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The Use of Antibodies to Study Cell Structure and Metabolism

FLOYD E. BLOOM, H. JAMES WEDNER* AND CHARLES W. PARKER*

*Laboratory of Neuropharmacology, Division of Special Mental Health Research, National Institute of Mental Health, St. Elizabeth's Hospital, Washington, D.C. and *Division of Immunology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri*

Introduction

HISTOCHEMISTRY seeks to analyze the chemical composition of cells by developing methods to localize cellular constituents *in situ* within tissue sections. The ideal reagent for this purpose would be one which combined a high degree of molecular specificity for the component being sought with an equally high degree of sensitivity. Antigen-antibody reactions exhibit the extreme degree of molecular specificity desired for histochemistry and such reactions have been exploited for this purpose for the 30 years which have elapsed since Coons *et al.* (26) demonstrated that antibody could be coupled with a fluorescent marker with full retention of the antibody affinity for antigen. Immunocytochemistry has thus far found its most intense application in the realm of cytopathology, exploring the presence of abnormal cellular constituents such as virus and bacterial antigens and abnormal proteins (see 9, 57). In this presentation we have reviewed briefly the concepts of immunocytochemistry which allow this technique to be applied critically to the analysis of normal cell structure and metabolism. Four classes of molecules have been successfully localized by this methodology: enzymatic intracellular macromolecules, nonenzymatic intracellular macromolecules (either structural proteins, secretory products, or other characteristic proteins of unknown function), cell surface macromolecules and receptors, and small intracellular synthetic products, such as the cyclic nucleotides. The latter subtopic will be covered most extensively, as it is the area in which we have most concentrated our efforts in immunocytochemistry. Interpretation of the technical requirements of small molecule localization can best be appreciated by a critical appraisal of the methods which have been applied for the localization of macromolecules with antibody reagents.

Applying Immunocytochemistry

Localization of Tissue Antigens. Several textbooks and an extensive review of the field of immunocytochemical staining (9, 57) make an exhaustive review of the principles unnecessary. With respect to the cytological analysis of cell structural and metabolic components, an antigen to be localized must either be capable of synthesis *de novo* or isolatable in sufficient purity to generate single bands by standard techniques of protein separation. Small molecules can be used as antigens by synthesis of hapten onto larger carrier molecules (see below). Two localization methods have become standard. In the "direct" method the antibody is labeled with a marker substance visible by light, fluorescence or electron microscopy. In the more generally applied indirect method the primary antibody to the antigen to be localized is not labeled, but is itself located through a second antibody bearing the label (*e.g.*, labeled goat antirabbit antitubulin). Recently, numerous other variations of these techniques have appeared. Wachsmuth and Lachmann (111) prepared antigen-antibody complexes with antibody excess, and separated the precipitin bands from an agar gel immunoelectrophoretogram. The partially purified and reacted

immunoglobulin complex retained vacant antigen binding sites which were then used to detect antigen (antidiuretic hormone, aminopeptidase, and antinuclear globulin) in tissue sections. The antigen-antibody complexes were then localized indirectly with a labeled antiglobulin antibody. A different sort of intermediate labeling was devised by Mason *et al.* (68) who made use of the antigenicity of the marker protein, horse radish peroxidase. They raised antibody to peroxidase in the same species used to raise antibody to the tissue antigen they wished to localize. An anti-immunoglobulin was then prepared in a second species to act as a bridge between the immunoglobulin against the tissue antigen and the homologous species antibody against the marker. Thus, antibodies to peroxidase and to growth hormone or chorionic gonadotropin were both raised in different rabbits. The antihormone immunoglobulin was applied to the tissue and this was reacted with unlabeled goat or sheep antirabbit globulin. On this set of molecules an additional exposure was made to rabbit antiperoxidase which was bound to the antirabbit globulin already attached to the section and finally the peroxidase was added to be bound by the final immunoglobulin. This sandwich of immunoglobulins, topped by the marker enzyme, was then localized by the histochemical reaction for peroxidase (42). Although complicated, the advantages accrue from the fact that it is not necessary to conjugate the marker to the antibody, possibly disturbing its specificity.

Sternberger *et al.* (103) have reported a similar localization method employing unlabeled antibodies in which the marking antibody (the third step reaction in the Mason procedure described above) was already reacted with peroxidase to form a soluble pentamer containing three peroxidase molecules and two antiperoxidase immunoglobulins. Sensitivity and specificity were said to have been increased approximately 2 to 3 orders of magnitude over routine immunofluorescence detection of tissue spirochete antigen.

Haemmerling *et al.* (45) described two additional modifications to improve the resolution of markers used for electronmicroscopic immunocytochemistry and to avoid the loss of antibody activity which can accompany direct immunoglobulin-marker conjugations. They prepared hybrid antibodies from the rabbit in which γ-globulin fragments specific for either the tissue antigen or for the antigen to be used as a marker were combined. In this way each immunoglobulin molecule reacting with the tissue antigen (in this case a surface alloantigen H-2^b) could also be reacted with a marker molecule. In addition to the use of ferritin as an ultrastructural label, Haemmerling *et al.* (45) demonstrated the usefulness of small plant virus particles such as southern bean mosaic virus as markers, since (a) these molecules are directly visualizable in the electron beam, (b) they are of an appropriate size and are strongly antigenic, and (c) they do appear to bind generally to cell surfaces. The virus particles thus represent an alternative ultrastructural marker to ferritin. Improved detection of ferritin can be achieved by staining with bismuth (1) or could be replaced with a radioactive immunoglobulin prepared by reaction with ^{3}H-acetic anhydride (84) or ^{125}I iodination and localized autoradiographically (see 56).

Localization of Immunoglobulin Labels. The standard general method to detect the immunoglobulin which has been used to identify the tissue antigen being sought is to attach a fluorescent marker, usually fluorescein isothiocyanate (FITC) or rhodamine, to the immunoglobulin. The improved sensitivity of the fluorescent marker over colored stains permitted Coons to succeed in his classic description of the method. The detection of FITC conjugates in tissue sections through the use of the most efficient light sources and filters has been discussed in great detail by Ploem (86). Although fluorescein is the most widely used marker dye for the purpose, problems arise from the fact that its excitation maxima (490 nM) is not too far from its emission peak (525 nM) and that the

standard illuminator used in this field (the 200 watt high pressure mercury lamp) emits very little energy in this portion of the spectrum, although it does have a rather sharp peak at 546 nM. This means that excitation is less than optimal despite the high amouut of energy absorbed by the tissue. The high illumination of unwanted light leads to "autofluorescence" of the tissue, which can introduce problems of interpretation, especially when low intensity staining is present. Ultraviolet energy also leads to more rapid decomposition of the FITC fluorophor and makes photomicrography difficult. All of these problems can be overcome with a more logical selection of filters and an illuminator appropriate for the area of the spectrum desired; these points are extensively discussed by Ploem (86).

The immunoglobulin can also be localized by conjugating a marker enzyme which can then be demonstrated histochemically. The peroxidase reaction of Graham and Karnovsky (42) has been employed most widely for this purpose (9, 57), but other markers such as glucose oxidase (43, 60), cytochrome *c* (28) and lactate dehydrogenase (20) have also been used as well as attachment of radioactive markers (84), ferritin (72, 77), or virus particles (45). The advantage of the enzyme marker or the radioactive marker is that the sensitivity can be increased over the fluorescence level, since autoradiographic exposure or enzyme histochemical incubation times can both be extended considerably without loss of selectivity. Both methods also produce markers which can be directly visualized by routine bright field microscopy and may be expected to remove the interpretive problem occasionally caused by "background" fluorescence. For those who will continue to employ the commercially available immunoglobulins and antisera already conjugated to fluorescent markers, several methods for simplified removal of excess fluorescein or labeled contaminant proteins may be of value (3, 16, 22, 79, 89).

Preparation of Tissue. When sections of tissue are used for the immunocytochemical localization, considerable thought must be devoted to the necessary compromises between adequate retention of tissue structures and preservation of antigen reactivity in its native location. For most purposes, cryostat sections of tissue freeze-quenched rapidly to the temperature of liquid nitrogen are used. These sections (4 to 20 micra thick) are then exposed to mild solvents which are also protein denaturants of varying potency (*e.g.*, chloroform-methanol). If the antigen to be localized is a structural protein (like collagen) or a particulate-bound secretory product (like anterior pituitary trophic hormones) it may be possible to apply standard chemical fixation methods with retention of antigenicity and no translocation of the antigen during the process (see 40, 53, 87). On the other hand, it cannot be assumed without experimentation that the antigen being sought does not migrate within the tissue water set free during thawing of the section unless freeze-dried sections are also examined. The use of lipid solvents is employed widely (25, 57) since these materials will extract lipid from the tissue while retaining some degrees of light microscopic structural details by denaturation. The removal of the lipids can actually improve the antigen-antibody reactivity (25). Hartmann *et al.* (47) have reported that extraction of tissue sections with chloroform-methanol and addition of Triton X to the primary immunoglobulin reagents and subsequent washes improves the sensitivity of dopamine β-hydroxylase within cryostat sections of brain. On the other hand, Kawamura (57) observes that treatment with chloroform-methanol destroys the antigenicity of Japanese encephalitis virus (JB) although progressive extractions of the sections with fluorocarbons such as carbon tetrachloride improved immunoreactivity. It is of interest that while immunocytologists go to considerable lengths to obtain purified antigenic materials and to purify the immunoglobulins raised against these antigens, few appear to have examined the effect of the solvents used to "fix" the tissue and its antigens on the reactivity of the immunoglobulin for the antigen *in vitro* (25). If the "contrast" and staining intensity of

the tissue section is to be used as the sole criterion for adequacy of antigen retention and reactivity, then it seems especially important to perform control staining experiments assiduously.

Immunocytochemical Controls. Criteria for successful localizations with immunocytochemistry were listed by Coons (25) (see also 32). Such criteria include blocking experiments in which incubation of the antisera or immunoglobulin preparation prevents the specific pattern of tissue staining, or the opposite control in which saturation of tissue antigen with unlabeled immunoglobulin prevents the attachment of the specific labeled immunoglobulin. In addition, one might add cytophysiological criteria to the point that staining intensity or distribution ought to correlate with changes in the tissue antigen (such as the effects of endocrine gland removal on the number of specific cell types storing trophic hormones in the anterior pituitary (81). Similarly, ability to retain specific patterns of cellular immunoreactivity with diluted immunoglobulin preparations implies specificity of the reaction, although this test is successful only infrequently.

Immunocytochemical Localization of Enzymatic Macromolecules

A wide variety of enzymes which can be highly purified have been used to raise immune sera which then can be used to localize the enzyme antigen in the tissues from which the enzyme was harvested. These localizations are summarized in table 1. Two aspects of such localizations are worthy of further discussion from a general theoretical viewpoint. One question is the nature of the changes which are undergone by the enzyme molecule during the course of its purification as an antigen and as an enzyme. It seems clear that while enzyme activity may be extensively enriched by protein purification steps, these same steps may induce changes in the tertiary structure of the molecule as an antigen which will influence the ability of the immunoglobulins to recognize and bind to the antigenic enzyme as it occurs in the tissue sections. Secondly, there is the question of

TABLE 1

Intracellular enzymes localized by immunocytological methods

Enzyme	Reference
Aldose reductase	21
Alkaline phosphatase	35
α-Glycerophosphate dehydrogenase	17
Amylase	116
Catalase	78
Cathepsin D	88
Chymotrypsinogen	34, 116
Creatine phosphokinase	73
Dipeptide naphthylamidase	54
Dopa decarboxylase	53
Dopamine-β-hydroxylase	38, 47, 53
Hexokinase	27
Ornithine aminotransferase	15
Phenylethanolamine-N-methyltransferase	53
Ribonuclease	32
Triosephosphate dehydrogenase	14, 17
Trypsin, trypsinogen	34, 116

enzyme families utilizing the same substrates for similar reactions but having different chemical compositions as disclosed by electrophoretic or chromatographic separation techniques. For example, crystallized horse liver alcohol dehydrogenase has five separable components (85), each of which can be used as an antigen. When this is done and the isozymes tested for immunological cross reactivity, it is found that two components cross react, while the other three do not. When purified bacterial aspartate transcarbamylase is used as an antigen (8), it is found that the intact enzyme reacts with the antisera more effectively than either of the two known subcomponents of the enzyme. This might well be expected since only the native antigen could possess all of the antigenic determinants. On the other hand, when enzymes which are mutually related, such as papain and chymopapain are used as antigens and cross reactivity tested (4), it is found that antigenic determinants shared by the two molecules can inhibit the enzyme activity of both enzymes better than the total immunoglobulins raised in response to chymopapain alone. This implies that some

immunoglobulins react with antigenic determinants close to the active site of the enzyme and others do not. Sanders *et al.* (91) have reported that peptic digests of chymotrypsin will react selectively to purified immunoglobulin raised to either trypsin and chymotrypsin, suggesting that at least for this class of enzymes, homologous chemical functions arise from a backbone of similar chemical structure. Such considerations may be especially pertinent when enzymes are harvested from organs in which the enzyme concentration is high in order to develop an antigen to provide an immunoreagent to localize the enzyme in tissues where the enzyme protein concentration is low. For example, it is common practice to purify dopamine β-hydroxylase from adrenal medulla, and apply the immune serum raised from this antigen against all tissues in which catecholamines can be assayed (38, 47, 53), or against the same enzyme activity in different species. While successful staining across tissue and species lines implies that such cross reactivities do not interfere with the localizations, it is difficult to conclude from such studies that shared antigenic determinants imply identical enzyme molecules and related enzyme parameters, such as K_M's, substrate and inhibitor specificities. Furthermore, when inhibition of enzyme activity is used as a test for cross reactivity with an antienzyme antibody, it seems worth noting that some enzymes are directly activated by exposure to the appropriate immunoglobulin (106), and that considerable immunoglobulin reactivity may occur with antigenic sites at some distance from the active site and thus not be indicated by enzyme inhibition (23).

Immunocytochemical Localization of Non-enzymatic Tissue Macromolecules

A long and growing list of secretory and structural proteins and antigenic lipids have been localized successfully by immunocytochemical methods. These macromolecules are summarized in table 2. Although the localization of immunoreactivity to the granular components of cells known to synthesize and secrete an antigenic polypeptide hormone may not be dramatic, such methods can be used to follow the synthesis of these molecules is an extremely specific manner. On the other hand, when a tissue yields several distinguishable protein antigens, these methods are particularly valuable in determining which cell types or cell components give rise to the identifiable antigens. Thus, by such methods it is possible to show that antibodies to the soluble brain protein

TABLE 2

Structural and biosynthetic cell antigens localized by immunocytochemistry

Antigen	Reference
Hypophysial hormones	7, 19, 29, 30, 48, 49, 58, 64, 74
Other hormones	81, 82, 108, 111
Calcitonin	18, 28, 110
Erythropoietin	37
Gastrin	65
Glucagon	84
Insulin, proinsulin	63, 115
Parathormone	46
Renin	52
Structural proteins	
Actin	5
Chromogranin	38
Chromomembrin	6
Collagen	66
14-3-2	24*
Fibrin/fibrinogen	2, 94
Hemoglobin	55
Myosin, meromyosin	62
S-100	24, 107*
Thrombesthenin	13
Tubulin	*
Other antigens	
Immune globulins	36, 61, 69, 90, 102, 104, 112
Calcium binding protein	109
Ceruloplasmin	93
Glycosphingolipid	67
Milk protein	117
N-acetylglucosamine	92
Transplantation antigens	39, 40, 76, 83

* K. L. Sims and B. W. Moore: Immunofluorescent studies on the localization of the brain specific proteins, S-100 and 14-3-2. In preparation.

S-100 (75) localize mainly to glia (24, 107)[1] while antibodies to another soluble acidic specific brain protein, 14-3-2[1] localize almost exclusively to neurons (24).[1] Similarly, antibodies to the protein of chromaffin granules, known as chromogranin (38) can be used to follow the release of intravesicular material during natural exocytotic events (98) and to stain the organelles in the tissue by immunocytochemistry (38). Recent developments of an antibody to the purified membrane of the chromaffin granule (6) may extend these observations. The formation of certain immunoglobulins has also been examined by immunocytochemical ultrastructural methods, in which the usually insurmountable problems of access of antigens and antibodies in fixed tissues embedded in resin for thin sectioning have been overcome through the use of hardy antigens (61, 66, 104), water soluble embedding materials (70), and methods for the amplification of the marker labels (1, 102, 105). Immunocytological examination of tissue sections may reveal the presence of antigenic components even when direct immunoassay of tissue homogenate extracts indicates that the tissue is low in content of this antigen. This situation arises because the immunocytological analysis can reveal a high concentration of antigen in a very small and restricted population of cells among a vast majority which do not possess the material. Thus an immunocytological approach may be essential to the localization of brain peptides which may function as neurotransmitters (see 10, for discussion).

Localization of Cell Surface Macromolecules by Immunocytology

Immunological methods have been extremely fruitful when applied at both the light- and electronmicroscopic level to the question of cell surface receptors, especially when these methods are applied to the nature of the process by which certain immune cells are triggered to form antibodies (31, 50, 80, 83, 90). In addition, these methods can be used temporally to examine the migration of molecules on cell surfaces (33, 56, 83, 96). With suspensions of thymocytes and other immune cells *in vitro*, Karnovsky and Unanue (56) have applied ultrastructural markers with conventional high resolution autoradiography and freeze-etching to assess the density and migration of cell surface receptors on lymphocytes for conconalvalin A, immunoglobulin, antilymphocyte globulin, and alloantigen H-2. These ultrastructural studies complement and extend other observations made with fluorescent markers (see 33) on the process by which antigen binds to receptors of a specific density on the surface of an immunocompetent cell, and which allows these bound antigens to be aggregated into caps and engulfed into the cell (33, 83). Such studies also bear directly on the nature of the fluidity of the intramembranous components of the plasma membrane allowing certain receptors to move independently of other receptors toward the pole of the cell. It seems feasible that similar methods could be employed to study the nature of the process by which new membrane components are constantly being added to the cell surface (see 97).

Immunocytochemical Localization of Cyclic 3′,5′-Adenosine Monophosphate

Cyclic adenosine monophosphate (AMP) triggers numerous intracellular physiological events in response to the actions of hormones. Most tissues giving such responses consist of multiple cell types, and unless cell separation and suspensions are performed prior to hormonal activation, it is not possible to determine which cell types give rise to the increase in AMP. Immunological methods have been employed to develop a sensitive immunoassay for cAMP (101) and extension of these methods to immunocytochemistry has permitted certain preliminary steps toward a cellular localization of cAMP (114).

[1] Sims, K. L. and Moore, B. W: Immunofluorescent studies on the localization of the brain specific proteins, S-100 and 14-3-2. In preparation.

Application of Anti-cAMP Immunoglobulin to Immunocytochemistry. Antisera to the antigen described above were prepared in rabbits, and the immunoglobulin subfractions (20 to 40 mg of protein/ml) were used as the primary immunoreagent, and detected with FITC labeled goat immunoglobulin raised against rabbit immunoglobulin (114). In order to obtain distinct and reproducible cellular patterns of staining, it was essential that the cryostat sections not be exposed to any solution after drying, and that immunoglobulin fractions be used as the immunoreagents. Formaldehyde fixation was found to be possible between the immunoglobulins, and improved the retention of cellular structure, particularly in brain (11).

Positive immunofluorescent staining with rabbit anti-cAMP immunoglobulin has been observed in specific cells of several different rodent organs. To verify that the staining observed indicated the presence of cAMP in these cells, several types of immunological and pharmacological experiments were performed. No staining was observed when the immunoglobulin fraction of unimmunized rabbits was used as the primary immunoreagent. Slight diffuse background staining was observed in preliminary experiments in which unfractionated naive sera were used. No staining was observed if the fluorescein-conjugated goat antirabbit IgG was reacted with the sections without prior exposure to rabbit anti-cAMP Ig. The positive discrete cellular staining patterns obtained with rabbit anti-cAMP Ig and goat fluorescein-conjugated antirabbit IgG could be blocked if the aliquot of the rabbit Ig was reacted with 10^{-3} or 10^{-4} M cAMP for 4 hr at 4°C before tissue exposure. Incubation of the rabbit anti-cAMP Ig with 5×10^{-3} M concentrations of adenosine triphosphate (ATP), 5′-AMP, succinate, cyclic inosine monophosphate, cyclic guanosine monophosphate, or cyclic uridine monophosphate for 4 hr at 4°C or incubation of the antiglobulin alone for 4 hr had no effect on the specific cellular staining. Furthermore, identical cytological patterns of positive staining were seen with antibodies prepared to cAMP antigens, by using either human serum albumin or keyhole limpet hemocyanin as the carrier protein. These results indicate that staining was not due to cross reaction between an unknown rodent protein and antibodies to the carrier proteins (11, 114).

Immunocytological Localizations of cAMP after Stimulations. To determine whether this indirect immunofluorescence method would detect increases in staining intensity or changes in cellular staining patterns under conditions in which cellular cAMP levels are increased (such as those which occur after hormonal stimulation), we have examined several different tissues (11, 95, 114).

cAMP Immunofluorescent Staining in Mouse Salivary Gland. Mouse salivary glands were examined for cAMP immunofluorescence before and after intraperitoneal injection of isoproterenol (0.8 ng/g). This treatment has been reported to produce a several-fold increase in tissue cAMP content within 10 min after injection (see 114). The staining patterns and intensity for cAMP immunofluorescence were compared between animals treated with isoproterenol and control mice given injections of pilocarpine (40 μg/g), a cholinolytic dialogue which does not affect tissue cAMP synthesis. Immunofluorescent staining was increased markedly in the salivary glands of isoproterenol-treated animals and a discrete pattern of cellular staining was observed. Only acinar cells and periacinar basket cells exhibit staining while ductules, blood vessels, and connective tissue stroma remain negative (114).

Patterns of cAMP Immunofluorescent Staining in Stimulated Lymphocytes. The ability to localize cAMP histochemically allows not only the localization of cAMP to individual cells but also the subcellular localization of the nucleotide within a single cell. This type of analysis is especially important in situations where there are differing effects on cellular metabolism under conditions where similar changes in cellular cyclic AMP occur. This is particularly true

for the human lymphocyte. Phytohemagglutinin (PHS), an extract of the Jack Bean (*P. vulgaris*), causes blastogenesis of lymphocytes when incubated with the lymphocytes under appropriate conditions (71). This blastogenic response has been linked to rises in intracellular cAMP, and it has been suggested that cAMP serves as the second messenger in the PHA response (99). Isoproterenol (ISO) and prostaglandin E_1 (PGE_1) are also potent stimulators of cAMP, and under appropriate conditions, incubations of lymphocytes with these agents can inhibit the blastogenic response to PHA (101).

We have carried out experiments with combinations of PHA and ISO or PGE_1 in short term incubations and measured the increase in whole cell cAMP.[2] In these experiments the increase in cAMP was additive thus suggesting that there may be separate adenylate cyclases responding to each of these pharmacological agents. These results led us to consider the possibility that there were spatial differences between the adenylate cyclases which could be demonstrated by localizing the cAMP generated by using the immunohistochemical techniques (113).

Lymphocytes were incubated with PHA, PGE_1, and ISO under conditions which had been determined previously to give the maximum increase in intracellular cAMP. After a 10-min incubation the cells were rapidly sedimented, resuspended in phosphate buffered saline, coverslip smears were made, and rapidly dried. The localization of cAMP was carried out as described above with the exception that the staining times were from 10 to 30 min with a washing times of 3 to 15 min. With this technique increases in cAMP could be demonstrated with each of the agents used. However, the localization of the increased cAMP was different for each of the agents used (fig. 1). When the cells were incubated with PHA, there was an increase in cAMP found in a spotted pattern along the plasma membrane. There was not a consistent increase found in the cytoplasm nor was there staining seen in the nucleus. The patchy pattern is seen in virtually 100% of the cells examined. In addition, studies were carried out at 0°, 25°, and 37°C and

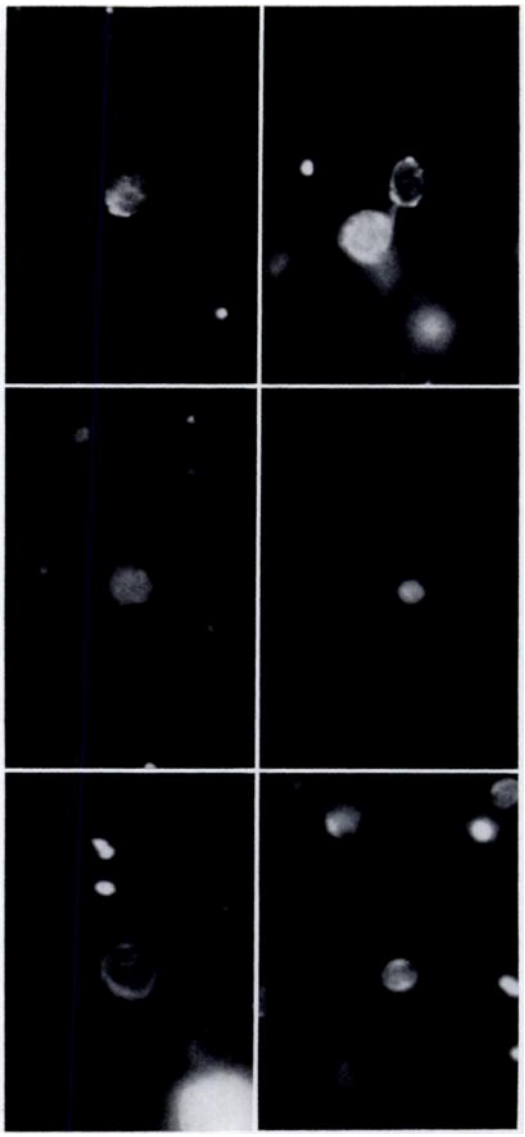

FIG. 1. Immunohistochemical localization of cAMP in human lymphocytes. Purified peripheral blood lymphocytes from a single donor were incubated with phytohemagglutinin, isoproterenol or prostaglandin E_1 (PGE_1) and cAMP was localized by the immunohistochemical technique as described in the text. The different patterns of cAMP localization for PHA (top), ISO (middle) and PGE_1 (bottom) are demonstrated.

[2] H. J. Wedner *et al.*: In preparation.

the pattern did not change. This indicated that the staining is not due to movement of surface molecules induced by the incubation of the cells with PHA (see below).

When the cells were incubated with PGE the pattern of localization was completely different. In this instance there is an increase found over the entire cytoplasm, but none is seen in the nucleus. Studies done with shorter incubations have failed to demonstrate movement of the cAMP from the plasma membrane into the cytoplasm. It should be pointed out, however, that because of the design of the experiments it is impossible to examine cells at time periods less than 150 sec which may be enough time for the cAMP to be generated at the plasma membrane and diffuse into the cytoplasm. The possibility exists, however, that some of the cAMP generated may come from adenylate cyclases found within cytoplasmic organelles.[3]

A third pattern was seen with isoproterenol. In this instance the localization was found over the entire cell but most important, there is prominent staining of the nucleus, not seen in the other two conditions. Since the nucleus occupies the major part of the cell volume, we propose that a large part of the increase in intracellular cAMP seen with isoproterenol must be nuclear. Confirmation for this observation comes from studies with isolated lymphocyte nuclei which have demonstrated an adenylate cyclase on the nuclear membrane which is responsive to isoproterenol.[4]

The result of these experiments when combined with the measurement of whole cell cAMP by radioimmunoassay indicate that there are most likely multiple adenylate cyclases spatially separated which, under appropriate conditions, are capable of generating cAMP into specific cellular compartments. The cAMP generated by one adenylate cyclase may then induce cellular metabolic events not induced by cAMP in another cellular compartment. This may account for the confusing results found in the lymphocyte where blastogenesis may be either induced or inhibited by agents thought to act *via* cAMP. In addition, it indicates that experiments in which the use of cAMP or its dibutyryl derivative to attempt to mimic the effect of pharmacological agents may fail due to the inability of this type of experiment to place the cAMP into the correct cellular compartment.

The use of the immunohistochemical localization of cAMP may also be effective in localizing the area within the cell where cAMP is generated. Such studies attempting to view the subcellular distribution in tissues such as intestine stimulated by cholera toxin and toad bladder stimulated by vasopressin are under way in our laboratory.

cAMP Immunofluorescent Staining in Stimulated Neurons. The brain is extraordinarily rich in hormonally sensitive adenyl cyclase, particularly in the cerebellum (see 11). On the basis of correlative histochemistry, electrophysiology, and pharmacology it has been suggested that the inhibition of one type of cerebellar neuron, the Purkinje cell, results from a synaptic pathway from the locus coeruleus which is mediated by norepinephrine and that this action of norepinephrine is in turn mediated by cAMP within the Purkinje neuron (12, 51). This cellular situation can be tested by application of the immunofluorescent staining method for cAMP to determine whether the staining pattern of Purkinje cells does change specifically under the influence of exogenous catecholamines or activation of the noradrenergic synaptic pathway. In order to determine whether there was in fact any discrete cellular staining pattern for cAMP in tissue sections prepared in the required way (see above), the postdecapitation rise in cerebellar cAMP level was used to produce a cAMP rich tissue source. When cerebellums were freeze-quenched within 90 to 150 sec after decapitation, discrete cellular immunofluorescent staining for cAMP was

[3] D. Snider and C. W. Parker: Unpublished observations.

[4] H. J. Wedner and C. W. Parker: In preparation.

observed only in two types of cortical cells: granule cells and Purkinje cells. Under these conditions of postdecapitation delay, approximately 80% to 90% of Purkinje neurons exhibited positive staining (fig. 2). Most reactive cells showed staining exclusively in the nucleus, while others showed staining of both nucleus and cytoplasm, and a few cells showed exclusively nuclear staining (11). In appropriately sectioned Purkinje cells, staining could be seen to extend within the apical dendrites into the molecular layer. Under similar physiological conditions, staining of cytoplasm was also observed in neurons of the reticular formation and the deep cerebellar nuclei (fig. 3). However, white matter and glia around Purkinje cells do not show positive staining.

Several immunological reactions establish the specificity of the indirect immunocyto-

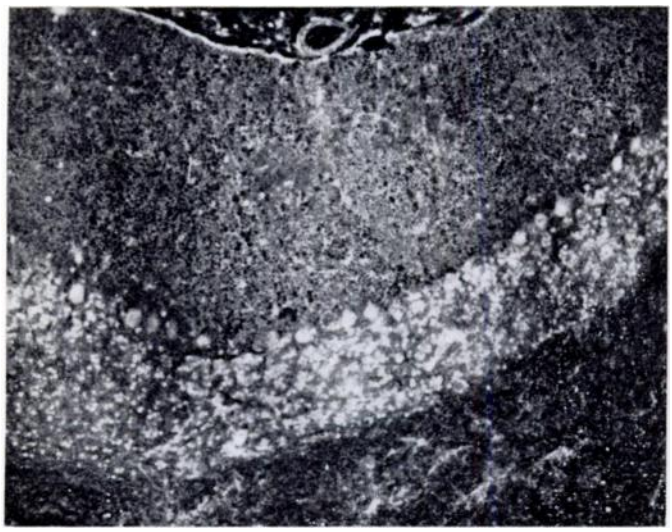

FIG. 2. Cerebellar cortex stained by indirect immunofluorescent method for cAMP. Note positive staining in large Purkinje neurons and smaller granule cells, and no staining in underlying white matter.

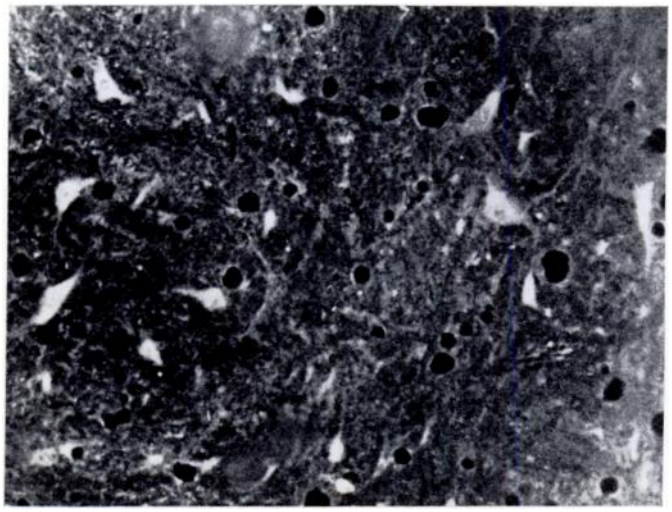

FIG. 3. Indirect immunofluorescent staining for cAMP in medullary reticular formation.

chemical staining and confirm that the staining observed in these neurons is attributable to their cAMP content. No staining of cerebellar sections was observed when the Ig fraction of non-immunized rabbits was used as the primary immunoreagent or when the fluorescein-conjugated goat IgG was reacted with tissue sections that had not been exposed to rabbit Ig against cAMP. The positive discrete neuronal staining patterns observed only when both immunoreagents were used could be blocked if portions of the rabbit Ig to be used for staining were first incubated with 0.1 or 1.0 mM cAMP for 4 hr at 4°C. The relatively high concentrations of cAMP required to block the immunocytochemical staining presumably reflect the large cAMP binding capacity of the undiluted anti-cAMP immunoglobulin.

When the rabbit Ig against cAMP was first incubated (4 hr, 4°C) alone or with 5.0 mM concentrations of ATP, succinate, adenosine 5′-monophosphate, or the cyclic monophosphates of guanosine, inosine, or uridine, there was no observable effect on the cell staining. Furthermore, identical cytological patterns of positive staining were seen with antibodies prepared to cAMP that was conjugated to either human serum albumin or keyhole limpet hemocyanin. This result indicates that positive staining could not be due to cross reactivity between an unknown brain protein of rodents and antibodies to the carrier proteins.

In order to determine the staining patterns in tissue samples which more closely reflected conditions *in vivo*, cerebellar biopsies were taken from anesthetized rats mounted in a stereotaxic headholder and exposed as for physiological experiments (12, 51). These samples and biopsies obtained from the cerebella of rats within 30 sec of decapitation showed similar cellular patterns of cAMP immunofluorescent staining, which differed from that seen in the long delay postdecapitation samples. In the anesthetized samples and brief delay postdecapitation samples, only 10% to 15% of Purkinje neurons show staining, and in these reactive cells the staining is often only a narrow rim of peripheral cytoplasm (11). In contrast to the marked changes in the frequency and intensity of Purkinje neurons staining reactivity, fluorescence in the granule cell layer was only slightly reduced in intensity and showed essentially no differences in the distribution of the stained elements.

The exposed cerebellar cortex of the anesthetized rat then was used as a physiological preparation to test the actions of applied neurohormones and synaptic pathway stimulation on the immunofluorescent staining patterns for cAMP (95). After topical application of norepinephrine (10 to 100 μM), but not after exposure to extremely high concentrations of other transmitter substances which also slow Purkinje neuron discharge (table 3), most Purkinje neurons showed highly positive immunoreactivity for cAMP. Lower concentrations of norepinephrine (1 μM) did not yield significant differences in staining pattern from controls. Samples taken during activation of the noradrenergic inhibitory pathway from locus coeruleus also exhibited a similarly high frequency of reactive Purkinje cells, but this increase was not observed when the electrode was inserted in the locus coeruleus and not electrically activated or when the locus coeruleus was stimulated in animals in which the cerebellar noradrenergic innervation had been destroyed by prior treatment with 6-hydroxydopamine (see 95; and table 3). The immunofluorescent histochemical technique thus provided evidence that the cAMP of Purkinje cells increases in response to norepinephrine (NE) or stimulation of the locus coeruleus, directly supporting the theory that the inhibitory noradrenergic response is mediated by cyclic AMP.

The specific cellular staining patterns and discrete intracellular changes which follow selective chemical activations in neurons and lymphocytes suggests that intracellular or intercellular translocation may be prevented by cAMP binding proteins (21a, 41, 44, 111a). In preliminary experiments, other

TABLE 3

Changes in proportion of cerebellar Purkinje cells showing immunoreactivity for cAMP after various treatments in vivo (see 95)

Treatment	Mean % Positive
Topical applications	
Ringer's solution	16
Norepinephrine 1×10^{-6} M	14
Norepinephrine 1×10^{-5} M	70*
Norepinephrine 1×10^{-4} M	68*
Norepinephrine 1×10^{-3} M	87*
γ-Aminobutyrate 2×10^{-3} M	20
L-Glycine 1×10^{-3} M	15
Serotonin 1×10^{-4} M	21
Histamine 1×10^{-4} M	20
Electrical stimulations	
Locus coeruleus	74*
Locus coeruleus (after 6-OHDA)	12
Locus coeruleus—sham	18
Locus coeruleus, adjacent brain	16

* $P < .001$.

neuronal models have been investigated to determine the cellular pattern of cAMP immunofluorescent staining after stimulation with catecholamines. In isolated pineal explants, exposure to norepinephrine produces marked increases in pinealocyte staining, but not in blood vessels.[5] In slices of bovine sympathetic ganglia, exposure to dopamine (59) produces marked staining of ganglioneuron cytoplasmic staining, but does not influence staining is satellite glia, blood vessels, or fibroblasts.[6] Thus this methodology appears capable of analyzing the sites in which cAMP and perhaps other small molecules may participate in interneuronal chemical communication.

Localization of Other Small Molecules by Immunocytochemistry. It is attractive to speculate that immunofluorescence localization techniques might be extended to small molecular weight substances other than cAMP. Antibodies are already available to prostaglandins and preliminary attempts at localizing prostaglandins by immunofluorescence (see 84) are in progress. Studies with histamine and the catecholamines would be of particular interest but the preparation of antibodies with appropriate specificity poses serious technical problems. In order to obtain antibodies to small molecules such as histamine, it is necessary to conjugate them covalently to protein. Conjugation is achieved easily through the amino nitrogen on the aliphatic side chain of histamine but if a simple amide bond is formed with protein carboxyl groups, the amino nitrogen will be uncharged and the antibodies obtained will bind more readily to histidyl residues on proteins than to histamine itself. Conjugation is possible through the imidazole ring of histamine but again this would so alter the structure of the immunogen that the antibody would not recognize free histamine. A method that might be successful would be to conjugate through the carboxylate group of histidine leaving both the amino and the imidazole groups or the molecule unsubstituted. Another possible approach would be conjugation through the aliphatic amino group with glutaraldehyde followed by reduction to create a positive charged secondary amino group (simulating the charged group of histamine). Even assuming antibodies to histamine can be obtained, because of its small molecular size, if histamine were bound to a receptor, the remaining area of the molecule might be too small to permit effective binding with antibody. With catecholamines, similar problems in preparing antibodies of suitable specificity and possible difficulties in obtaining effective interaction with tissue bound hormone can be assumed. However, we[7] have succeeded recently in obtaining antibodies with weak binding affinity for epinephrine so it seems likely the problem of obtaining the necessary antibodies can be solved. Nonetheless, alternative approaches to tissue localization may still be needed. Aside from the obvious approach of using labeled catecholamines or

[5] F. E. Bloom, E. F. Battenberg and F. Klein: Unpublished observations.

[6] F. E. Bloom, E. F. Battenberg, J. W. Kebabian and P. Greengard: Unpublished observations.

[7] C. W. Parker: Unpublished observations.

histamine for radioautographic studies (which, however, has serious problems in interpretation), the use of hormone-protein conjugates (coupled so as to retain affinity for tissue receptors) might be considered. The hormone-protein conjugates could be radiolabeled and localized in tissue by radioautography or by their ability to interact with fluoresceinated antibody specific either for the protein or the hormone.

Conclusions

Immunocytological methods have been profitably employed as specific stains for identifiable tissue antigens. Large macromolecules which resist translocation and contain sufficient antigenic determinants to retain antigenicity during chemical fixation have been localized most successfully. If employed as haptense to raise specific antisera, smaller molecular metabolites may also be localized within cells by immunological techniques, providing methods are used to restrict their diffusion within the tissue sections. Ultrastructural immunocytological experiments have been successful in analyzing the distribution of protein receptors on the surfaces of cells in suspension and in the intracellular synthesis of immunoglobulins. Increased collaborative efforts between immunologists and histochemists may be expected to continue this growing trend toward the biological synthesis and application of specific immunohistochemical reagents.

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