MODERN METHODS IN PHARMACOLOGY

Volume I

Editors

Sydney Spector

Nathan Back

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Sydney Spector

Roche Institute of Molecular Biology
Nutley, New Jersey

Nathan Back

State University of New York
Buffalo, New York

Alan R. Liss, Inc., New York

Address all inquiries to the Publisher
Alan R. Liss, Inc., 150 Fifth Avenue, New York, NY 10011

Printed in the United States of America.

ISSN 0732-7218 • ISBN 0-8451-2500-I

Preface

Advances in the field of pharmacology, as is true for all scientific disciplines, are very much dependent on the development and introduction of new methods.

A citation list of instruments used in pharmacologic studies would contain enormous numbers of citations for such routinely used instruments as the spectrophotometer, spectrophotofluorometer, or the beta or gamma counter. As advances are made in instrumentation, investigators generate procedures to study biological phenomena utilizing these often more sophisticated and sensitive new instruments.

Pharmacologists have come a long way from the not too-distant "smoked drum" era, although we do not minimize the contributions made by investigators who have used or still use the "smoked drum" technique or modifications thereof. However, within the past few years the introduction and application of new methods have become increasingly apparent and have provided compelling reasons to assemble information about new biochemical pharmacologic techniques and their application in one reference source. We also felt that a book series is the best and most convenient format for disseminating this current information and that such a series publication would provide the stimulus for future books on the subject.

As editors we express our sincere appreciation to those authors whose contributions appear in the series' first volume for their efforts, and for sharing our belief in the timeliness of such a publication.

Sydney Spector

Nathan Back

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Antigen-Defined Immunocytochemistry

LARS-INGE LARSSON

BACKGROUND

Immunocytochemical techniques are able to localize a wide variety of molecules with a high degree of precision at both the cellular and subcellular level. Biologically important molecules thus localized have varied in size from biogenic monoamines to large proteins, polysaccharides, and nucleic acids. Secretory molecules have been localized not only at their sites of synthesis and storage, but also at their sites of action—an accomplishment made possible through novel immunocytochemical modifications resulting in improved sensitivity, specificity and precision [1]. No doubt, these techniques will be exploited in the future for the tracing of destinations and intracellular fate, not only of endogenous molecules, but also of exogenously administered agents—the fate of which at present can only be partially followed by autoradiographic methods, which are not always able to differentiate between intact and degraded forms.

Technically, immunocytochemical techniques are easy to master. Unfortunately, this has led to more emphasis on what could be labeled "stain technology" than on an understanding of the chemical-immunological background. If this background is ignored immunocytochemical techniques are of no more avail than standard histological techniques when it comes to understanding the localization, processing, transport, and fate of defined molecules.

The first and most important problem in immunocytochemistry concerns specificity. How can immunocytochemical specificity be defined and to what extent can the staining obtained be assumed to represent a single type of molecule? Since the size of an antigen-combining site on an antibody can be determined rather precisely and since numerous studies on the interactions of antibodies with polyamino acids have been carried out, we can state fairly confidently that the size of a region of a protein reacting with an antibody (the antigenic site) averages three to eight amino acids. If the amino acids constituting the antigenic site are arranged in a way unique to the protein of which they

form a part and if the antibody recognizes only this unique arrangement, it can be assumed to be monospecific for that particular protein. This is, however, rarely the case. Thus, proteins are often synthesized in the form of larger molecules which, posttranslationally, are cleaved to smaller fragments, and, therefore, antibodies may not be able to differentiate between precursor and product forms. Moreover, microheterogeneity of proteins, involving genetically determined heterogeneity, may occur. Most important, however, is the fact that the sequences and structures of only a minority of proteins have been determined. Since proteins and peptides often show sequence homologies, we may never be able to unequivocally state that a given antibody is monospecific for a certain protein at the immunocytochemical level.

Some of these points are illustrated in Figure 1, showing a hypothetical protein, reacting with different types of antibodies. As shown by Atassi and co-workers [2–4], an antigenic site may be either continuous or discontinuous, ie, built up by a continuous sequence of amino acids or of amino acid sequences brought close together for conformational reasons. Different amino acids of the antigenic site may be of varying importance for antibody binding. Thus, as shown in Figure 1, cross-reactivity may be due either to the occurrence of the entire antigenic site as part of another protein, or may be due to the presence of an amino acid sequence resembling, but not being fully identical with the antigenic site.

In the absence of other supportive evidence, localization results obtained with a single species of antibody are therefore always open to question.

As also indicated in Figure 1, a protein or peptide may possess multiple antigenic sites. In large proteins with an ordered structure such sites usually form part of the exposed regions of the molecule, whereas in proteins and peptides with a random coil conformation, theoretically all parts of the molecule could elicit an antibody response. Even in the latter case, however, certain regions seem to be more immunogenic than others. It has, however, proven possible to raise antibodies to also poorly immunogenic regions of a molecule, eg by immunizing with synthetic fragments.

Antibodies recognizing different regions of the peptide or protein to be studied constitute valuable tools for increasing immunocytochemical specificity [5,6]. Thus, combined use of such antibodies will allow immunological mapping of a larger portion of the molecule investigated. The probability that cross-reacting molecules should possess multiple antigenic sites in common with the appropriate antigen diminishes with the number of antigenic sites that can be mapped. Region-specific immunocytochemistry has by now been applied to many antigens [7–12]. The principles are illustrated in Table I, using the hypothetical protein of Figure 1. As shown in Table I, certain cell types (y and y) react with either one of the two region-specific antibodies A and B, but only one cell type (x) reacts with both region-specific antibodies. From these

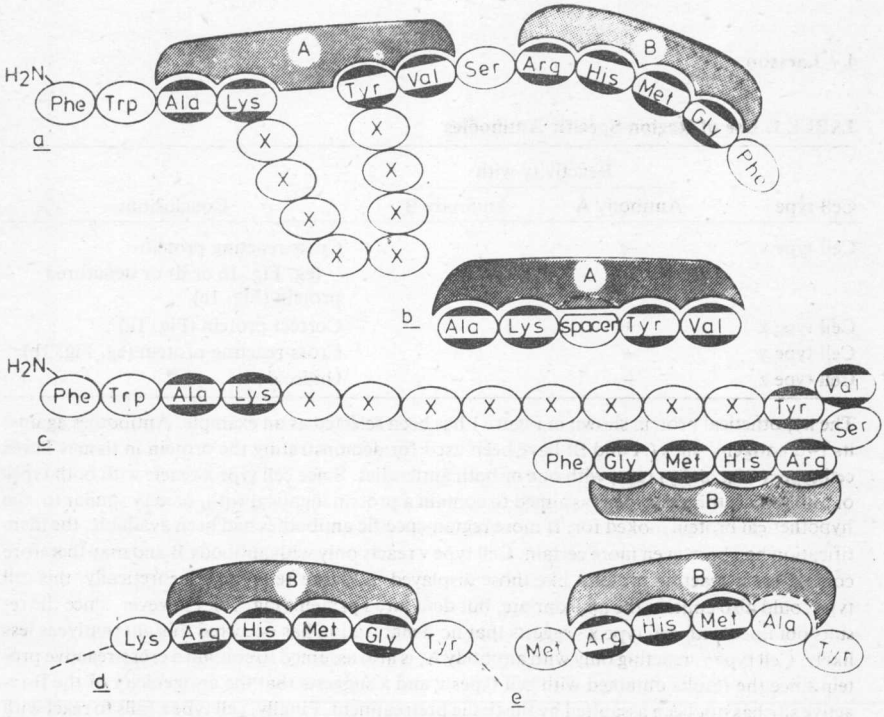

Fig. 1. Antigenic determinants in peptides and proteins. In a, two antigenic sites of a hypothetical protein are illustrated. Site A, reacting with antibody A (shaded area), is discontinuous and, hence, dependent upon the conformation of the protein. The site B, reacting with antibody B, is continuous. In b, a synthetic peptide mimicking the discontinuous antigenic site A is shown. As demonstrated by Atassi and coworkers [2–4], surface-simulation synthetic may provide such artificial peptides, that may cross-react with antibodies made against the native antigenic site. Note the presence of a spacer function (e.g., a neutral amino acid) to give the right distance between the two components of the discontinuous antigenic site. Although not an integral part of the true antigenic site, such spacers have a decisive impact on the antigen-antibody reaction. This is also true for natural antigenic sites where, for example, the size, charge, or hydrophobicity of amino acids surrounding an antigenic site may be very important for antibody binding. In c, the protein shown in a has been denatured. Note that the two components of the discontinuous site (A) are now spaced apart and unable to react with antibody A. In contrast, the antigenic site B has been unaffected by this denaturation and is still able to bind antibody B. Thus, denaturation may affect different antigenic sites in the same protein to various degrees. If the denaturation involves chemical cross-linking by a fixative, continuous antigenic sites containing reactive amino acid residues may be adversely affected, whereas sites containing no such residues may be unaffected. Discontinuous sites may be affected either through direct chemical attack or through conformational changes. It is, hence, futile to argue that a certain fixative invariably preserves (or destroys) antigenicity of a particular protein or peptide, since this will depend upon the region-specificity of the antibodies employed. In d and e, two types of protein sequences, potentially cross-reactive with antibody B are shown. In d, the entire sequence of antigenic site B exists as an integral part of another protein. In e, three of the four amino acids are identical to antigenic site B, whereas the fourth is distinct. Both d and e may or may not cross-react with antibody B, depending upon the type of amino acid substitution in e and upon the influences of adjacent amino acid residues in both.

TABLE I. Use of Region-Specific Antibodies

Cell type	Reactivity with Antibody A	Reactivity with Antibody B	Conclusions
Cell type v	–	+	Cross-reacting protein (eg, Fig. 1e or d) or denatured protein (Fig. 1c)
Cell type x	+	+	Correct protein (Fig. 1a)
Cell type y	+	–	Cross-reacting protein (eg, Fig. 1b)
Cell type z	–	–	Undecided

The hypothetical protein shown in Figure 1 has been selected as an example. Antibodies against its two antigenic sites (A and B) have been used for demonstrating the protein in tissue. Three cell types (v, x, and y) react with one or both antibodies. Since cell type x reacts with both types of antibodies, this cell type is assumed to contain a protein identical with, or very similar to, the hypothetical protein looked for. If more region-specific antibodies had been available, the identification had been even more certain. Cell type v reacts only with antibody B and may therefore contain cross-reacting proteins like those displayed in Figure 1e and d. Theoretically, this cell type could also contain the appropriate, but denatured protein (Fig. 1c). However, since the results obtained with cell type x suggests that no denaturation has occurred this alternative is less likely. Cell type y, reacting only with antibody A, is also assumed to contain a cross-reactive protein since the results obtained with cell types v and x suggests that the antigenicity of the B-reactive site has not been assaulted by the tissue pretreatment. Finally, cell type z fails to react with either antibody A or B. This is not enough to conclude that this cell type may contain low concentrations of the protein searched for. All we can decide is that cell type x contains larger amounts of this protein, whereas cell types v, y and z may contain amounts of the protein searched for in amounts too low to be detected by the immunocytochemical procedure used. Thus, great care must be exerted both when positive and negative results are interpreted. Since different immunocytochemical procedures may differ in sensitivity by several orders of magnitude great caution is recommended.

results it is reasonable to assume that only cell type x contains the protein searched for, whereas cell types v and y contain other, but immunologically related, proteins. With three or more region-specific antibodies, the identification of the protein would be even more certain. Such problems are met with frequently in everyday immunocytochemistry. Thus, as an example, the two cybernins gastrin and cholecystokinin (CCK) contain identical COOH-terminal antigenic sites, whereas the rest of the two molecules differ (Fig. 2). Antibodies directed to these molecules will react with either one or both, depending upon their region-specificity. Table II summarizes some of our findings with different region-specific gastrin and CCK antibodies. Note in this table that if only Abs. 4562 or 2609 (which were raised against gastrin) had been used, TG cells, CCK cells, and caerulein cells would have been interpreted as gastrin cells. This serves to illustrate that with increasing numbers of defined region-specific antisera, immunocytochemical specificity increases.

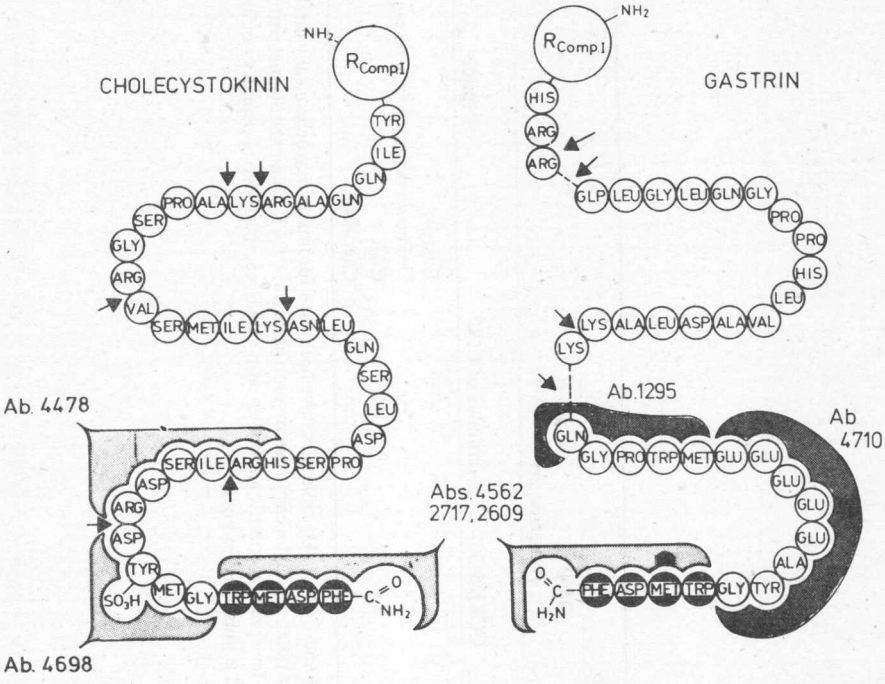

Fig. 2. Amino acid sequences of cholecystokinin (CCK) and gastrin and region-specificity of different antibodies to these peptides (cf. Table II). Note the presence of a common COOH-terminal pentapeptide sequence in both gastrin and CCK. The main form of gastrin (gastrin-17) corresponds to the 17 COOH-terminal residues shown in the gastrin sequence, whereas CCK-33 constitutes the 33 COOH-terminal amino acids of the CCK sequence.

Use of such antisera may also result in the discovery of new and potentially important molecules, like the novel peptide occurring in the TG cells [8]. Since this molecule apparently contains a C-terminus identical with, or strongly resembling, that of gastrin and CCK and since it occurs in an endocrine cell type, future studies of it are worthwhile, not least in view of the fact that the COOH-terminal part of gastrin and CCK determines the biological activity of these two cybernins.

The advantages of region-specific immunocytochemistry are many and obvious. Much effort must, however, be spent with the production and evaluation of such tools. Thus, antisera are mixed in the sense that they contain many different immunoglobulins directed not only against the antigenic region desired, but also against other regions of the immunogen, against contaminants of the immunogen, autoantibodies, etc. Since region-specific immunocytochemistry requires pure detection reagents of determined specif-

TABLE II. Use of Region-Specific Antibodies

	Antibody no.					
	4562, 2609	4710	1295	4698	4478	
	Antigenic site					
Cell type	G[14–17] = =CCK[30–33]	G[6–13]	N-terminus of G	CCK[25–30]	N-terminus of CCK	Peptide
G cells	+	+	+	–	–	Gastrin
TG cells	+	–	–	–	–	Unknown peptide yet to be sequenced
I cells	+	–	–	+	+	CCK
Caerulein cells	+	–	–	+	–	Caerulein

A set of antibodies reacting with different regions of the gastrin [1–17] and CCK [1–33] molecules have been used for immunocytochemically differentiating between gastrin cells, CCK cells, TG cells and cells producing caerulein (a potential phylogenetic precursor to gastrin and CCK). Note the distinct pattern of antibody reactivity for each of the peptides searched for. Note also that had fewer region-specific antibodies been employed, the different peptides would have been confused with each other. The region-specificity of the antibodies used is illustrated in Figure 2.

icity, purification of antisera is necessary. If the contaminating antibody populations are of known specificity, they can easily be removed through immunoabsorption against their corresponding antigens, coupled to a solid phase, like cyanogen bromide-activated Sepharose 4B beads. Most often, however, only the nature of the wanted antibodies is known. Purification of these may be accomplished through immune affinity chromatography, utilizing the desired (synthetic) antigenic site coupled to a solid support as an immunosorbent [13,14]. However, elution of the desired antibodies, which have bound to the immunosorbent, poses problems. Thus, antibodies of high avidity (high binding energy) are difficult or impossible to elute under non-denaturing conditions and many immune affinity purified antisera are, therefore, relatively enriched in antibodies of low to medium avidity, often with a concomitant fall in titer [1]. Since high avidity antibodies are the ones least prone to dislocation or removal during immunocytochemical procedures, and, frequently, of the highest specificity, they are often the ones most desirable to use. For this reason immune affinity chromatography, although quite helpful, does not represent the ultimate answer to immunocytochemical purification problems and has the added disadvantage of consuming quite large amounts of pure antigens.

A very attractive alternative is provided by the recent development of monoclonal antibodies [15,16]. In this technique single antibody-producing cells are fused to mouse myeloma cells. Clones of such hydridoma cells, producing the wanted antibody population, are selected and, when injected into the peritoneal cavity of a mouse, produce large amounts of ascites fluid, rich in monoclonal antibodies. Accordingly, such antibodies will stem from a single clone of immunoglobulin-producing cells.

Since current immunocytochemical techniques are almost exclusively directed against detection of guinea pig or rabbit antibodies by indirect labeled or unlabeled procedures, routine use of monoclonal antibodies, which almost exclusively are of mouse origin, requires development of new and sensitive reagents for their visualization. Incidentally, the labeled antigen detection techniques, to be described below, are, in contrast to other immunocytochemical techniques, independent of the species producing the primary antibodies, and may therefore advantageously be used in conjunction with monoclonal antibodies. A second problem is that the technology for producing monoclonal antibodies is quite advanced and available only to laboratories equipped with cell culture techniques and large animal facilities. Already by now, however, commercial firms have made their way into market with some 20–30 different monoclonal antisera. An explosive development in this area can probably be foreseen.

Recently, two new methods have been developed that possess the ability to

select only specific high-avidity antibodies from mixed antisera. Both techniques depend on the fact that IgG molecules possess two equivalent antigen-combining sites. Thus, it is possible to let one of these two sites react with a labeled antigen molecule, leaving the remaining site free to react with tissue-bound antigen [12,17]. The site of reaction between the antibody and tissue is marked by the labeled antigen molecule. The labeling of the antigen may be carried out with either radioactivity, enzymes (like horseradish peroxidase), fluorescence (like fluoresein isothiocyanate or fluorescamine), or with particles (like iron-dextrans, ferritin, or colloidal gold). In the two so far developed, generally applicable, labeled antigen detection methods—the RICH (radioimmunocytochemical) [17] and the GLAD (gold-labeled antigen detection) [12] techniques—antigens have been labeled with either radioiodine (RICH) or colloidal gold (GLAD). The labeled antigen detection methods are highly sensitive and can advantageously be used to select specific antibodies from mixed antisera, allow use of optimally fixed and contrasted specimens, and are the only ones documented to allow simultaneous detection of multiple antigens at the electron microscopical level.

In the following, we will describe these two techniques in more detail. Before we commence, a note of caution should be inserted, however. Many immunocytochemists may feel that once pure antibodies have been obtained, either by monoclonal antibody techniques or selected by the RICH or GLAD techniques, such antibodies should be monospecific. From what has been said above, however, monospecificity of an antibody can never be taken for granted. Thus, any pure antibody is liable to cross-react with other proteins or peptides containing identical or similar antigenic sites. In the test-tube or by immunodiffusion techniques the presence of such cross-reacting molecules can sometimes be excluded. Such data are, however, not universally applicable to the situation in tissue sections, where uncertainty remains whether all molecules capable of reacting with antibodies can be extracted (solubilized). Therefore, when either monoclonal antibodies and/or the labeled antigen detection techniques are used, it is essential to keep in mind that only in combination with region-specific immunocytochemistry can true localization of the intended antigen be approached.

The futility of uncritically applying data from test-tube experiments to tissue sections is also illustrated by the unfortunate and frequent practice of citing radioimmunoassay data as proofs for immunocytochemical specificity. Thus, some papers state that antisera, which in radioimmunoassays show low or unmeasurable cross-reactivity with certain peptides, will not cross-react with these peptides at the immunocytochemical level either. This is entirely unjustified. Thus, in radioimmunoassays, the degree to which an antibody reacts with a labeled (radioiodinated) antigen (tracer) is measured. Other antibodies, which may well be present, but are unable to bind the tracer, are, hence, never detected. Such antibodies may, however, well be detected, when

conventional labeled or unlabeled immunocytochemical methods, which detect all antibodies that bind to tissue sections, are employed. In contrast, the labeled antigen detection techniques employ tracers similar to those used in radioimmunoassays and we have found that specificity evaluation of antisera by radioimmunoassays and by labeled antigen detection techniques corresponds rather closely, whereas the situation with conventional immunocytochemical techniques is different. Thus, our antigastrin antibody 2604 is fully specific for gastrin in a radioimmunoassay system, employing an ^{125}I-labeled synthetic human gastrin I tracer, and, hence, shows negligible cross-reactivity with the related hormone, cholecystokinin (CCK). At the immunocytochemical level, however, the same antibody stains both gastrin and CCK cells if indirect immunofluorescence or the peroxidase-antiperoxidase (PAP) technique is employed. Immunocytochemical absorption of Ab. 2604 with either gastrin or CCK abolishes staining of both gastrin and CCK cells. However, when radioimmunocytochemical (RICH) complexes are made with this antibody using ^{125}I-synthetic human gastrin I, the complexes will react only with gastrin cells and not with CCK cells [18]. We interpret these observations to mean that when limiting amounts of labeled antigens are added to an antibody, these will be bound by antibodies having the highest binding energy (the closest "fit")—these antibodies will therefore be the ones most likely to react with their corresponding antigen (gastrin) in tissue sections, also with the highest binding energy. Thus, at least in this case, the specificity of the RICH method resembles the specificity of radioimmunoassay techniques much more closely than it resembles the specificity of conventional immunocytochemical techniques.

Recent data also indicate that the specificity of pure antibodies may vary with the pH of the reaction. Thus, in a study of monoclonal antibodies directed against chicken erythrocytes, Mosmann et al. [19] found that the specificity of the same antibody varied with pH. Thus, pH could change the specificity of a monoclonal antibody from a unique antigenic site to a cross-reacting site. With some antibodies this was brought about by an increase in pH, with others by a decrease [19]. The specificity also seemed to be affected by the temperature (4°C or 37°C) of the reaction.

Since the antibodies used were monoclonal [19] the changes in pH must influence the binding properties of the antigenic sites. At certain pH values a cross-reacting site may bind the antibodies more strongly than at other pH values [19]. It is possible that observations made in radioimmunoassay systems of variable pH optima for different combinations of antigens and antibodies may have a similar background. These important observations indicate that for comparisons between immunological systems to be made conditions should be identical. The possibility of changing the apparent specificity of antibodies in immunocytochemistry by testing at different pH values seems well worth exploring.

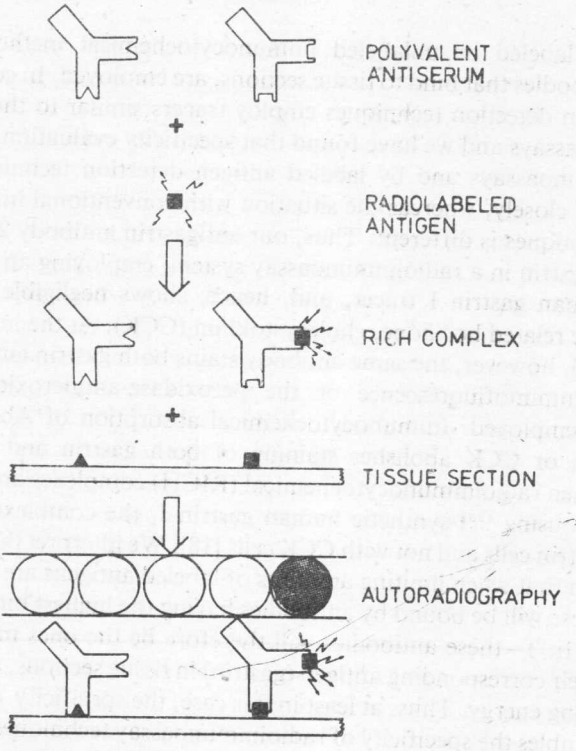

Fig. 3. Schematic drawing illustrating the principle of the radioimmunocytochemical (RICH) technique. Note that antibodies unable to bind pure, radiolabeled tracer may well react with tissue, but will not be autoradiographically detected.

THE RADIOIMMUNOCYTOCHEMICAL (RICH) TECHNIQUE

Principle

Antibodies of the IgG class possess two equivalent antigen-combining sites. When mixed with radioiodinated antigen in the zone of antibody surplus, only one of the two combining sites per antibody will bind radioiodinated antigen, leaving the remaining site free to react with tissue-bound antigen [17] (Fig. 3). Titration experiments, using a fixed amount of antibody added to varying amounts of radioiodinated antigen (or vice versa), allow determination of the concentrations at which a preponderance of antibodies, binding only one antigen molecule per antibody (RICH complexes), occur. Such RICH complexes are then applied to tissue sections, where their remaining, free, antigen-combining sites will combine with tissue-bound antigen (Fig. 3). The site of

reaction is revealed by autoradiography [17]. Since unspecific antibodies will not be able to bind specific, radioiodinated antigen, they may well bind to tissue sections, but will not be autoradiographically detected. Unspecific absorption of RICH complexes to tissue is prevented by pretreatment of the sections with high concentrations of serum from the same species as that donating the primary antiserum.

Methodology

Requirements. The technique requires access to facilities for γ-counting, radioiodination of antigens and autoradiography.

Radioiodination. Peptides are iodinated using carrier-free $Na^{125}I$ (NEN or Amersham) and chloramine T, lactoperoxidase or iodogen, according to techniques established for preparation of tracers for radioimmunoassay work. Since different peptides are variously sensitive to iodination and differ in their requirements for purification after iodination, the reader should consult the radioimmunoassay literature for details on the particular peptide or protein intended for investigation (a good general introduction is given in references 20 and 21).

Following iodination, free iodine and other low molecular weight reactants may be removed by gel filtration or by absorption of the peptide onto leached silica glass or cellulose (when applicable) [20,21]. It is desirable to obtain monoiodinated tracers (ie, one ^{125}I molecule per antigen molecule) and high specific activities. This can be achieved by further purifying the tracer, separating "cold" antigen and diiodinated antigen from the desired monoiodinated antigen. Such purification, which is desirable, but not always necessary, is usually achieved by ion exchange chromatography or polyacrylamide gel electrophoresis [20–23]. Only in the case of very small peptides can the added extra mass of 125 daltons be expected to allow purification of monoiodinated tracers by size separation (like gel filtration).

Both the purification and subsequent storage of the tracer depends upon the properties of the protein or peptide studied. Many tracers are unstable and have a useful shelf-life of only 2–4 weeks. Moreover, the halflife of ^{125}I is 60 days. These facts should be taken into account when experiments are planned.

After iodination and purification, the quality of the tracer must be checked. One useful control is that a surplus of the antibody should be able to bind all or nearly all ($> 90\%$) of the tracer. If this is not the case, the peptide may either have been damaged during iodination or may be of low specific activity, so that the added amount of radioactive tracer is contaminated by large amounts of "cold" (unlabeled) molecules. In either case the situation should be rectified by improved purification of the tracer and/or modification of the iodination procedure. Of paramount importance for the specificity and success of subse-

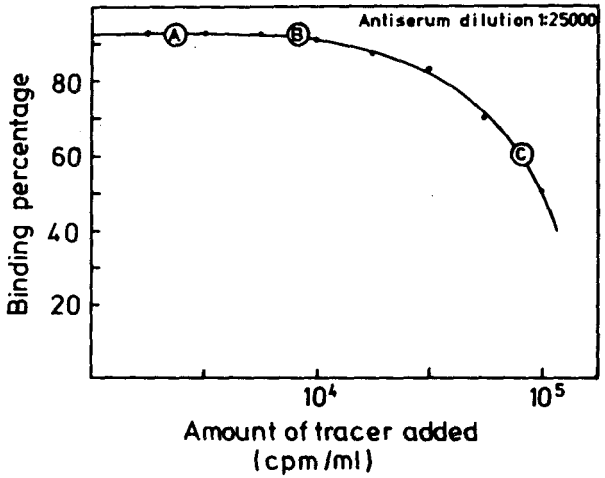

Fig. 4. Graph used for deciding optimal concentrations of tracers and antibodies for preparing immunocytochemically useful RICH complexes. In this example, a constant antibody concentration is titrated against variable concentrations of radiolabeled antigen (tracer).

quent manipulations is that the molecules used for iodination are pure (preferably synthetic).

Preparation of RICH complexes. We usually prefer to test one or two dilutions of the antibody (eg, 1:5000 and 1:50000) against varying amounts of the tracer (expressed in counts per minute, cpm). Alternatively, a fixed amount of radioactive tracer may be added to various concentrations of antibody. In either case, a set of tubes containing antibody and tracer are incubated for 24–48 hours at 4°C. Dilutions of tracers and antibodies are conveniently made in conventional immunocytochemical buffers (eg, 0.05 M sodium phosphate buffer pH 7.5, containing 0.15 M NaCl (PBS) and 0.1–0.5% bovine serum albumin). The pH optima for most antigen-antibody reactions are between 7–8.5. Albumin is added to prevent nonspecific absorption of tracer (and antibodies) by glassware and sections. Following incubation, free and antibody-bound tracer are separated by any of the many systems available (dextran-coated charcoal, ethanol precipitation, second antibody precipitation, polyethylene glycol, resin absorption; cf. refs. 20–23), and the supernatant and precipitate are counted in a γ-counter. Tubes containing tracer, but no antibody, should be included for determining the efficiency of the system separating bound and free tracer. From these values the percentage bound versus free tracer is calculated and a graph constructed (Figs. 4 and 5) [17,20]. As mentioned in the introduction the graph can either represent binding percentage (B%) on the y-axis and varying amounts of tracer added