The clinical pharmacology of biotechnology products
M M Reidenberg, editor

DEVELOPMENT OF ANTAGONISTS FOR IFNγ AND TNF

MICHAEL STEINMETZ
Central Research Units, F.Hoffmann-La Roche Ltd, CH-4002 Basel, Switzerland

Multicellular organisms use peptidic and non-peptidic molecules to transmit signals between organs (hormones) and cells (cytokines). These molecules exert their functions by binding to polypeptidic receptors located on the target cell surface or within the cell. Many useful drugs act by binding to these receptors and thereby altering their biochemical and biophysical properties. So-called agonists evoke a response similar to that produced by the natural ligand, while antagonists block physiological triggering of the receptor.

MANIPULATION OF THE IMMUNE SYSTEM TO TREAT DISEASE

Our understanding of the immune system that protects higher vertebrates against infectious organisms and transformed cells has grown steadily over the past twenty years. Today the essential cellular components, most if not all of the molecules involved in antigen recognition, and many but certainly not all of the cytokines regulating the cellular interactions in the system have been characterized.

The enormous progress that has been made is mainly due to the application of recombinant DNA technology which enables us to identify, clone and produce even rare polypeptides in large amounts, such that their activities can be studied *in vitro* and *in vivo*. In fact, many of the clinically relevant recombinant proteins, already on the market or presently in clinical trials, are factors which play a role in the activation, growth and differentiation of cells of the immune system. These recombinant drugs are currently being used primarily to enhance immune responses against tumor cells and to replenish cells after chemotherapy.

Another family of factors, currently only incompletely characterized, downregulates immune responses to prevent harmful effects on the host itself. Some of these factors might be important for the establishment of tolerance to self components. Although the aetiology of local and systemic autoimmune diseases like multiple sclerosis and rheumatoid arthritis, respectively, is currently not understood,

one can assume that mechanisms which normally downregulate immune responses are not functioning properly.

INTERFERENCE WITH IMMUNE RECOGNITION AND EFFECTOR FUNCTIONS

Several possibilities exist to block immune reactions deliberately. Some of these are summarized in Fig.1. One could try to block antigen uptake or processing, interfere with antigen presentation by class I and class II molecules of the major histocompatibility complex (MHC), block recognition of the antigen/MHC complex by T cell receptors, or inhibit effector reactions like cytokine release, expression and binding of adhesion molecules, or binding of cytokines to their corresponding cell surface receptors.

In the following I will concentrate on our efforts to develop antagonists for interferon γ (IFNγ) and tumor necrosis factor (TNF), potentially useful for the treatment of autoimmune diseases. Primarily papers from our own laboratories will be cited, more complete reference lists can be found in these publications.

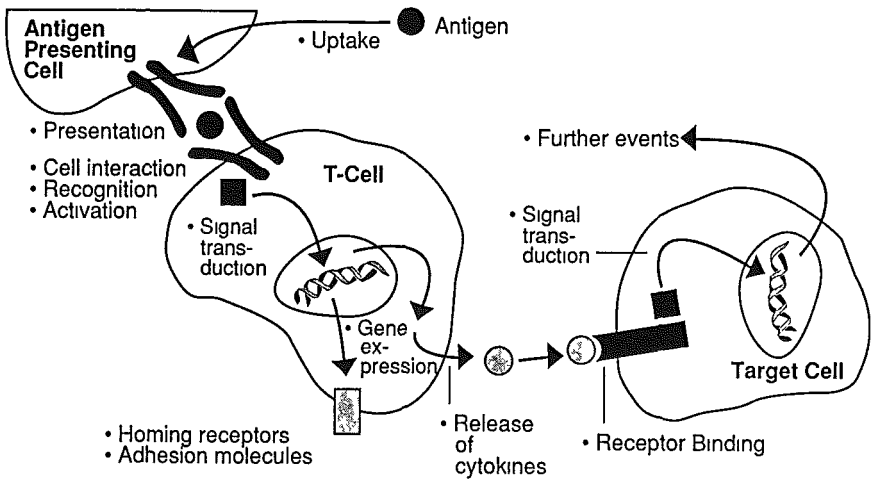

Fig.1 Immune recognition and response reactions provide multiple targets for interfering with autoimmune disease

APPROACHES TO THE IDENTIFICATION OF IFNγ and TNF ANTAGONISTS

We are using both random drug screening and rational drug development programs for the identification of antagonists. All programs involve the use of recombinant forms of the receptors and their ligands. For random screening of natural and synthetic compounds, a binding assay with purified recombinant receptor and ligand is being used (Fig.2). For rational drug development, the molecular interactions in the receptor/ligand complex are being analysed so that small molecules able to block binding can be designed (Fig.3). Other approaches to the identification of receptor or ligand antagonists are also possible, some of which will be mentioned below.

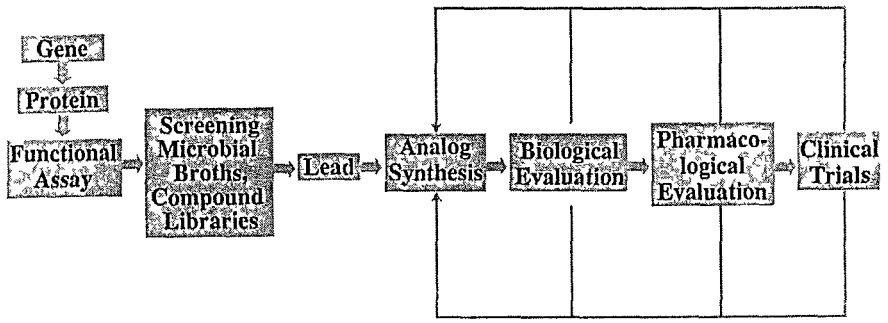

Fig.2 Random drug screening approach for finding low molecular weight antagonists

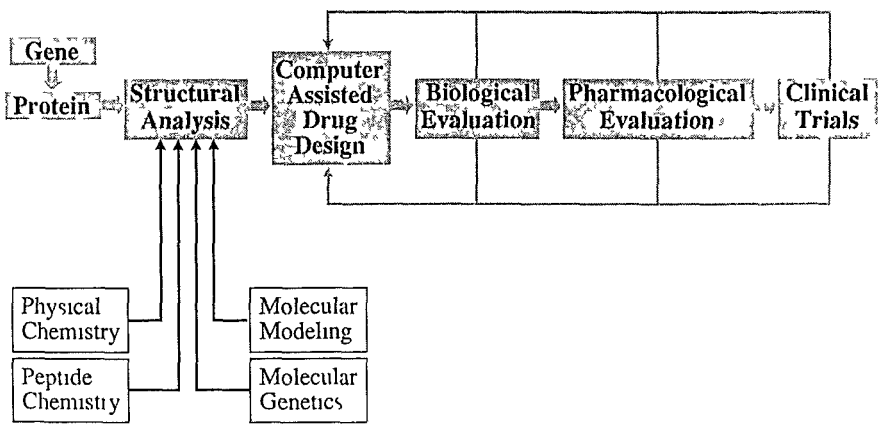

Fig.3 Rational drug development of low molecular weight antagonists

IFNγ ANTAGONISTS MIGHT BE USEFUL FOR THE TREATMENT OF ACUTE AND CHRONIC INFLAMMATORY DISEASES

The rationale for the development of IFNγ and IFNγ receptor antagonists relies on experimental observations made in animal models of various human diseases. In these models it was shown that antibodies neutralizing IFNγ could be used to prevent or treat certain inflammatory diseases or to prolong the rejection of organ transplants. These findings are relevant for certain clinical syndromes as shown in Table 1.

TABLE 1
POTENTIAL USE OF IFNγ ANTAGONISTS IN ACUTE AND CHRONIC INFLAMMATION

Experimental findings in animals with anti-IFNγ antibodies	Relevant clinical syndrome	Reference
Inhibits LPS-induced inflammation	Local gram-negative infections	Heremans et al. (1)
Prevents LPS-induced shock	Septic shock	Heremans et al. (2)
Prevents cerebral malaria	Cerebral malaria	Grau et al. (3)
Decreases lupus-like nephritis and adjuvant arthritis	Autoimmunity	Jacob et al. (4,5)
Delays rejection of tumor, skin and heart allografts	Organ transplantation	Landolfo et al. (6) Didlake et al. (7)

IFNγ EXERTS ITS BIOLOGICAL EFFECTS BY BINDING TO A SINGLE CHAIN CELL SURFACE RECEPTOR

Human IFNγ is a glycosylated protein of 143 amino acids in length, encoded by a single gene on chromosome 12. It has no similarity to IFNα or ß and acts in a species-specific way. IFNγ produced by T cells is the major macrophage activating molecule. Through this and other activities it potentiates immune responses. However, IFNγ has direct antiviral and antiproliferative activities as well.

IFNγ induces a biological response by binding to a single chain cell surface receptor of 90 kD in molecular weight (8). The human IFNγ receptor shows ubiquitous expression with 10^3-10^4 copies per cell surface (9). It is encoded by a single gene located on chromosome 6q and is composed of 472 amino acid residues which are about equally divided between the extra- and intracellular space (10). The receptor binds human IFNγ in a species-specific way with a dissocation constant of 0.1 nM.

A SOLUBLE FORM OF THE HUMAN IFNγ RECEPTOR IS USED FOR DRUG SCREENING AND THREE-DIMENSIONAL STRUCTURE DETERMINATION

We have characterized the entire extracellular region of the human IFNγ receptor by epitope mapping (11) and expressed it in *E.coli* (12) and insect cells (13). Large amounts of recombinant, soluble receptor can be expressed in *E.coli* and purified to homogeneity. After renaturation the recombinant receptor binds IFNγ with an affinity that is only about 10-fold lower than that of the native, membrane-bound receptor. With the recombinant material from *E.coli* we have developed a solid phase receptor binding assay which is currently being used to screen small synthetic and natural compounds for antagonistic activity (14).

From insect cells, infected with a recombinant baculovirus coding for the extracellular region of the human receptor, milligram amounts of recombinant, soluble receptor can also be isolated. The recombinant receptor from insect cells is glycosylated (although differently as compared to the native receptor) and binds IFNγ with essentially the same affinity as the native receptor. This material will be used for crystallization purposes to elucidate the three-dimensional structure of the IFNγ binding site and to start a drug design program.

AN ANIMAL MODEL TO TEST POTENTIAL HUMAN IFNγ ANTAGONISTS

Because of the species specificity it is unlikely that a potential antagonist for human IFNγ, identified by drug screening or drug design, will antagonize murine IFNγ. To be able to test drug candidates in small animals, we are developing a transgenic mouse model. Transgenic mice have been obtained with the human IFNγ receptor gene and shown to express the human receptor on spleen cells and to a lower extent on thymocytes. The transgenic receptor binds human but not mouse IFNγ as expected. Binding, however, is not sufficient to induce a response in transgenic cells as measured by antiviral activity and induction of class I and class II MHC gene expression.

These findings are in agreement with earlier reports showing that the expression of the human IFNγ receptor on mouse cells, achieved by somatic cell hybridization (15) or gene transfer (10), did not confer responsiveness to human IFNγ although binding was obtained. It seems that at least one additional polypeptide, apparently encoded on human chromosome 21, interacts with the human receptor in a species-specific way and is required for signal transduction (15). Once the cloning of the signal transducer has been achieved we will introduce the human gene into our receptor transgenic mice to develop an animal model that can be used to test potential IFNγ antagonists.

CAN THE SOLUBLE RECEPTOR ITSELF BE USED AS AN IFNγ ANTAGONIST?

To test the feasibility of using the soluble receptor itself as an antagonist, we have cloned the mouse IFNγ receptor (16) and expressed the entire extracellular region of the mouse IFNγ receptor using the baculovirus expression system (13). The recombinant receptor has been purified to homogeneity in milligram amounts and shown to bind mouse IFNγ with the same affinity as the membrane-bound receptor (17). The soluble receptor is able to block the induction of an antiviral response by mouse IFNγ in cultured cells.

We are currently testing *in vivo* stability, immunogenicity and clearance of the soluble receptor after injection into mice. We will then assess its ability to block B and T cell responses, graft-versus-host reactions, graft rejection and the generation of autoimmune diseases in murine models. Once transgenic mice with a functional human IFNγ receptor have been developed as discussed above, we will also be able to test the soluble human receptor in a small animal model for its potential use in the clinic.

BLOCKING TNF ACTIVITY MIGHT BE BENEFICIAL FOR ACUTE AND CHRONIC INFECTIOUS AND INFLAMMATORY DISEASES

TNFα and TNFß are two structurally related cytokines primarily produced by activated monocytes, macrophages and T cells. Both cytokines play a role in host defense reactions by a variety of activities. It has been shown, for instance, that TNFα induces the expression of adhesion and MHC class I molecules, plays an important role in granuloma formation in mycobacterial infections, causes the necrosis of certain mouse tumors and triggers antiviral activity in synergy with interferons. TNFα and ß bind to the same two TNF receptors (see below) and in general appear to induce the same spectrum of activities. There is substantial evidence that the active form of both cytokines is the trimer.

The efficacy of TNF as an anti-cancer agent is currently investigated in clinical trials. TNF antagonists might be useful for a variety of infectious and inflammatory diseases. Studies with cultured cells and animal experiments have indicated that TNF is a mediator enhancing tissue-injury in rheumatoid arthritis, bacterial meningitis, multiple sclerosis, cerebral malaria and graft-versus-host disease. Systemic TNF plays a role in malaria and septic shock. TNF also induces HIV expression in latently infected T cells and thus may play a role in the spread of the virus. Antagonists for TNF or TNF receptors therefore might find many applications in the clinic.

TWO HUMAN TNF RECEPTORS ARE STRUCTURALLY DISTINCT

We have used biochemical, serological and molecular genetic studies to show that human cells express two distinct cell surface receptor molecules which bind TNF. It was postulated from initial crosslinking experiments that a 55 kDa TNF receptor existed on human Hep2 cells, while HL60 cells were found to express both the 55 and a 75 kDa TNF receptor (18). Subsequently, monoclonal antibodies with specificity for either the 55 or the 75 kDa receptor were raised against partially purified receptor preparations and used to confirm and extend these initial findings (19). Finally with the help of these monoclonal antibodies, the two TNF receptors were purified to homogeneity and partial amino acid sequences were obtained (20). Oligonucleotide primers, synthesized according to the amino acid information, were used to amplify fragments of the corresponding receptor genes by polymerase chain reaction and full-length cDNA clones for both receptors were isolated using these fragments as probes (21,22).

Both human TNF receptors are transmembrane proteins containing 426 (55 kDa) and 439 (75 kDa) amino acid residues with extra- and intracellular regions of similar sizes. Sequence comparisons show that the extracellular regions of both receptors are structurally related to each other and to the nerve growth factor receptor and other cell surface antigens. The most characteristic structural unit is a cysteine-rich sequence motif, repeated four times in the extracellular regions of both human TNF receptors. In contrast , the intracellular regions of the two receptors, which are rich in proline and serine residues, are not related to one another nor to any other known protein. It is therefore possible that the two receptors connect to different signalling pathways and are functionally distinct.

Using the specific monoclonal antibodies described above as tools, clear-cut evidence has been obtained for the differential regulation of the two TNF receptors (22-25). Mitogen activation of human peripheral blood lymphocytes, for instance, strongly induces the expression of the 75 kDa, but not of the 55 kDa receptor (22). The expression of the 75 kDa receptor was found to be induced also in activated B cells on time scales which depended on the type of stimulus (24,25). In one study of activated tonsillar B cells a relatively late induction of the 55 kDa receptor was observed (24).

There is compelling evidence that both receptors are functional and transduce different signals. For instance, while B cell proliferation upon IgM treatment for 72 h could be blocked by antibodies directed against the 75 kDa receptor, antibodies directed against the 55 kDa receptor had no effect (24). Clearly, molecular genetic studies are needed to further dissect the functions of the two TNF receptors.

TNF ANTAGONISTS

In experiments similar to those described above for IFNγ antagonists, we are using recombinant forms of the human TNF receptors to develop antagonists. The extracellular region of the 55 kDa TNF receptor has already been expressed in soluble form using eukaryotic expression systems. The recombinant receptor will be used for random drug screening and crystallization. Expression experiments are also underway to produce soluble forms of the 75 kDa TNF receptor. In addition to our attempts to find small molecular weight antagonists, we will test the soluble TNF receptors for their potential clinical use. Given the recent success in the development of random peptide libraries (26), one can also start a search for small peptides able to block binding of TNF to its receptors.

SUMMARY

In this short communication I have summarized our approaches to the development of IFNγ and TNF antagonists for clinical use. Three different avenues are being followed. First, a high-flux receptor-based assay is being used to search for non-peptidic small molecular weight antagonists. Second, structural analysis of receptor/ligand interactions should allow us to embark on rational drug design programs. Finally, soluble forms of the receptors are being tested in animal models of human diseases for their potential clinical use. The observation that soluble forms of IFNγ and TNF receptors are found in the serum and urine of febrile patients suggests that these molecules act as physiological antagonists.

ACKNOWLEDGEMENTS

I would like to thank the members of the IFNγ and TNF receptor groups in our department for their enthusiastic research in the areas described here and Drs. Gianni Garotta, Werner Lesslauer and Richard Pink for comments on the manuscript. Ms Anne Iff is gratefully acknowledged for typing the manuscript.

REFERENCES

1. Heremans H, Dijkmans H, Sobis H, Vandekerckhove F, Billiau (1987) J Immunol 138:4175-4179
2. Heremans H, van Damme J, Dillen C, Dijkmans R, Billiau A (1990) J Exp Med 171:1853-1869
3. Grau GE, Heremans H, Piguet P-F, Pointaire P, Lambert P-H, Billiau A, Vassalli P (1989) Proc Natl Acad Sci USA 86:5572-5574
4. Jacob CO, van der Meide PH, McDevitt HO (1987) J Exp Med 166:798-803
5. Jacob CO, Holoshitz J, van der Meide P, Strober S, McDevitt H (1989) J Immunol 142:1500-1505
6. Landolfo S, Cofano F, Giovarelli M, Prat M, Cavallo G, Forni G (1985) Science 229:176-180
7. Didlake RH, Kim EK, Sheehan K, Schreiber RD, Kahan BD (1988) Transplantation 45: 222-223
8. Fountoulakis M, Kania M, Ozmen L, Lötscher HR, Garotta G, van Loon APGM (1989) J Immunol 143:3266-3276
9. van Loon AGPM, Ozmen L, Fountoulakis M, Kania M, Haiker M, Garotta G (1990) J Leukocyte Biology, in press
10. Aguet M, Dembic Z, Merlin G (1988) Cell 55:273-276

11. Garotta G, Ozmen L, Fountoulakis M, Dembic Z, van Loon AGPM, Stüber D (1990) J Biol Chem 265:6908-6915
12. Fountoulakis M, Juranville J-F, Stüber D, Weibel EK, Garotta G (1990) J Biol Chem 265:13268-13275
13. Gentz R, Hayes A, Damlin K, Fountoulakis M, Lahm HW, Ozmen L, Garotta G (1990) submitted for publication
14. Ozmen L, Fountoulakis M, Stüber D, Garotta G (1990) Biotherapy, in press
15. Langer JA, Pestka S (1988) Immunology Today 9:393-400
16. Hemmi S, Pehini P, Metzler M, Merlin G, Dembic Z, Aguet M (1989) Proc Natl Acad Sci USA 86:9901-9905
17. Fountoulakis M, Juranville JF, Gentz R, Schlaeger EJ, Manneberg M, Lahm HW, Ozmen L, Garotta G (1990) submitted for publication
18. Hohmann H-P, Remy R, Brockhaus M, van Loon APGM (1989) J Biol Chem 264:14927-14934
19. Brockhaus M, Schönfeld, HJ, Schläger EJ, Hunziker W, Lesslauer W, Lötscher H (1989) Proc Natl Acad Sci USA 87:3127-3131
20. Lötscher HR (1990) In: Aggarwal BB, Vilcek J (eds) Tumor necrosis factors: Strucutre, function and mechanism of action. Marcel Dekker, New York, in press
21. Lötscher H, Pan YCE, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W (1990) Cell 61:351-360
22. Dembic Z, Lötscher H, Gubler U, Pan YCE, Lahm HW, Gentz R, Brockhaus M, Lesslauer W (1990) Cytokine, in press
23. Hohmann HP, Brockhaus M, Baeuerle, P, Remy R, Kolbeck R, van Loon APGM (1990) J Biol Chem, in press
24. Heilig B, Mapara M, Brockhaus M, Möller A, Dörken B (1990) submitted for publication
25. Erikstein BK, Smeland EB, Blomhoff HK, Funderud S, Lesslauer W, Espevik T (1990) submitted for publication
26. Scott JK, Smith GP (1990) Science 249:386-390

Discussion - DEVELOPMENT OF ANTAGONISTS FOR IFN-GAMMA AND TNF

M.M. Reidenberg

You mention using antibodies as tools to see if antagonism would be useful. In many cases of acute illness, where one or two doses of drug might suffice, wouldn't the antibody or the FAB fragment of an antibody potentially be a useful therapeutic agent, avoiding the need to develop a small molecule?

M. Steinmetz

As long as one considers an acute response, I would think an antibody might be useful. However if you talk about chronic inflammatory diseases then of course you will not be able to use the antibody.

P. Tanswell

Could you comment on any particular advantages of this expression system SF9 baculovirus?

M. Steinmetz

In our hands the SF9 system is a good system for rapid expression of a molecule which requires a higher eukaryotic expression system. It takes only a few weeks in order to get reasonable amounts of soluble receptor, as in our example, or any other protein which you might wish to express. If you are interested in very high level expression in the end then probably the CHO system is better, but it takes several months to amplify the introduced gene and develop a stable cell line secreting large amounts of the protein of interest.

L. Gauci

Do you think, from the molecular biologist's point of view, that there is going to be any difference in being able to interact with a receptor at the level of a tiny molecule as opposed to a relatively specific large protein?

M. Steinmetz

It is very difficult to answer this question at this point in time. I would think one should be able to develop a small molecular weight molecule which will block binding of the ligand even if the ligand is a high molecular weight protein.

But our attitude toward the use of proteins as therapeutic agents has changed in recent years. For instance, four or five years ago many people were convinced that multiple injections of monoclonal antibodies would not be possible. Therefore, it would seem logical to gather evidence of the usefulness of protein antagonists before embarking on complex chemical programs.