"Magic Bullets:" From Muskets to Smart Bombs!!!

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The Holy Grail of in vivo targeting for the immunologist is the "magic bullets" of Paul Ehrlich (*1*). Although these magic bullets may ricochet (*2*) from time to time and on occasion completely miss their mark, their exquisite specificity, affinity and malleability have ushered in a new era in diagnosis and therapy of various disease processes. In the initial imaging trials with antibodies, whole serum was utilized which was quickly replaced by an IgG-enriched fraction of the serum (*3*). This was improved by the advent of affinity-purified antibodies for immunodiagnosis (*4*). The development of monoclonal antibody technology (*5*) has further accelerated the field of radioimmunoscintigraphy. Concurrent progress in radiolabeling has also contributed to the in vivo success of monoclonal antibodies. Although the application of monoclonal antibodies in tumor imaging enjoys a preeminent position, its application in nononcologic fields has also increased proportionately.

TARGETING WITH ANTIBODIES

The success of antibody-based in vivo targeting of an antigen is directly related to: (1) the capacity of the antibody to bind to the antigen (i.e., specificity and affinity), (2) the residence time of the antibody in the circulation, and (3) the accessibility of the antibody to the antigen (i.e., vascular component).

Specificity and Affinity of Antibodies

Specificity of an antibody is of paramount importance in immunoscintigraphic applications. Absolute specificity, however, for a chosen target may not always be achievable. For instance, antibodies used in tumor imaging are specific for tumor-associated surface antigens, but these tumor-associated antigens are also commonly present in nonmalignant normal tissues. Similarly, utilization of antifibrinogen antibodies for detection of fibrin clots was not entirely successful due to the inability of the antibody to differentiate fibrin in the clot from the ubiquitous fibrinogen in the circulation. Likewise, monoclonal antimyosin antibody used for clinical detection of myocardial necrosis cross-reacts with skeletal muscle myosin. Since skeletal muscle necrosis is not usually expected to be associated with myocardial infarction, myocarditis or heart transplant rejection, a lack of absolute specificity is relatively unimportant. A total lack of specificity, on the other hand, would altogether negate any chances of in vivo targeting utilizing the specific antigen-antibody interaction.

Apart from specificity, the affinity of a chosen antibody may command a more important partnership in radioimmunoscintigraphy. The affinity of an antibody is governed by the law of mass action and hence its capacity to bind the antigen. Assuming that the binding sites of the antibody are uniform and noncooperative in action, the following equation can be deduced:

$$Ag + Ab \underset{k'}{\overset{k}{\leftrightarrow}} AgAb. \qquad \text{Eq. 1}$$

At equilibrium, the rate of formation of AgAb (to the right) ($R_1 = k \times [Ag][Ab]$) is the same as the rate of formation of Ag and Ab (to the left) ($R_2 = k' \cdot [AgAb]$). Therefore,

$$k \times [Ag][Ab] = k' \times [AgAb]. \qquad \text{Eq. 2}$$

Since the *intrinsic association constant* K_a, which represents the affinity of the antibody binding site, is the ratio of the *association constant k* and the *dissociation constant k'* ($K_a = k/k'$), Equation 2 can be written as:

$$K_a = k/k' = [AgAb]/[Ag][Ab], \qquad \text{Eq. 3}$$

that is,

$$[AgAb]/[Ab] = K_a \times [Ag]. \qquad \text{Eq. 4}$$

[AgAb]/[Ab] is in effect the target-to-background ratio (T/B) (*6*). Therefore

$$T/B = [Ag] \times K_a. \qquad \text{Eq. 5}$$

Since target visualization hypothetically depends on the concentration of the antigen and the affinity of the antibody

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(Equation 5) and the concentration of the antigen in a given clinical context is not usually variable, higher affinity of a specific antibody may be the only other determinant that can be manipulated relatively easily to enhance target visualization. Assuming that there are 10^6 tumor-associated antigens per tumor cell (*7*), and there are 10^9 cells per gram of tumor (i.e., ~1 ml) (*8*), there should be 10^{15} antigens per gram of tumor. The number of antigens divided by Avogadro's number provides the mole concentration of the Ag, i.e., $10^{15}/6.023 \times 10^{23} = 1.66 \times 10^{-9}$ moles or 1.66×10^{-6} molar. Therefore, to obtain a target-to-background ratio of 10:1 utilizing Equation 5, the minimal Ka required to provide that target-to-background ratio can be calculated as:

$$10 = 1.66 \times 10^{-6} \times Ka$$

or

$$Ka = 6.024 \times 10^6 \text{ liters/mole.}$$

This situation assumes that all antigenic sites are bound and that there are no conformational antigenic variations on the tumor cells. In practice, these variations have been documented, and if only 1% of the antigen is assumed to be available for binding, a 100-fold higher Ka will be required to provide target-to-background ratios of 10 ($Ka = 6.024 \times 10^8$ liters/mole).

On the other hand, nononcologic immunoscintigraphic applications may have more favorable outcomes, such as imaging of acute myocardial necrosis with radiolabeled antimyosin antibody (*9*). For instance, if we assume that 80% of the myocardial mass is water and that only 30% of the total myocardial mass is myosin, then 1 g of myocardium would have 60 mg or 1.2×10^{-7} moles of myosin per gram of myocardium, or 1.2×10^{-4} molar concentration of myosin. Since there are two homologous antigenic determinants per molecule of myosin (one on each heavy chain of myosin), 2.4×10^{-4} molar concentrations of antigen are available for binding to monoclonal antimyosin antibody. Assuming again that only 1% of this antigen can be bound in vivo, an antimyosin antibody with Ka identical to that of the tumor antibody ($Ka = 6.024 \times 10^8$ liters/mole) will theoretically result in a significantly higher target-to-background ratio.

By utilizing Equation 5

$$T/B = Ka \times [Ag]$$

the following is obtained

$$T/B = (6.024 \times 10^8 \text{ liters/mole}) \times (2.4 \times 10^{-6})$$

or

$$T/B = 1446.$$

The above ratios were calculated assuming that the antibody used in each case is not cleared from the circulation and that there is no limitation to the number of passages of antibody into the target zone. However, in practice, the residence time and accessibility of the antibody to the target tissue are additional factors that will further decrease the target-to-background ratios.

Residence Time of Antibodies

Antibodies are cleared from the circulation in vivo relative to their size and species origin of the antibodies. Intact antibodies of the IgG class of human immunoglobulins have a half-life of about 21 days in man (*10*). $F(ab')_2$ fragments would clear faster and Fab would clear even faster. In canine models, murine IgG has a half-life of about 2–3 days, with 8 hr for $F(ab')_2$ and about 4 hr for Fab (*11*). In humans, the effective half-life of murine monoclonal Fab was approximately 8 hr; with a half-life of 0.8 hr for the fast component and 12 hr for the slow component (*12*). Due to the slow elimination of intact antibody from the circulation, the absolute concentration of the antibody at the target sites is usually the highest relative to $F(ab')_2$ or Fab of the same antibody (*13*). However, the nontarget activity is also significantly higher than those obtained with fragments. Therefore, a balance between absolute antibody target concentration, target-to-background ratios and the longest waiting time that could be tolerated to obtain an optimal image must be considered in the selection of an antibody. For tumor imaging, the waiting time may not be too important, but it could be critical for the detection of acute myocardial infarction. Apart from the residence time of antibodies, the antibody size may also determine the relative immunogenicity of xenoproteins. Intact antibodies and $F(ab')_2$ are more immunogenic than Fab.

Antigenic Accessibility to Antibodies

The concept of accessibility of antibodies to target antigens for successful in vivo targeting is obvious. The major difficulties encountered in tumor detection and therapy largely result from nonuniform expression of target tumor markers, irregular tumor vasculature and aberrant microdiffusion dynamics (*14–17*). Nonuniform disposition of malignant cells within the tumor mass, the variable expression of surface markers on tumor cells and the continuous release of tumor-associated antigens restrict the homogeneous accessibility of specific antibodies (*14,18*). In addition, tumor vascular architecture is comprised of variably perfused regions (*16,19*). Necrotic areas in the tumor mass are relatively avascular and intervascular distances may be large in other areas. Whereas ultrastructurally leaky vessel walls in tumors (*20*) and an enlarged hydrophilic interstitium may theoretically facilitate extravasation of macromolecules (*17*), exaggerated interstitial pressures resulting from compression of infinitely growing tumor (*16*) and the lack of lymphatic drainage (*18*) contribute to the restriction and delay of monoclonal antibody diffusion.

In the cardiovascular system, delivery of antibodies to intravascular targets should permit better access to target antigens as compared to the extravascular antigens encountered in necrotic myocytes in acute myocardial infarction with persistent coronary occlusion. In practice, the reverse appears to occur. In acute myocardial infarction, residual blood flow, collaterals, diffusion and the existence

of a large antigen excess, which acts as an antigenic sink, enhances monoclonal antibody targeting despite the apparent lack of regional myocardial blood flow. A very large antigenic sink drives Equation 1 to the right. On the other hand, the apparent unimpaired access of the antibody into the target region for intravascular thrombi imaging should permit high antibody localization. However, the extent of antigen concentration and the structure of the target (accessibility) as well as the clearance rate of the antibody determine absolute antibody concentration within these targets. With totally occlusive thrombi, the antigen on the thrombus may be less accessible to antibodies than tumor centers. Kanke et al. (*21*) reported an inverse relationship between pulmonary embolus size and the percent injected dose of antifibrin antibody incorporated per gram of clot, which indicates that large thrombi have greater accessibility problems.

IMMUNOSCINTIGRAPHY: FUTURE DIRECTIONS

The future of immunoscintigraphy may depend on the development of smaller antibody fragments or complexes of small fragments of antigen binding regions. These should retain a sufficiently high affinity of the parent antibody. They should also possess optimal circulation time (i.e., long enough for sufficient antigen targeting and short enough to quickly reduce background activity). These fragments with antibody activity, such as Fv, CDR and mimetics, may be genetically engineered (*22–25*). Furthermore, immunogenicity of these ultrasmall antibody fragments should also decrease or eliminate the potential of a HAMA response. Attempts which involve manipulation of antibody characteristics or use of secondary targeting procedures are also being made to decrease nonspecific background activity. Improvements in radiolabeling procedures and the development of instant kit labeling procedures should obviously enhance utilization of antibody-based diagnostic imaging.

High Tech Antibody Production

Since the initial report of fusion of murine spleen cells to murine myeloma cells (*5*), cross-species hybridization such as murine-rat (*26*), murine-human (*27*), as well as human-human (*28,29*) hybridization have been tried. In vivo immunization, the traditional approach to eliciting a specific humoral response to the immunogen, has been expanded to include in vitro immunization techniques. A recent development may permit production of monoclonal antibodies without the need for immunization and mammalian hybrid cells. Lerner and his colleagues (*30*) have successfully developed a method to produce the whole antibody "repertoire" of an animal. The whole repertoire was then expressed in *Escherichia coli*, and specific antibody producing bacterial clones were then selected and expanded to produce the desired monoclonal antibody. This method dubbed the *combinatorial library method* of fabricating monoclonal antibodies utilized recombinant DNA techniques to accrue a complete gene library of human or mouse immunoglobulins. PCR-amplified light- and heavy-chain genes are combined in a viral vector to produce inordinate combinations of light- and heavy-chain genes. The virus containing the vectors can be used to infect bacteria which can then be cultured to produce light and heavy chains of the immunoglobulin genes. As the virus replicates, the host bacteria are killed and form plaques in cultures. The antibodies are released from the dead bacteria. The plaques containing the antibodies can be screened for specific antibody activity and the viral phages possessing the desired antibody specificity can be recovered and amplified to produce theoretically larger quantities of antibodies. An alternative approach is to start with an in vivo immunization of a chosen antigen. This process would increase the number of B-cells producing the desired antibody; they will then contain increased concentration of mRNAs for the light and heavy chains of the antibody. These mRNAs can be amplified and the cDNA of the L and H genes can be incorporated into viral vectors and expanded.

With further refinement and manipulation, it has been possible to produce *Fab abzymes*, which are antibodies with enzymatic activities. Others have taken the molecular biological approach to develop Fv fragments as *single-chain* antibodies. The carboxy terminal region of V_L was ligated to a *peptide spacer* or *linker* which was ligated to the n-terminus of the V_H (*22,23*). The Fv or single-chain antibodies are one-half the size of Fab but possessed identical antigenic specificity and affinity. On the horizon of in vivo antibody utilization are several smaller immunological reagents. Complementarity determining regions (*24*) with sufficiently high affinity and specificity for target antigens may one day supplant Fv, just as Fvs are poised to supplant Fab in cardiovascular disease diagnosis. Further down the line, mimetics (*25*), which are synthetic molecules with engineered binding sites complementary to the CDR region, may one day rule the realm of in vivo targeting. These smaller sized portions of antibodies should be less immunogenic. However, they will also have faster blood clearance and therefore less residence time in the circulation and less absolute target accumulation. The lower residence time should not be a problem in the clinical context of antimyosin scintigraphy since the concentration of myosin in necrotic myocardium is substantial. Furthermore, decreasing the waiting time between intravenous administration and target detection will be highly desirable.

Reduction of Nonspecific-Nontarget Activities

Detectability of a target by radioimmunoscintigraphy (Equation 5) depends not only on absolute antibody localization ([AgAb]) but also on background activity ([Ab]), assuming that the antibody is freely accessible and that the antigen-antibody reaction is in equilibrium at the time of assessment. For an antibody of a given affinity with a constant [Ag] in a particular system, it appears that the only way to increase the target-to-background ratio is to decrease the background ([Ab]). In practice, the background concentration of the antibody ([Ab]) is made up of

free Ab ([Ab_f]) and Ab associated with the nontarget organ by nonspecific interactions ([Ab_{ns}]). Therefore, in our system of imaging acute myocardial infarction, the target is infarcted myocardium and the background is a combination of blood-pool activity and normal myocardial activity. Hepatic and renal activities have not been included into this consideration for simplicity of calculation, and because they empirically would immediately and automatically decrease the administered total antibody concentration, i.e., [AgAb] + [Ab], leaving only the effective antibody concentration. Equation 5 can therefore be rewritten as:

$$[AgAb]/[Ab_f + Ab_{ns}] = T/B = [Ag] \times Ka. \quad \text{Eq. 6}$$

By utilizing Equation 6, one can readily surmise that if the [$Ab_f + Ab_{ns}$] is decreased without affecting the [AgAb], the target-to-background ratio would be increased. The [Ag] on the right hand side of the equation cannot be increased, and the affinity (K_a) of the antibody cannot be increased without molecular-biotechnological intervention. Therefore, we have attempted to minimize nonspecific-nontarget uptake of the antibody ([Ab_{ns}]) by manipulating the antibody's characteristics. Although the reduction of [Ab_{ns}] cannot directly affect the Ka of the antibody, we have proposed that the practical affinity (K_p) of that antibody can be manipulated. Although [AgAb] may concomitantly contain specific and nonspecific AgAb interactions, [$AgAb_{ns}$] is negligible relative to specific [AgAb]. Therefore, the [$AgAb_{ns}$] factor can be ignored. Thus, [AgAb] = T can be considered to be constant for a particular antibody-target system, and [Ag] is also constant. Therefore, Equation 6 could be rewritten as the following when monoclonal antibodies are considered for radioimmunoscintigraphy:

$$[AgAb]/[Ab_f + Ab_{ns}] = T/B = [Ag] \times K_p,$$

where K_p is inversely proportional to [Ab_{ns}]. When [Ab_f] approaches zero due to blood clearance, the role of [Ab_{ns}] in the determination of target-to-background ratios becomes increasingly important. Therefore, if [Ab_{ns}] can be decreased, target-to-background can be increased.

We have recently reported that imparting a high negative charge to antibodies can reduce nonspecific uptake of antibody by nontarget tissue (*31*). This hypothesis was based on the premise that the positive charge inherent in the basic antibody molecule may contribute to ionic interaction with acidic zeta potentials of the reticuloendothelial system macrophages and promote higher nontarget organ activities. Furthermore, the interaction between the positive charge of the antibody and the negative charge of cell surface residues and heparin sulfate proteoglycans of the extracellular matrix may provide high nonspecific background activity in the normal (nontarget) tissues. This hypothesis also assumes that the affinity of the antibody subjected to charge modification is not altered. Accordingly, antimyosin Fab (AM_n-Fab) was modified with a synthetic polymer (DTPA-PL), which provided two distinct advantages. First, it imparted a high negative charge to the antibody which resulted in lower background and nontarget organ activities. Second, the use of the synthetic polymer provided a means for coupling a large number of DTPA ions which in turn enabled chelation of a large number of ^{111}In ions to provide preparations of high specific radioactivity (40–50 moles of ^{111}In/mole AM-Fab). Because of the high specific activity of the radiolabeled modified AM-Fab and the decrease in nonspecific radiation exposure to a limiting nontarget organ such as the kidneys, the radioactivity dosage may be increased to provide better image characteristics, such as higher photon flux at the target sites.

Another approach which can enhance target-to-background ratios is by increasing the clearance rate of the blood pool activity of the radiolabeled antibody. However, since [Ab_f] is dependent on the molecular size of the Ab, any attempt to decrease Ab_f by reducing the size of the Ab (i.e., increase blood clearance) would also affect the [AgAb]. This limitation can be overcome by the use of bispecific antibodies. Bispecific antibodies can be made chemically or by hybrid hybridization. These antibodies demonstrate antibody specificity towards the chosen antigen as well as a chelating agent (*32,33*). The nonradiolabeled antibody complex is injected in vivo to permit target antigen binding followed by a period of waiting for clearance of the bispecific antibody from the circulation. A radiometal-chelate complex that is recognized by the other half of the bispecific antibody can then be administered. By utilizing Equation 6, ([AgAb]/[$Ab_f + Ab_{ns}$] = T/B = [Ag] × Ka), such an approach should provide an [Ab_f] of almost zero due to the blood clearance prior to tracer administration. Since the radiotracer-chelate complex is expected to be cleared from the circulation quickly and no antibody exists in the systemic circulation, Ka should be inversely proportional to [Ab_{ns}]. In this situation, [Ab_{ns}] is not affected and will depend on the nonspecific interaction of chimeric compounds with nontarget organ tissues. This approach should enable the use of a radiotracer with a short half-life since it is no longer necessary to match the half-life of the radiotracer to that of the antibody.

The use of a combination of biotinylated antibodies, avidin and biotinylated-radiotracers has also achieved the desired improvement in in vivo visualization of target organs (*34,35*).

Antibodies as a Role Model for Newer Imaging Agents

Although the use of antibodies has not reached the technological wizardry of smart bombs, they have nonetheless introduced a new dimension to noninvasive scintigraphic diagnosis of various diseases. They have also helped in the understanding of the pathophysiological basis of various disorders. It is worthwhile to note that affinity targeting spearheaded by antibodies should generate other radioaffinity targeting reagents such as neurotransmitters, cytokines, growth factors and adhesion molecules. The wealth of information gathered during the 50 yr of in vivo immu-

notargeting has laid the foundation for the development of high tech affinity targeting reagents for the 21st century.

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