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Antibodies as molecular mimics of biomolecules: roles in understanding physiological functions and mechanisms

Rodney A. Hill,¹ David J. Flint,² and Jennifer M. Pell³

¹Department of Animal and Veterinary Science, University of Idaho, Moscow, Idaho; ²Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde, Glasgow; and ³The Babraham Institute, Cambridge, United Kingdom

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Hill RA, Flint DJ, Pell JM. Antibodies as molecular mimics of biomolecules: roles in understanding physiological functions and mechanisms. *Adv Physiol Educ* 32: 261–273, 2008; doi:10.1152/advan.90130.2008.—Physiologists have routinely used understanding of the immune system to generate antibodies against regulatory molecules, growth factors, plasma membrane receptors, and other mammalian molecules in the development of analytical tools and assays. In taking this notion further, antibodies have been used in vivo to modulate physiological systems and to improve our understanding of their molecular interactions. To develop antibodies with physiological activity (efficacy), physiologists have worked with immunologists in developing interdisciplinary insights, requiring basic knowledge of immune system function in designing strategies to generate antibodies that interact with endogenous molecules of physiological interest, in vivo. Antibodies in different physiological systems have been shown to enhance or inhibit endogenous molecular functions. Two approaches have been used: passive and active immunization. Antibodies in these contexts have provided tools to develop further insights into molecular physiological mechanisms. Perhaps surprisingly, enhancing antibodies have been developed against a diverse set of target molecules including several members of the growth hormone/insulin-like growth factor-I axes and those of the β_2 -adrenoceptor axis. Antibodies that inhibit the actions of somatostatin have also been developed. A further novel approach has been the development of antibodies that interact with adipose cells in vivo. These have the potential to be used in therapeutic antiobesity approaches. Antibodies with efficacy in vivo have provided new insights into molecular physiological mechanisms, enhancing our understanding of these complex processes.

growth hormone; insulin-like growth factor-I; somatostatin; β_2 -adrenoceptor; adipocyte

WHEN WE THINK about the immune system, we naturally think about its role in fighting disease. The immune system in animals and humans has evolved to form a complex network of biochemical pathways and cellular interactions that are designed to attack, repel, and destroy microorganisms and parasites and their secreted molecules associated with invasion of the body. However, modern technology has enabled us to understand many of these processes, and we are now able to precisely direct immune function against a range of molecules, both naturally produced and completely synthetic. This article addresses the ways in which we have applied our understanding of the immune system to direct some of its actions toward molecules that occur in the body. In this context, antibodies have been used as tools to interact with and modulate the actions of physiological pathways. They have been used to

provide knowledge about the mechanisms of action of a range of classes of biomolecules including peptide hormones, receptors, and certain specific cellular targets. This approach to understanding physiological mechanisms involves redirecting immune function to recognize specific “self-antigens” and modulate physiological processes. We will provide examples of how antibodies can be used to inhibit, enhance, or mimic several important physiological processes.

Understanding the Role of the Immune System in Generating Physiologically Active Antibodies

If we assume that it is possible to evoke an immune response against a biomolecule with physiological activity, the technology provides several advantages:

1. The amount of a substance needed to evoke a humoral immune response is very small. Thus, significant antibody production is induced by immunization of animals with as little as 10–50 μg of the active agent. This represents 100- to 10,000-fold less than required for pharmacologically active compounds commonly used in animal studies.

2. Because immunization results in a relatively long-lasting response, biological effects may be elicited and studied over an extended period of time.

3. Immune modulation provides a tool that may be more specific than pharmacological agents. For example, pharmacological agents developed to recognize one receptor subtype may also interact with additional receptor subtypes, having (undesirable) side effects. Antibodies, which have high specificity, often recognize more discrete molecular domains.

We will address the elements required to stimulate an appropriate immune response and consider the ways in which antibodies have been used to both inhibit and stimulate specific molecular axes. Our treatment of the fundamental immunology is not comprehensive. It is designed to provide the reader with the concepts that underlie immune recognition and responses to homologous antigens, a process that is normally actively suppressed to avoid inappropriate autoimmune responses.

Stimulating the immune system to recognize self-proteins. A key hurdle to be overcome is the ability of the immune system to recognize the self and remain recalcitrant to an autoimmune response when many of the antigens used in this work are self-proteins or domains of self-proteins. Fortunately, our understanding of the cellular and molecular mechanisms that regulate the humoral immune response has been extensively studied in both rodent models and humans, and many of the underlying principals apply or are largely relevant to many species.

Target proteins. Immune modulation of physiological axes requires antibody recognition (and thus binding) of a target

Address for reprint requests and other correspondence: R. A. Hill, Dept. of Animal and Veterinary Science, Univ. of Idaho, PO Box 442330, Moscow, ID. 83844-2330 (e-mail: rodhill@uidaho.edu).

molecule. The characteristics required for a target molecule *in vivo* are that it must be accessible to the circulatory system and/or extracellular space such that humoral access for molecular interaction is possible, and it must be “immunogenic.” In this context, we define “immunogenic” as a molecule that will interact with molecules and cells of the immune system. Thus, target molecules are almost exclusively proteins or peptides and may be hormones/growth factors, specific hormone-binding proteins, hormone receptors, or other cell-associated molecules. In the cases of hormones/growth factors and binding proteins, these are soluble, circulating forms of target proteins, and the primary, secondary, and tertiary structures are usually well described. Thus, there are few problems in identifying suitable domains that may be targeted for antibody recognition.

Directing the immune response. Our aim is to evoke an immune response in an animal such that the antibodies produced will recognize an endogenous target protein in its native conformation to modulate its activity. Thus, for a hormone, targeting a hydrophobic domain (which is folded to an internal part of the molecule) would be futile. Similarly, for a receptor, targeting an intracellular domain would also be inappropriate. Figure 1 shows the conformation of a receptor subtype that is bound by epinephrine and norepinephrine *in vivo*, the β_2 -adrenoceptor (β_2 -AR), and highlights the features of a domain that is a suitable antigen (48). In this example, the antigens were synthetic peptide analogs of an extracellular domain of the native protein. For large molecules such as receptors, the use of a synthetic peptide analog has several advantages: first, a suitable domain may be selected. Using the whole molecule as antigen would produce antibodies that recognize different domains of the receptor, many of which would be unsuitable. Second, a synthetic peptide representing a suitable domain is easily prepared, whereas purification of the receptor to homogeneity is both technically difficult and expensive. This approach has been used successfully in a wide range of examples (7).

Generation of an immune response. It is not possible to evoke an immune response simply by injecting a protein or

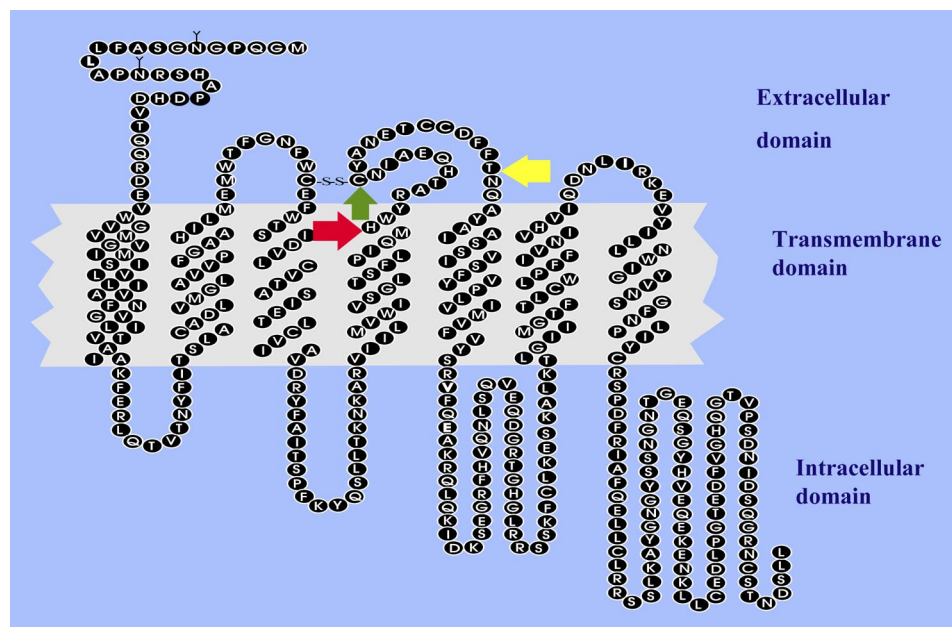
peptide containing epitopes that are recognized by B cells. This is true for any protein, be it a subunit antiviral vaccine, a “foreign” native protein, or a homologous hormone. It is necessary to emulate the features of an invading pathogen to stimulate the appropriate elements of the immune system. All vaccines rely on optimizing antigen presentation to evoke a sustained immune response. In the following sections we will not describe the role of adjuvants, but the reader is referred to the latest edition of Roitt et al. (89) for greater detail.

An antigen is required in more than one form to stimulate antibody production. The antigen needs to contain conformational epitopes that confer configurational specificity, and it also needs to provide T cell epitopes first to activate T helper cells and then to induce T helper cell interactions with antigen-activated B cells.

To enhance this process, when attempting to stimulate B cell recognition and a response against a small peptide antigen (e.g., a hormone or receptor peptide analog), an additional protein is chemically reacted with the target protein. This conjugation of the target protein with another protein is designed to increase the number of T helper epitopes available. There is genetic restriction in the range of T helper epitopes recognized by an individual of any species, and this is related to the major histocompatibility complex (MHC) (15). A range of proteins have been characterized for MHC recognition, most thoroughly in the mouse and human. Only relatively few are known for other species. Only class II MHC molecules in the so-called “exogenous pathway” are involved in the humoral response leading to antibody production. Again, we refer the reader to the latest edition of Roitt et al. (89) for greater detail.

Antibody production: the importance of a sustained response. In the context of generating a clear physiological response, it must be remembered that the active agent is the specific antibody generated by an appropriate immunization protocol. Thus, a central goal of the protocol is to generate antibodies that will be maintained above some unknown but critical blood concentration and to maintain this concentration for the required period of time. One of the great challenges for

Fig. 1. Model of the bovine β_2 -adrenoceptor (β_2 -AR). This membrane-bound receptor has been the target for a number of studies of immunomodulation (see references). In the study of Hill et al. (48), two synthetic peptide analogs, representing part and all of an outer loop of the β_2 -AR, were used as antigens to raise antibodies. The larger peptide contained 24 amino acid residues, shown here commencing with His¹⁷² (red arrow) through Thr¹⁹⁵ (yellow arrow), and the smaller peptide contained 13 amino acid residues, commencing with His¹⁷² through Cys¹⁸⁴ (green arrow). Although antibodies were raised against both antigens, only antibodies against the longer peptide recognized (and activated) the β_2 -AR. This illustrates the importance of conformation in antigen design. [From Ref. 89a.]



this technology is to be able to control antibody titers over prolonged periods. Advances in understanding the roles of complex molecular and cellular interactions are building our understanding. The use of specific recombinant cytokines is an example of a fruitful avenue of investigation that will likely lead to more sustained antibody responses (3, 57).

Important Concepts for the Determination of Antibody Action

Antibodies conventionally sequester antigen and inhibit antigen function. However, it is now established that, under specific circumstances, antibodies have the surprising action of potentiating or enhancing peptide hormone activity *in vivo*. Thus, the strategy for antibody design will depend on the functions of the target molecule and the desired outcome of the study in terms of whether the desired function may be inhibition, potentiation, or some other form of modulation of the target.

Active versus passive immunization. Two approaches are available for antibody administration: passive or active immunization. In passive immunization, antibodies are generated in a host animal, harvested, and, therefore, may be semipurified and assessed before administration to the target animal, i.e., the target animal is "treated" with defined immunoglobulin and does not need to stimulate its endogenous immune system. These antibodies are cleared from the body over a period of weeks. In active immunization, the target animal is immunized directly with antigen and generates its own antibodies. Three key differences therefore exist between passive and active immunization:

1. Passive immunization may be very controlled (dose and number of treatments), whereas the response to active immunization is less predictable.
2. Passive immunization is immediate, but active immunization takes longer as antibodies must be generated (several weeks to months).
3. Passive immunization is readily reversible, but active immunization may not reverse for many months. Thus, it is very important to consider the time scale of the target production parameter when designing immunization strategies.
4. Passive immunization requires larger amounts of preprepared antibody, whereas active immunization requires minute amounts of antigen.
5. Passive immunization carries an increased risk for the host due to potential for reactivity to donor antibodies (anaphylaxis).

Control of titer. As outlined above, maintenance of the antibody dose at an effective titer may be important in achieving a physiological response. In passive immunization, the amount of immunoglobulin administered to the target animal is regulated, and, therefore, titer can be controlled. However, as the immunoglobulin must be injected (usually subcutaneously, intraperitoneally, or infused intravenously), the volume that can be physically administered is restricted, and, therefore, antibody titer in the target animal will be limited. However, the only restriction in active immunization is appropriate stimulation of the immune system, which, in theory, should generate higher titers than by passive strategies. When the target protein is an endogenous peptide or protein, it is necessary to develop strategies for the generation of antibodies against self-mole-

cules [for a review, see Meloen et al. (76)]. Strategies to achieve this goal include conjugation of the endogenous target protein to an immune enhancer, or a carrier molecule. In addition, the immune system is also usually stimulated by presenting the immunogen in an adjuvant, a strategy also used to stimulate the immune response to more usual immunogens. To date, few systematic studies varying the dose of antigen, the nature of the carrier molecule (e.g., ovalbumin, keyhole limpet hemocyanin, or tetanus toxoid), and the immunostimulant (e.g., Freund's complete adjuvant or aluminium hydroxide) have been published. However, responsiveness even to a single-dose combination of these three components may be very variable. For example, anti-placental lactogen antibody titer varied by 10-fold between two subsequent studies that used similar immunization protocols (65). In contrast, even when the dose of antigen has been varied, antibody responsiveness has not (60). Clearly, much work is needed to define immunization strategies. For full optimization, these may be specific for each antigen.

Identifying Important Parameters of Antibody Interactions in Physiological Processes

Importance of antibody affinity. For this discussion, we will use the example of antibodies raised against IGF-I. IGFs (IGF-I and IGF-II) are important growth factors that are present in high concentrations in the circulation. Their actions are largely influenced by interactions with a family of binding proteins [IGF-binding proteins (IGFBPs)] and associated molecules that may bind IGFs with high affinity (36, 56). Studies of IGF action have extensively evaluated the roles of IGFBPs, especially in the context of the anabolic actions of IGF-I and the physiological responses associated with IGF-IGFBP interactions (90).

Studies of immune modulation of the IGF axis have included both active and passive immunization approaches (10, 44, 45, 47, 49, 50, 58, 62, 72, 73, 83–86, 92, 93, 98, 100) (detailed below). Some of these reports have found an inhibitory effect of specific antibodies on IGF-I actions (58, 62, 98), although many of those cited above have described IGF-I-enhancing activity. One of the factors that has an important influence upon inhibition or enhancement of IGF-I actions is the affinity of the antibody for IGF-I, although other factors, such as the range of epitopes recognized, are also important. When the affinity of the antibody for IGF-I is similar to that of the IGF receptor for IGF-I, there appears to be an IGF-enhancing effect *in vivo* (44, 45, 84–86, 100) and *in vitro* (86). In contrast, when the affinity of the antibody for IGF-I is considerably higher than that of the IGF receptor for IGF-I, its actions appear to be inhibitory both *in vivo* (58, 62) and *in vitro* (72, 73).

The dynamics of these interactions are complex. If we assume perhaps the simplest model in which there is complete steric hindrance of antibody, IGFBP, and IGF receptor binding to IGF-I, the distribution of bound IGF-I will be proportional to both the affinities of each of these interactions and the concentrations of each of the components (Fig. 2). All of these interactions will be in dynamic equilibrium.

A further important factor is illustrated in this case. The *in vivo* studies in which IGF-I-enhancing actions were demonstrated above all used polyclonal antibodies, which would

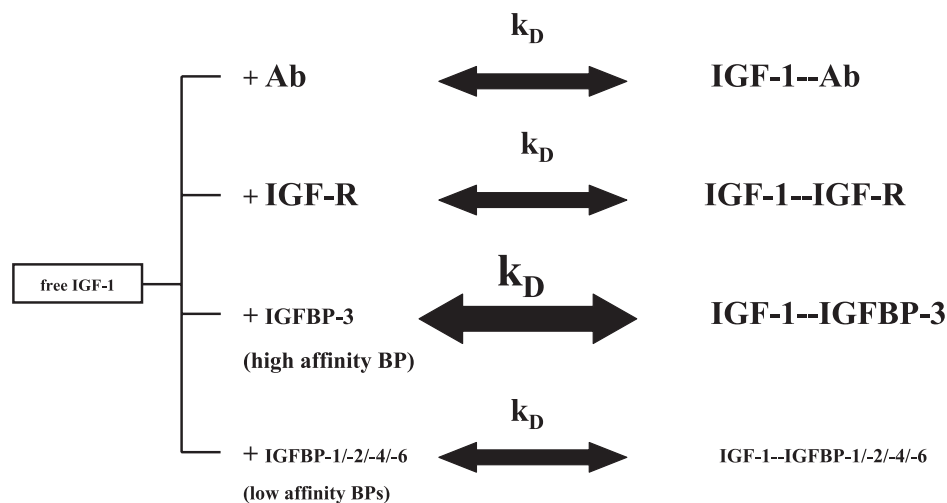


Fig. 2. Interactions of IGF-I with antibody (Ab), specific IGF-binding proteins (IGFBPs), and the IGF receptor (IGF-R). The distribution of bound and free IGF-I depends on the concentration of each of the components and the affinities of each of the interactions. In IGF-I-immunized animals, this system will be in dynamic equilibrium in vivo. As physiological and metabolic changes influence each of the components, shifts in the distribution of each of the components will occur. The size of the font used for each component shows the notional distribution of each component. Thus, there will be little free IGF-I. Although the affinity of the high-affinity binding protein (BP) is much greater than those of the IGF-R and Ab, when the concentrations of the IGF-R and Ab exceed the concentration of IGFBP-3, the distribution of bound IGF-I among these forms will be similar. In reality, other complex factors modify this simplistic model, which serves to illustrate the relative importance of concentration and affinity. [From Ref. 89a.]

normally be generated following immunization with the whole IGF-I molecule (composed of 70 amino acid residues, molecular weight: 7,649 Da). Thus, determination of the affinity of the antibody interaction with IGF-I represents a weighted average value of each of the epitopes recognized, with the immunodominant epitopes being proportionally represented. In some of these studies, there was a single dominant epitope. Thus, the affinity of the interaction was described most accurately in this case.

Most peptide hormone receptors have affinities for their ligands in the region of 10^{-9} – 10^{-8} M, whereas antibody affinities can vary from as low as 10^{-6} – 10^{-10} M. It is generally assumed that antibodies with a higher affinity for the target peptide hormone than its cognate receptor will inhibit the activity of the peptide hormone by effective competition for and sequestration of the peptide ligand. Alternatively, if the antibody has an affinity for the peptide hormone that is equivalent to or less than that of the receptor, then the receptor may be able to compete effectively for the pool of antibody-associated peptide. Furthermore, the pool of available hormone could also be expanded simply because of the presence of a large pool of low-affinity antibody. In this situation, the antibody may appear to be enhancing. Surprisingly, these hypotheses have not been formally tested. However, good analogous examples can be found in the relationship between IGF-I activity and its binding proteins. IGFs are associated with a family of six specific binding proteins (IGFBPs), which can be modified by controlled proteolysis, association with the extracellular matrix or cell membranes, or phosphorylation. In each situation, these modifications can induce changes in the affinity of the IGFBP for IGF-I. High-affinity IGFBPs are inhibitory, whereas low-affinity IGFBPs are potentiating, for IGF-I activity (for a review, see Ref. 56). As outlined above, antibodies that potentiate IGF-I activity in vivo have a modest affinity for IGF-I, similar to that of the IGF type 1 receptor (as discussed in Ref. 100).

Circulating immune complexes. Even though antibody titers are measured in serum samples, this may not be their only site of action. If the aim of the administered antibody is to inhibit the activity of a circulating physiological regulator, these measurements are appropriate. Similarly, if enhancing antibodies exert their effects simply by prolonging the half-life of the target regulatory molecule, serum titers are most relevant (discussed below). The degree to which these target molecules are complexed to antibodies will depend on their relative concentrations and the affinity of the antibody; however, even relatively low-affinity IGF-I-enhancing antibodies certainly complex IGF-I (100). Immunoglobulins, particularly those of the IgG class, gain access to interstitial and pericellular spaces within tissues (89, 118). Indeed, peripherally administered therapeutic antibodies against amyloid β -peptide are found in the central nervous system (8) and enhancing antibodies to porcine growth hormone (GH) change the distribution of tissue GH (107, 114). An intriguing observation in antibody enhancement of peptide hormone activity is that only a proportion of exogenous hormone needs be complexed to antibody for potentiation to occur (51, 88).

The way in which the immune system deals with circulating immune complexes is also important in our understanding of the efficacy of immune modulation of physiological pathways. After binding to an antigen, an IgG molecule changes conformation, and recognition sites along the molecule, which were previously hidden, become exposed. These sites bind cell-associated proteins expressed by cells of the reticuloendothelial clearance system. (Details of this mechanism are not completely understood, and the reader is referred to Ref. 89 for greater detail.) In the case where IgG is bound to a circulating hormone, the reticuloendothelial clearance system provides an alternative clearance mechanism. Figure 3, from a study by Hill et al. (45), clearly shows that in the presence of specific antibody, the mechanism of clearance of IGF-I was radically shifted from a renal to a hepatic mechanism. Thus, the liver is

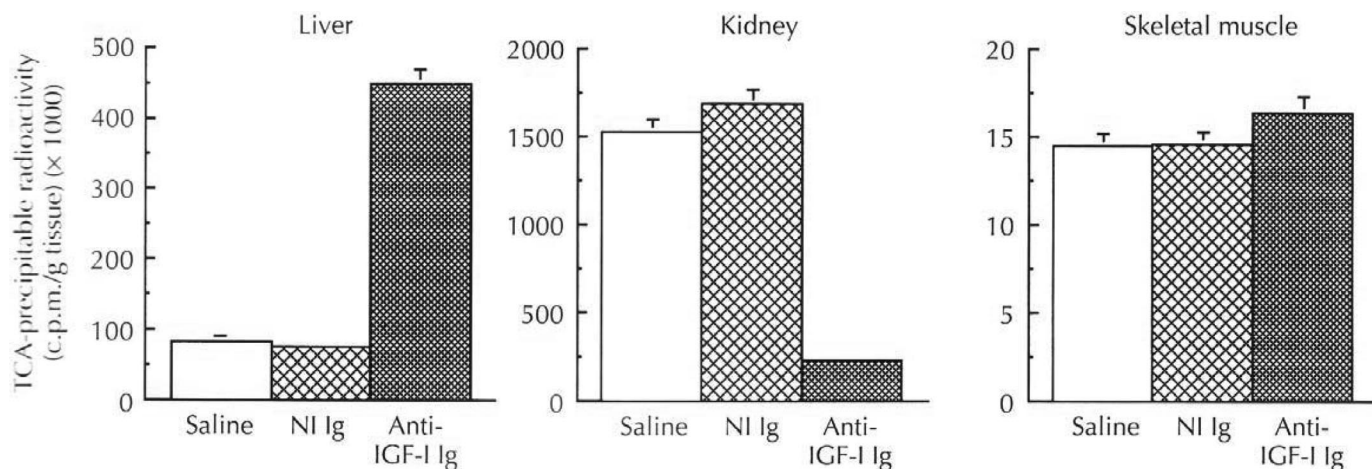


Fig. 3. Clearance of IGF-I from dwarf rats pretreated with sheep anti-IGF-I Ig, nonspecific Ig [nonimmune (NI) Ig], or saline. ^{125}I -labeled IGF-I (^{125}I IGF-I) was used in this experiment as a tracer and shows the behavior of endogenous IGF-I. The huge effect of anti-IGF-I Ig on the clearance mechanism is clear. In the two control groups, the great majority of IGF-I is taken up by the kidneys, degraded, and excreted. In anti-IGF-I Ig-treated rats, much of the IGF-I is complexed with antibody. These "immune complexes" are cleared through the reticuloendothelial system active in the liver. Interestingly, skeletal muscle tended to contain more IGF-I in IGF-I Ig-treated rats, suggesting that this could provide a mechanism for improved muscle growth. [From Ref. 45.]

an organ in which reticuloendothelial clearance is highly active. As stated above, this mechanism is not completely understood and is also affected by factors such as the affinity of the specific IgG for the hormone. Thus, the typical rapid off rates of low-affinity interactions may mean that this mechanism is not highly effective at clearing IgG-bound hormone. Furthermore, the way in which the body handles IgG-bound hormone is very complex, and the mechanism of clearance and affinity of interaction are just two factors.

Kinetics of immunoglobulin-associated peptide hormones. A conventional role of antibodies in infection is to facilitate the clearance of antigens. However, several studies have demonstrated that antibody-associated self-peptide hormone is cleared less rapidly and with different kinetic properties than nonantibody-associated hormone (11, 45, 75, 113). Subcutaneous injection of porcine GH that had been precomplexed to an enhancing monoclonal antibody (MAb) delayed absorption of porcine GH into the circulation and prolonged exposure of specific tissues to GH (113). Hill et al. (45), using an intravenous infusion of ^{125}I -labeled IGF-I precomplexed to enhancing anti-IGF-I polyclonal immunoglobulin, observed unusual IGF kinetics. When examined via a two-phase model, the clearance of IGF-I was decreased by almost twofold by the enhancing immunoglobulins (compared with nonimmune immunoglobulin), but the half-life of IGF-I was decreased in the (quantitatively greater) slower-decaying phase (Fig. 4). Furthermore, the IGF-I degradation rate was significantly decreased by

25–50% in the presence of enhancing immunoglobulins. These findings suggest that the anti-IGF-I immunoglobulin was protecting IGF-I from proteolysis, leading to a larger plasma pool of peptide that could be transferred readily to tissues. Enhancing antibodies can also change the interaction of a hormone with its receptor. Thus, prolonged receptor occupancy by GH has been observed when it is precomplexed to an enhancing MAb, and increased receptor activation, measured as prolonged stimulation of IGF-I and IGFBP-3 synthesis, was observed (11, 74).

Therefore, a key mechanism of antibody potentiation of hormone activity may be by prolonging the presence of bioavailable peptide hormone. This is supported by studies in which GH clearance was reduced by cotreatment with GH-binding protein (21) or by generation of a long-acting form of GH by conjugation with polyethylene glycol (20), which yielded stimulation of the biological response to GH of similar magnitude to that of enhancing antibodies. In addition, Mockridge et al. (77) observed that simple chemical cross-linking of GH enhanced its biological activity. However, a key observation is the demonstration of enhancement of IGF-I activity in vitro, in conditions when hormone levels are maintained, suggesting that mechanisms other than clearance must also be important (86). A prolonged hormone-receptor interaction in the presence of enhancing antibody is compatible with current in vivo and in vitro observations.

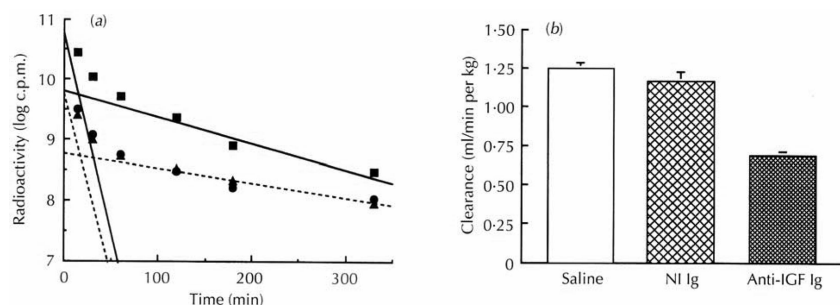


Fig. 4. A: decay curves of IGF-I from plasma of dwarf rats. ^{125}I IGF-I was used in this experiment as a tracer and shows the behavior of endogenous IGF-I. Dwarf rats were pretreated with anti-IGF-I Ig (■), NI Ig (●), or saline (▲). B: clearance of IGF-I in dwarf rats pretreated with anti-IGF-I Ig, NI Ig, or saline. Anti-IGF-I treatment reduced the clearance rate of IGF-I, and more IGF-I remained in the circulation over time. Thus, anti-IGF-I Ig treatment increased the total pool of IGF-I. [From Ref. 45.]

Site of antibody binding to target. One reason underlying the normally observed inhibitory actions of polyclonal antisera is the multiple binding of antibodies to several sites on the antigen. In terms of peptide hormone action, it is likely that the active site, i.e., the receptor-binding site on the peptide hormone, will be sterically hindered in the presence of specific antibodies, preventing receptor interaction and activation. Even though the sites of binding for all enhancing antisera have not been characterized, all findings to date suggest that enhancing antisera bind to peptide hormones at sites distant from the receptor-binding domain (13, 49, 86, 87). This is shown for IGF-I in Fig. 5. Thus, when designing hormone antigens, it is an advantage if the receptor-binding site is known so that appropriate antigenic peptides can be designed.

Physiological Effects Using Passive Immunization Models

Advantages and disadvantages. Passive immunization has the advantage in that the amount and nature of the immunoglobulin administered is known. Thus, passive immunization may be seen as having a distinct advantage, as the treatment may be more precisely controlled in terms of dose and period of administration. Every animal in the treatment group may be dosed precisely, and variations in the individual response seen in active immunization are removed. In practice, this has meant

that passive immunization studies have been widely used to investigate the mechanisms of action of immune modulation. Its main disadvantage is that the amount of antiserum to be administered is limited. Furthermore, it may need to be administered regularly to maintain an effective dose. Repeated treatment with heterologous antibodies is not possible because of the risk of anaphylaxis or anti-immunoglobulin responses in the recipient. However, recent advances in antibody technologies may well overcome this limitation.

MAbs. An array of exciting new technologies has paved the way for the use of MAbs as tools for passive immunization. MAbs are derived from a single clone and, therefore, generate just one immunoglobulin that defines a single epitope on the antigen, usually conformational rather than to a linear peptide sequence. Thus, a part of the structure of the antigen is accurately mirrored, but this can be very difficult to define by techniques such as epitope scanning (13). A further benefit of MAbs is that their affinity will be precisely known.

A massive advance in MAb technology was the development of genetically engineered antibodies (for reviews, see Refs. 42 and 54). In effect, this means that mouse antibodies can be "engineered" to be identical to the form of those from the "target" species, avoiding counterimmune responses. In theory, antibodies could be engineered for administration to any species. Antibody engineering and phage display techniques thus have huge scope to modify and adapt antibody action, for example, to change the half-life of defined antibody fragments (18), to define minotopes for subsequent active immunization (110), or to change antibody affinity (19). The major impact of this technology has been for therapeutic purposes in humans, for example, for cancer therapy (55, 91), rheumatoid arthritis (23, 108), and anaphylactic shock (43). An important example of a potentially useful therapeutic, humanized MAb was reported by Leckie et al. (64). In this case, a novel anti-IL-5 MAb was administered to human subjects intravenously with the hope of "curing" hyperallergic responses. The objective was to diminish eosinophilic-associated airway hyperresponsiveness. Both airway (sputum) and blood eosinophils were reduced to very low levels. Despite this encouraging result, airway hyperresponsiveness remained and was, at first, unexplained. Later research indicated that it was likely lung tissue resident eosinophils (which were not accessible to the antibody therapy) and not airway eosinophils that remained and may have still had significant effects on airway responsiveness in asthmatics treated with the antibody. This case points out an important consideration of targeted cell and tissue-specific delivery when attempting to use antibodies as therapeutics.

To date, most "humanized" MAbs have been inhibitory, for example, designed to block TNF- α activity in autoimmune disease. However, this technology could be applied to the development of designer antibodies or peptides for the immunological enhancement of hormone activity.

Physiological Effects Using Active Immunization Models

This is the form of immunization with which we are most familiar from everyday life. As children, we are immunized against a range of childhood diseases. In this example, it takes multiple immunizations to confer protective immunity to the child. Protective humoral immunity is only conferred when the

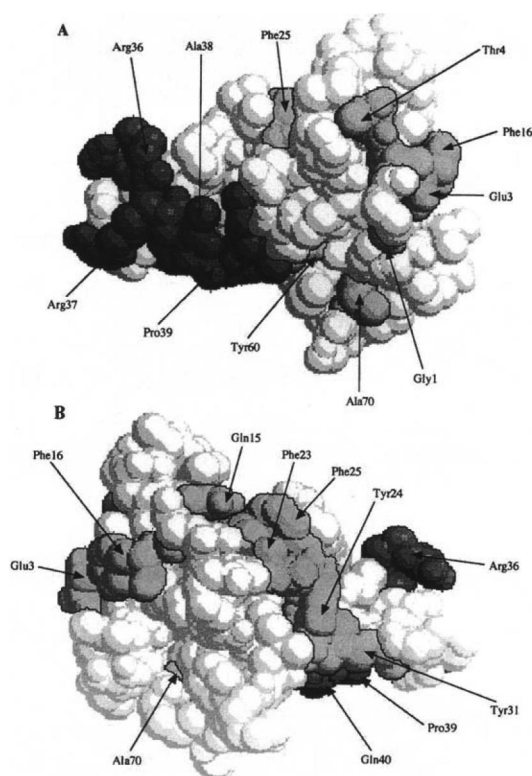


Fig. 5. The structure of IGF-I, showing the regions that interact with IGFBPs, anti-IGF-I Ig, and the IGF-R from two different perspectives. Residues important for IGFBP interactions (Glu³, Thr⁴, Gln¹⁵, and Phe¹⁶), and IGF-R binding (Tyr²⁴, Tyr³¹, and Tyr⁶⁰), and the terminal residues (Gly¹ and Ala⁷⁰) are shown by the light gray shading. Residues that have been epitope mapped and interact with growth-enhancing anti-IGF-I Ig (Arg³⁶ to Ile⁴³) are shown by the dark gray shading. This evidence indicates that it may be possible for the antibody to simultaneously bind IGF-I while it is also bound to the receptor or binding proteins. [From Ref. 49.]

blood antibody titer is sufficiently high to neutralize invasion of a pathogenic microorganism. The mechanisms that determine this pattern of response are outlined in *Stimulating the immune system to recognize self-proteins*. We will not describe the typical immunization strategies that are used, as there are many specific texts that provide this information in detail.

In the case of immunization using a self-protein, again, multiple doses are required. A biological effect is only observed when the titer of the specific antibody is sufficiently high that the antibody concentration is similar to either the circulating concentration of the hormone or, in the case of hormone receptors, sufficiently high that the antibody can interact and either block or activate the receptor in the presence of the cognate hormone. In this respect, hormones are attractive targets as they often circulate in picomolar or even femtomolar concentrations.

One of the issues that makes active immunization problematic is the individual variation in the immune response. Thus, the individual effective dose may be different in each animal in a treatment group. This problem makes interpretation of physiological responses difficult. In *Experimental Approaches to Immune Modulation*, we provide a discussion of specific examples of immune modulation of physiological axes in animals and the physiological responses achieved.

Experimental Approaches to Immune Modulation

Examples of functional inhibitors of physiological mechanisms. ANTIBODIES AGAINST SOMATOSTATIN. Somatostatin (SST) is secreted by a range of tissues, including hypothalamic, gastric, and pancreatic sources. Circulating SST provides a feedback mechanism that downregulates GH secretion by the pituitary gland. As such, SST might be expected to be a target for immunoneutralization to understand the regulation of growth processes in vivo. In fact, SST was one of the earliest hormones studied in this way. Arimura et al. (2) described the blockade of a stress-induced decrease in blood GH by anti-SST in rats. Since then, many studies have been conducted on the underlying mechanism of SST action using antibodies. Early studies in an unselected sheep breed showed large improvements in feed efficiency and growth following immunization against SST (95–97, 99). The mechanism appears to be related to SST action on gut peptides, improving digestion, although in many cases both serum GH and IGF-I concentrations increase following SST immunization. Subsequent studies in sheep that were selected for high growth either failed to show these effects (52) or the effects were smaller (9, 63, 105, 106).

These effects have also been demonstrated in other species including chickens (94) and piglets for which the sow had been SST immunized (32). SST immunization has also improved pregnancy rates in both sheep and pigs (61). Thus, it appears that immunization against SST results in hormone immunoneutralization and its subsequent clearance from the circulation.

OTHER INHIBITORY ANTIBODIES. A number of strategies have been attempted to invoke antibodies against adrenocorticotrophic hormone or cortisol. The underlying theory of this strategy is that antibodies could be used to neutralize these elements and reduce the effects of stress in animal models (115–117).

There are several examples of immunological intervention for the control of reproduction that have been trialed in live-stock. An immunization protocol was developed against gonadotropin-releasing hormone and commercialized in 1990 (53). This strategy has been tested in several species and has been shown to be effective for reversible castration of colts and spaying of fillies (26) and for eliminating boar taint and improving growth performance in boars (28).

Examples of functional enhancers of physiological mechanisms. GH-RELEASING FACTOR. GH-releasing factor (GRF) is a 40- to 44-residue peptide that has a role in the stimulation of pituitary GH synthesis and secretion. Therefore, potentiation of its activity could induce increased GH secretion and hence increased GH-stimulated growth. It is known that the receptor-binding region of GRF resides in the NH₂-terminal 29 residues (22). Specific site-directed anti-peptide antibodies were therefore generated against either the NH₂- or COOH-terminal domains of GRF (87); their effects (with and without preincubation with GRF) were examined in vitro (GH release from cultured primary pituitary cells) and in vivo (circulating GH concentrations). Intriguingly, antibodies directed against the NH₂-terminal region of GRF inhibited GRF activity, whereas antibodies directed against the COOH-terminal region enhanced GRF activity. These studies therefore suggest that site of binding of the antibody is indeed important for subsequent hormone activity and that the phenomenon of antibody-mediated enhancement of peptide hormone activity may occur both in vivo and in vitro.

ANTIBODIES AGAINST IGF-I. Most studies to date have examined the effects of antibodies against IGF-I by passive immunization. Initial studies, using both polyclonal antibodies and MAbs, observed either negligible (58, 98) or short-term inhibitory effects (62) on the growth-promoting activity of IGF-I in vivo. However, subsequent specific antibodies raised against IGF-I in sheep potentiated the ability of IGF-I to stimulate growth in GH-deficient dwarf mice (100). Similar to previous studies, passive administration of anti-IGF-I antibodies increased total circulating IGF-I concentrations.

As IGFs are potent growth factors, their activity is tightly regulated by a family of six high-affinity IGF-BPs (for a review, see Ref. 56). The affinity of the enhancing anti-IGF-I antibody reported by Stewart et al. (100) was similar to that of a potentiating IGF-BP ($\sim 2 \times 10^{-8}$ M), and, therefore, it was proposed that the antibody in some manner maintained a pool of bioavailable IGF peptide. A subsequent radiotracer study (45) examining IGF-I pharmacokinetics in the presence and absence of enhancing antibodies supported this hypothesis.

As previous investigations into the properties of other peptide hormone-enhancing antibodies have suggested that the site of antibody binding to the peptide is important, the IGF-I epitopes recognized by the enhancing antibody were determined using epitope scanning techniques. In this approach, multiple short and overlapping peptides (typically hexamers or octamers) are synthesized that span the entire sequence of the peptide under investigation; these are then incubated with the antibody, and the sites of interaction are detected by ELISA techniques. The enhancing anti-IGF-I antiserum recognized a very specific region of IGF-I: residues Ser³³ to Cys⁴⁷ (49); moreover, antibodies that did not potentiate IGF-I activity did not recognize this region (86). Interestingly, the domain recognized by the enhancing antibody is situated on the opposite

face of IGF-I to the receptor-binding site, consistent with the location of antibodies that potentiate both GH and GRF activities.

ANTIBODIES AS ENHANCERS OF GH ACTIVITY. The biological function of antibodies is most usually considered as neutralization of antigenic molecules, and, for example, administration of polyclonal antibodies against GH neutralize hormone activity (35, 81). However, certain MABs were surprisingly shown to increase rather than inhibit GH action, as first demonstrated by Holder et al. (51). This effect upon GH action could be enhanced by precomplexing it with MABs before administration in an animal model (4, 6). This antibody-mediated enhancement of human GH activity confirmed earlier experiments that had demonstrated the enhancement of EGF, insulin, and other polypeptide hormones by antibodies (for a review, see Ref. 5).

In an attempt to improve the prospects for the development of a peptide vaccination strategy, epitope mapping studies of the GH molecule were undertaken to identify regions of the GH molecule associated with enhancement. Previous GH peptides were principally selected on the basis of computer algorithms that predicted immunogenic regions within the GH molecule. This resulted in the selection of many peptides that were contained in loop structures within the GH molecule and, based on the published crystal structure of GH, were accessible on the surface of the molecule. Antibodies generated from such peptides may be more likely to bind to native protein than those raised against peptides that represent elements of the secondary structure within a protein, and initial epitope mapping studies, using a technique that identifies linear epitopes, showed that polyclonal antibodies raised against bovine GH contained major epitopes that were present in all of the loops joining the four α -helices as well as in the nonordered COOH-terminus of the protein (12). Although many MABs could not be epitope mapped using this technique, presumably because they recognized discontinuous epitopes, one anti-ovine GH-enhancing antibody, Mab OA15, did bind to a continuous epitope lying between residues 91 and 102 of bovine GH in a loop between helices 2 and 3 of GH (13). The peptide epitope to which this enhancing Mab bound was used to produce a polyclonal antiserum, and this was shown to be capable of enhancing ovine GH activity in a dwarf mouse model (77).

Most hypotheses regarding the mechanism of enhancement have concentrated on in vivo effects, including increased half-life of the hormone-antibody complex, increased GH receptor occupancy time by hormone-antibody complexes, and targeting of the hormone by the antibody into specific target tissues (e.g., the liver). Evidence has been provided to support some of these hypotheses (75, 107, 113).

ANTIBODIES AGAINST THE GH RECEPTOR AS RECEPTOR AGONISTS. The concept of identifying the site on a hormone receptor to which the hormone binds and using this to produce antibodies that bind to the same site as the hormone has been explored (Fig. 6). GH activates its receptor [GH receptor (GHR)] by a homodimerization mechanism involving two sites on the hormone molecule interacting with two different receptor proteins, resulting in a 1:2 (hormone-receptor) complex (24). Such a mechanism of GHR interaction suggested that reception activation might be achieved by the binding of appropriate bivalent anti-receptor antibodies to two receptors.

Targetted antibodies to hormone receptors should mimic the hormone

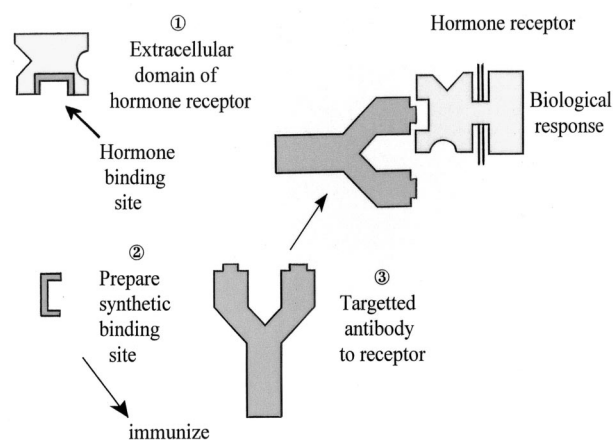


Fig. 6. Using anti-receptor antibodies as pseudohormones. The mechanism of action of anti-receptor antibodies is thought to involve antibody binding to extracellular domains of the receptor, not necessarily the hormone-binding site. This principal has been demonstrated for both the growth hormone receptor and for β -ARs. Synthetic peptide mimics of an outer domain of the receptor are used to immunize animals and evoke antibodies that bind to this receptor domain. On antibody binding, a change in the receptor conformation is induced. This leads to an intracellular response using the usual second messenger pathway. [From Ref. 89a.]

Such receptor-activating antibodies have already been described for the prolactin receptor involving the stimulation of mammary gland casein synthesis in vitro and in vivo (29, 30). These antibodies also had the ability to inhibit prolactin activity and, on their own, showed inhibitory or stimulatory activity depending on the dosage (30). To date, no reports of anti-GHR antibodies that stimulate the GH axis in vitro or in vivo have been published. Epitope mapping studies of the bovine GHR extracellular domain (14) may assist in the selection of candidate receptor peptides to use for the generation of anti-receptor antibodies. In the case of such anti-receptor antibodies, however, the appropriate dimerization of the receptors, as is induced by GH binding, may not be replicated by antibody-receptor engagement, and, under these circumstances, no signal transduction may be effected. Indeed, such antibodies may prove to be antagonists at the GHR and may form the basis of an approach for the treatment of GH-excess syndromes such as acromegaly. An alternative strategy has been adopted where putative peptide epitopes (e.g., from rat GHRs) are presented within the framework of the GHR derived from another species (e.g., sheep). When sheep are immunized with such peptides, the response to the chimeric protein should be largely targeted toward the rat sequences. As outlined above, the use of such recombinant proteins as vaccines may overcome problems associated with the appropriate presentation of peptides during vaccination. Initial studies using chimeric rat/ovine GH-binding protein molecules have indicated the feasibility of this approach (1).

ANTIBODIES AS ENHANCERS OF β -AR ACTIONS. There is an extensive body of research reporting on antibodies raised against elements of the β -AR axis. These include mechanistic and fundamental studies and clinical studies, particularly as there is at least one human idiopathic pathology that appears to be associated with the presence of anti- β -AR antibodies in serum (66, 112). A feature common to many of these studies is that

the antibody action can be clearly demonstrated in vitro and in model species. The mechanism appears to be consistent across species and β -AR subtypes and consistent with the proposed underlying theory (outlined in ANTIBODIES AGAINST β -AR LIGANDS).

ANTIBODIES AGAINST β -AR LIGANDS. Several laboratories have investigated antibodies against β -AR ligands and, subsequently, anti-idiotypic antibodies directed against β -ARs (67). These phenomena were investigated further by a number of groups using MAbs. Following their group's earlier work in rabbits, Chamat et al. (17) raised antibodies in mice to alprenolol-BSA conjugate. Four clones were studied, with reported affinities for alprenolol ranging between 5.3×10^{-7} and 4.2×10^{-8} M. Antibodies also showed stereoselectivity, having a higher affinity for the (*l*)-enantiomer of alprenolol than for the (*d*)-enantiomer. Guillet et al. (39) then reported a novel method for raising anti-idiotypic MAbs that involved immunizing mice with fixed hybridoma cells bearing a specific antibody for the β -AR. Three anti-idiotypic antibodies were able to bind the β_2 -AR. One MAb was capable of generating adenylate cyclase activity, which was inhibited by propranolol, a β -antagonist (40). Fine detail of the nature of the anti-idiotypic response was later reported (16), as was the diversity of V_H and V_L genes encoding the anti-alprenolol antibodies (79). Several review articles by Strosberg and colleagues (67, 101–104) have also described much of this work. None of these studies reported in vivo effects of these antibodies; however, in a later study (60), rats immunized against clenbuterol developed titers, but no biological effects were demonstrated.

ANTIBODIES AGAINST SYNTHETIC PEPTIDE ANALOGS OF β -AR. Synthetic peptide antigens are only useful if the primary, secondary, and, preferably, tertiary/quaternary structures of the protein in question are known. To attempt to generate peptides corresponding to a (likely) antigenic region of a protein, features such as the position of the sequence on the protein in its native conformation should be considered; i.e., if the sequence occurs on the outer surface of the native protein, or if it forms part of a hydrophilic loop, it is much more likely to be seen by antibodies than a sequence that occurs in a hydrophobic or an internal domain. Fortunately, the β -AR structure has been elucidated. Theveniau et al. (109) raised antibodies in rabbits to two extracellular domains of the hamster β_2 -AR. Antibodies recognized the receptor in immunoblots and precipitated radiolabeled β_2 -ARs.

In a series of studies, Magnusson et al. (69–71) investigated the occurrence of autoantibodies in patients with idiopathic dilated cardiomyopathy. Because of the limitations of low titers of antibodies in the human serum they studied, anti-peptide antibodies were raised in rabbits and served as a model. The antigens used were 26-amino acid sequences corresponding to outer loop 2 of the β_1 -AR and β_2 -AR. The main focus of these studies was directed to the β_1 -AR as this is the predominant subtype in the heart. Anti- β_1 -AR antibodies incubated with C6 glioma cell membranes (rich in β_1 -ARs) were able to displace the agonist (isoprenaline) affinity to higher values. However, anti- β_2 -AR antibodies did not show any effects on ligand binding when incubated with A431 cell membranes rich in β_2 -ARs (68). In a following study (69), β_1 - and β_2 -peptides were used as antigens in an ELISA to screen the sera of patients with idiopathic dilated cardiomyopathy and from healthy control individuals; 14 of 42 patients and 4 of 34

controls were found to carry antibodies to the β_1 -sequence. Only affinity-purified antibodies from ELISA-positive patients had an inhibitory effect on conventional ligand binding to the β_1 -AR in C6 glioma cell membranes. These antibodies also recognized the receptor protein in immunoblots and bound in situ to human myocardial tissue. The authors concluded that the antibodies could serve as a marker of an autoimmune response with physiological and/or pathological implications. Another report (70) from this group described the functional effects of antibodies at the β -AR. Rabbits immunized with the β_1 -sequence mentioned above developed antibodies that decreased β -antagonist binding in a similar fashion to that described for antibodies from patients with idiopathic dilated cardiomyopathy. Antibodies incubated with transformed *Escherichia coli* inner membranes (expressing the human β_1 -AR) or C6 glioma cell membranes caused a decrease in the number of ligand-binding sites. The decrease was dependent on the concentration of both antibodies and magnesium ions, and antibodies appeared to bind to the receptor with high affinity. Interestingly, the antibodies also produced an agonist-like effect in an in vitro bioassay. Spontaneously beating, isolated neonatal rat cardiomyocytes were incubated with the antibody, which caused a positive chronotropic effect. The effect of the antibodies was blocked by a preincubation with the β_1 -peptide and was antagonized by the β_1 -selective antagonist bisoprolol but not by the β_2 -selective antagonist ICI-118,551. This group also investigated the immune response of mice to the analogous β_2 -peptide (41) and found that the 26-amino acid peptide induced a T cell-mediated humoral response in three mouse MHC haplotypes. The T cell epitope was located in the NH_2 -terminal region of the peptide.

These findings of antibody activity having a biological effect (at the β_1 -AR) led another group to study the same mechanism at the β_2 -AR. Hill et al. (48) were able to produce anti- β_2 -AR antibodies by immunizing rabbits with a similar peptide preparation. These antibodies also had biological activity in bovine smooth muscle, increasing the affinity of a β -antagonist and causing a leftward shift in the concentration-response curve for isoproterenol-induced bovine smooth muscle relaxation. There were a number of possible mechanisms by which the antibodies might exert their effects at the β_2 -AR. First, the antibodies might behave similarly to the conventional agonists, by causing a conformational change in the receptor protein upon binding, although the precise sequence of interactions would be different. It has been proposed that conventional agonists bind to side chains of amino acid residues within the cleft formed by the seven transmembrane domains of the receptor (see Fig. 1). However, the mechanism of antibody binding and activation of the β_2 -AR is likely to be different. Sterically, antibodies would be precluded from binding to the conventional ligand-binding site deep within the receptor. (The molecular mass of conventional agonists is $\sim 1/1,000$ th that of IgG). Assuming that the antibodies bind to the sequence corresponding to the synthetic peptide used as the antigen, their effects must be mediated via the second outer loop, which joins transmembrane domains 4 and 5 (Fig. 1). Both of these domains play a role in conventional ligand binding. This evidence suggests that the binding of antibodies to outer loop 2 may induce a conformational change in the receptor, analogous to that which follows the binding of a conventional agonist. This proposed conformational change may then lead to

receptor activation. This mechanism is consistent with that proposed for the activation of the β_1 -AR in human subjects outlined above.

Examples of antiobesity strategies. ANTIBODIES TO ADIPOCYTE PLASMA MEMBRANES. Another novel approach to immune regulation of physiological processes is in the use of antibodies in their more classical role of targeting molecules and cells for destruction by the immune system, for example, via complement-mediated cell lysis, but in this case the targeted cells are adipocytes. Antibodies directed against the plasma membranes of adipocytes have been used to induce their destruction and thereby limit the storage capacity for fat. Successful depletion of fat has been achieved in rats, sheep, and pigs in both passive and active immunization.

PASSIVE IMMUNIZATION (ADMINISTRATION OF ANTIBODY). Early studies demonstrated lysis of isolated adipocytes *in vitro* (34), and these antibodies were also effective *in vivo* (37). There were also unexpected effects: appetite, protein deposition, and food conversion efficiency all increased after treatment (82). There were, however, also side effects, including reduced food intake on the day of treatment and transient sedative effects lasting several hours. Activation of the immune system, along with depletion of serum complement, occurs rapidly after treatment and could explain these side effects. Subsequent studies were undertaken in sheep, pigs, chickens, and rabbits. In sheep, passive immunization against adipocyte plasma membranes produced modest effects, reducing both fat content and liveweight gain (78, 80). In rabbits, more dramatic effects were achieved 1 wk after treatment, but there was an ability to replete fat stores 2 mo later (27). The most profound effects have been achieved in pigs, where subcutaneous injections produced total fat depletion at injection sites that lasted for over 3 mo (59). Intraperitoneal injection of antiserum also produced a 30% decrease in back fat thickness, a 25% decrease in fat content of the forelimb, and an increase in muscle mass.

ACTIVE IMMUNIZATION. Active immunization, using adipocyte plasma membranes as an immunogen, to provoke an autoimmune response against adipose tissues has been assessed in rats, sheep, and pigs. Although reductions in adipocyte cell numbers were achieved in rats, the effects were offset by compensatory increases in adipocyte volume, reducing the effect on adipose tissue mass. In sheep, one study failed to produce any significant effects, although a second study produced a decrease in fat and lean tissues. In contrast, in pigs, immunization produced significant decreases in body fat content.

MECHANISM OF ACTION OF ANTI-ADIPOSE ANTIBODIES. An early study (34) demonstrated that antibodies could induce lysis of adipocytes *in vitro* solely in the presence of complement, whereas an *in vivo* study (37) involving cellular infiltration of adipose tissue suggested that cell-mediated responses might be responsible. A study (38) in complement-deficient rats clearly demonstrated that antibodies were completely ineffective in the absence of complement, demonstrating that complement activation is critical.

DEVELOPMENT OF AN ANTI-OBESITY THERAPY. It appears possible that this approach could be considered for clinical use to treat obesity. The antibody, however, would need to be a MAb, probably with absolute specificity for adipocytes. Approval of product licences for the treatment of obesity has arrived with the recognition that obesity is not simply a cosmetic problem but a

considerable, and increasing, health risk to the population in general based on the strong association between obesity, diabetes, and cardiovascular disease. However, it is clear that antiobesity treatments will not be licensed for use if they only achieve weight loss. This must be accompanied by improvements in comorbidity factors such as blood lipid profiles and insulin sensitivity. There are also concerns that the mobilization of triglyceride in obese individuals could produce new problems, particularly in terms of cardiovascular disease and plaque formation.

To address some of these considerations, studies were conducted in the "cafeteria-fed" rat as a model of obesity. After consuming a high-fat diet and increasing their body weight by ~50%, animals were treated with antisera to adipocytes. The response to treatment was a 10% decrease in body weight and a 30–40% reduction in body fat that lasted for at least 3 mo after treatment (33) (Fig. 7). There was also evidence of improvement in a factor associated with obesity, serum leptin, since concentrations were significantly decreased from the elevated levels present in untreated obese rats.

The use of human antibodies in the clinical setting may allow an approach in which they can be used over a prolonged period in a controlled fashion. (This is because repeated use of heterologous antibodies evokes an antibody response resulting in neutralizing of the effect, whereas homologous antibodies will not do so.) This could remove concerns about immediate side effects like those apparent in animals given high doses of antiserum in a "single-shot" strategy.

The search for such human antibodies has been considered via use of a human antibody library (111) in which suitable antibodies can be identified. A panel of over 100 different human single-chain Fv (scFv) antibodies binding human adipocyte plasma membranes have been isolated (31). Three of these scFvs were used in a subsequent study based on their additional cross-reaction with rat adipocytes. Each scFv was reformatted as a rat chimaeric IgG_{2b}, and the ability to induce lysis of rat epididymal adipocytes *in vitro* and the reduction of serum complement levels *in vivo* were determined. Each of these antibodies, both singly and in combination, was able to

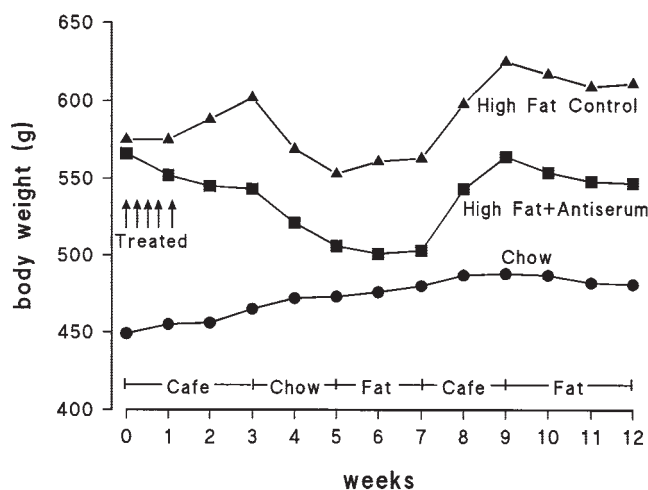


Fig. 7. Changes in body weight after treatment (arrows) with antibodies to adipocytes in rats fed a cafeteria diet (cafe). Diets were switched to a low-fat diet (chow), an intermediate-fat diet (fat), and then back to a high-fat diet (cafe), showing that the loss of weight was maintained independently of the diet. [From Ref. 89a.]

induce adipocyte lysis in vitro, and they were also effective in vivo, where they significantly reduced serum complement levels indicative of adipocyte lysis (25). Taken together, these results suggest that single MAbs could be used therapeutically and that massive adipocyte lysis occurring over several weeks is well tolerated in severely obese rodents, suggesting that at least some of the concerns regarding lipid mobilization during the weight loss period are unfounded.

Conclusions

This article has addressed the concepts of immune modulation of physiological processes in vivo and in vitro and the potential role of antibodies as models to understand physiological functions. Science has developed a sophisticated understanding of the complexities of both the immune system and physiological regulatory systems and how we might study and manipulate their interactions. Despite this, our understanding of these processes and interactions is incomplete.

For some physiological regulatory axes, we now understand some of the underlying mechanisms by which immune modulation may regulate specific processes. Enhancing antibodies against IGF-I clearly increase the available pool of the growth factor and decrease its rate of clearance from the circulation. Factors that contribute to these actions are related to the affinity of the antibodies and the binding site on the growth factor that is recognized by enhancing antibodies.

To develop immune modulation to its full potential, advances in understanding of the immune system and molecular physiological processes are required.

Immune modulation provides scientists with an alternative tool to study physiological processes in vivo. This constitutes an area of research that will provide greater understanding of fundamental physiology in the future; however, basic understanding of the underlying mechanisms must be improved before these benefits may be fully realized.

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