Fab that has binding specificity for a human carcinoma cell marker. The finding that *E. coli* can be engineered to secrete a foreign heterodimeric molecule builds on the earlier report that intrachain disulfide bonds can form correctly in proteins secreted into the periplasmic space of *E. coli* (*10*). One useful application for bacterially produced Fab molecules will be in tumor imaging in vivo (*11, 12*) [tumor marker–specific Fabs produced proteolytically from whole antibodies have already been used for this purpose (*13–15*)]. A great advantage of the engineered bacterial Fab is that the protein heterogeneity that results from nonspecific cleavages and differences in the susceptibility of anti-

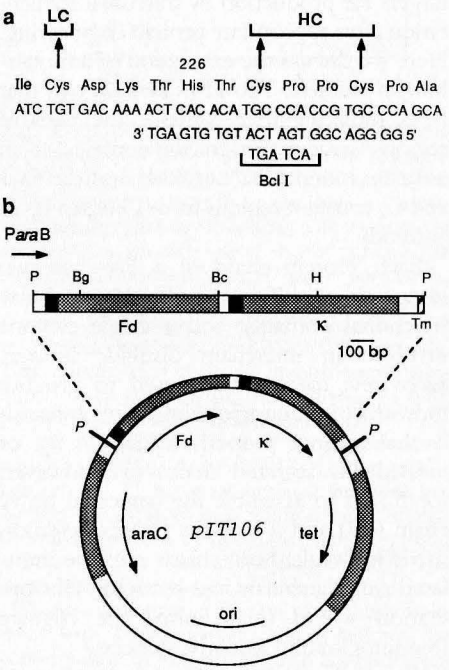

Fig. 1. (**a**) Strategy for introduction of a stop codon and Bcl I restriction site at amino acid 228 in the chimeric L6 heavy chain cDNA clone. An M13mp19 subclone containing a portion (V_H, C_H1, C_H2) of the chimeric L6 γ gene, pING1400, was used as a substrate for site-directed mutagenesis with the primer shown. Arrows indicate the cysteine residue involved in disulfide linkage with light chain (LC) and those involved in linkage with heavy chain (HC) in an intact IgG. (**b**) Map of plasmid pIT106 and a schematic view of the relevant Fab operon placed under the control of the *S. typhimurium* ara*B* promoter (P*ara*B). The 300-bp *pel*B leader sequence cassette including the 22–amino acid *pel*B leader and 230 bp of upstream DNA was derived from pSS1004 (*7*). Gene fusions were constructed with *pel*B and both Fd and κ genes. *Erwinia carotovora* DNA sequences 5′ to the −48 position were removed by means of an Nde I restriction site, and the coding sequences for these genes were assembled [in the *ara*B expression plasmid pIT2 (*17*)] into a single transcription unit with 70 bp separating the two genes. The pectate lyase ribosome binding site is located adjacent to each gene. In addition, the DNA sequence from −48 to the *pel*B:antibody gene junction is a direct repeat preceding the Fd and κ genes. The 28-bp *trp*A transcriptional terminator (Pharmacia) was cloned at the end of the transcription unit (Tm). Restriction endonuclease sites are as indicated: P, Pst I; Bg, Bgl II; Bc, Bcl I; and H, Hind III. The solid

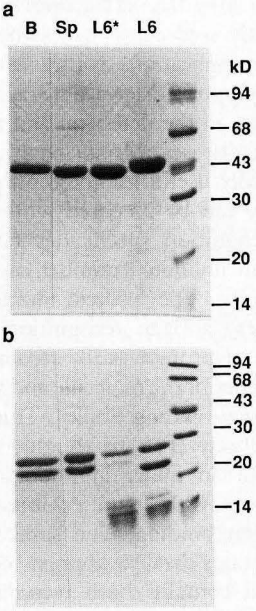

Fig. 2. SDS-PAGE comparison of bacterially (B) and Sp2/0-produced (Sp) Fab to papain-produced mouse L6 and chimeric L6* Fab. Mouse L6 and chimeric L6 antibodies were digested with papain (*18*), and Fab was purified by S-Sepharose chromatography. Each protein was examined by SDS-PAGE on a 10% gel under nonreducing conditions (**a**) and on a 12% gel under reducing

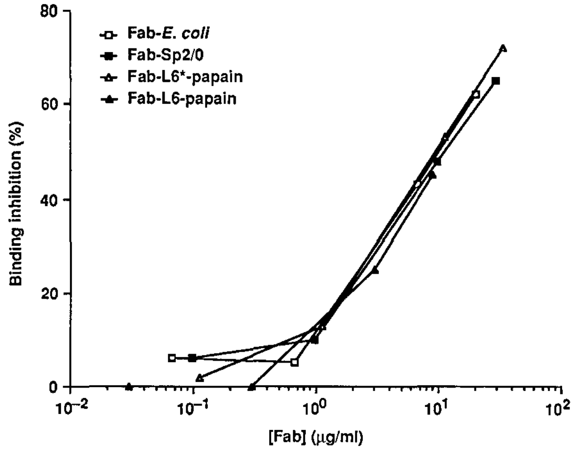

Fig. 3. Binding inhibition of bacterial Fab. Bacterial Fab, Sp2/0 Fab, and proteolytically produced chimeric L6 Fab (L6*) and mouse L6 Fab were used to inhibit FITC-labeled mouse L6 antibody binding to the surface of antigen-positive C3347 colon carcinoma cells.

bodies to protease cleavage will be obviated; a consistent, homogeneous preparation can be produced. Of additional interest is the relative ease with which the Fab cDNA genes can be modified before expression in bacteria. For example, modifications of the primary structure of either the Fd or κ chain (or both) that are useful for subsequent conjugation of imaging or therapeutic agents or fusion to other peptides (*16*) can be introduced by site-directed mutagenesis techniques. We found that *E. coli* can properly assemble a functional two-protein unit with a complex pattern of intra- and interchain disulfide linkages and that sufficient quantities of this material may be prepared for eventual use as a human diagnostic and therapeutic reagent.

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19. We thank K. E. Hellstrom and co-workers for help performing Fab assays, R. Kodori for the *pelB* gene cassette, R. Rahbar and B. Haselbeck for technical assistance, and R. Wall and G. Wilcox for comments on the manuscript. This work was supported by INGENE.

13 January 1988; accepted 18 March 1988

Technical Comments

Carcinogenic Risk Estimation

In their widely publicized and popularized article "Ranking possible carcinogenic hazard," Bruce N. Ames *et al.* (17 Apr. 1987, p. 271) conclude that "analysis on the levels of synthetic pollutants in drinking water and of synthetic pesticide residues in foods suggests that this pollution is likely to be a minimal carcinogenic hazard relative to the background of natural carcinogens" and thus that the "high costs of regulation" of such environmental carcinogens are unwarranted. These conclusions reflect both flawed science and public policy.

Although Ames *et al.* challenge the validity of animal carcinogenicity data for quantitative estimation of human risk, they nevertheless use such extrapolations, based on the percentage *H*uman *E*xposure dose/*R*odent *P*otency dose (HERP), for ranking carcinogenic hazards. Apart from the fact that HERP rankings are based on average population exposures excluding sensitive subgroups, such as pregnant women, the derived potencies of Ames *et al.*, doses inducing 50% tumors in "synthetic pollutants," such as trichloroethylene, are derived from bioassays in which lowest doses are large fractions of the maximally tolerated dose (MTD), whereas potencies for more extensively studied "natural carcinogens," such as aflatoxins, are generally derived from titrated doses, orders of magnitude below the MTD. Since dose-response curves are usually flattened near the MTD (*1*), potencies derived from high-dose testing yield artificially low risk estimates; HERPs for "synthetic" carcinogens are thus substantially underestimated compared with many "natural carcinogens."

Compounding this misconception, Ames *et al.* maintain that carcinogenic dose-response curves rise more steeply than linear curves and that tumor incidences increase more rapidly than proportional to dose. At high doses, dose-response curves are usually less steep than linear curves (*1*), as also recognized elsewhere by Ames and his colleagues (*2*). Thus at MTD doses, large further dose increases may induce only small tion and cytotoxicity (*3*); linear extrapolations from high-dose tests thus underestimate low-dose risks.

For Ames *et al.*, the term "carcinogen" heterogeneously includes direct and indirect influences, including promoting and modifying factors and mutagens. Caloric intake is considered "the most striking rodent carcinogen." However, no correlations have been established between food intake and tumor incidence among animals eating ad libitum, despite wide variations in caloric intake and body weight (*4*), nor have correlations been established between obesity and most human cancers. In the statement by Ames *et al.*, "at the MTD a high percentage of all chemicals might be classified as 'carcinogens'," toxicity and carcinogenicity are confused. However, among some 150 industrial chemicals selected as likely carcinogens and tested neonatally at MTD levels, fewer than 10% were carcinogenic (*5*). Many highly toxic chemicals are noncarcinogenic, and carcinogen doses in excess of the MTD often inhibit tumor yields. While Ames *et al.* revive the discredited theory that chronic irritation causes cancer, most irritants are noncarcinogenic, and there is no correlation between nonspecific cell injury and carcinogenic potency (*6*).