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Conjugated Avidin Identifies Cutaneous Rodent and Human Mast Cells

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Avidin conjugated to the fluorescent dyes rhodamine or fluorescein binds to mast cell granules in rodent and human skin. Sequential staining of tissue mast cells first with conjugated avidin, and then with a metachromatic stain demonstrated that both techniques identify the same mast cell granules. Specificity for mast cells was confirmed by the absence of avidin-positive cells in the skin of mast cell-deficient (W/W^v) mice. Binding of conjugated avidin to mast cells was inhibited by pretreating tissue specimens with unconjugated avidin but not by pretreating conjugated avidin with biotin, indicating that avidin does not bind to biotin or a biotin-like molecule. Within murine dermis, unique patterns of mast cell distributions were observed, with a prominent perivascular localization in ear skin, and a complete absence of mast cells underlying the scales in tail skin. In tissue sections of guinea pig skin undergoing basophil hypersensitivity reactions and in murine and human skin specimens infiltrated with eosinophils, conjugated avidin selectively stained only dermal mast cells, demonstrating specificity for mast cells in sites of inflammation. Conjugated avidin also readily stained rat peritoneal mast cells, demonstrating its utility for identifying extracutaneous mast cells. Unlike the metachromatic stains, avidin binding to mast cells in tissues is not limited by methods of fixation or special embedding and cutting procedures. Thus, mast cell identification with conjugated avidin is a reliable, specific, and simple method with important clinical and investigative applications.

The egg white protein avidin and naturally occurring biotin interact in an unusual stochiometric way, forming strong, noncovalent bonds that exhibit an exceptionally small dissociation constant of approximately 10^{-15} M [1]. The strength and specificity of this association has permitted the use of avidin and biotin as mutually seeking ligands for the detection and quantitation of tissue substrates. One commonly used strategy employs biotin-conjugated molecules that bind to appropriate tissue substrates [2–4]. The location of biotin conjugates are detected by avidin labeled with an enzyme or a fluorescent dye. These techniques have been employed successfully using avidin conjugated to the dyes fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) or to the enzyme horseradish peroxidase (HRP). During the course of work with each of these reagents, we observed that conjugated avidin in the absence of exogenous biotin specifically identified the granules of mast cells in cutaneous tissues and in isolated cell preparations. In this report we present data that characterize this unique specificity of avidin binding to rodent and human mast cells.

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Abbreviations:
CBH: cutaneous basophil hypersensitivity
FITC: fluorescein isothiocyanate
HRP: horseradish peroxidase
IFA: incomplete Freund's adjuvant
OA: ovalbumin
PBS: phosphate-buffered saline
PMNs: polymorphonuclear cells
TRITC: tetramethylrhodamine isothiocyanate

MATERIALS AND METHODS

Animals

BALB/cCum mice 8–12 weeks old were obtained from Cumberland View Farms, Clinton, Tennessee. Mast cell-deficient W/W^v mice and their sex- and age-matched +/+ controls ($WBB6F_1/J-++$) were purchased from Jackson Laboratory, Bar Harbor, Maine. Sprague Dawley rats, barrier-derived and specific pathogen-free, weighing 150–200 g were obtained from Camm Laboratories, Wayne, New Jersey. Inbred female strain 2 guinea pigs, weighing 300–400 g, were obtained from Crest Caviary, Raymond, California.

Reagents

Avidin, biotin, and ovalbumin were purchased from Sigma Chemical Co., St. Louis, Missouri. Incomplete Freund's adjuvant (IFA) was purchased from DIFCO Labs, Detroit, Michigan. Giemsa stain and clophonium were obtained from Fisher Scientific Co., Dallas, Texas. Fluorescein isothiocyanate conjugated avidin (FITC-avidin), tetramethylrhodamine isothiocyanate-labeled avidin (TRITC-avidin), and biotinylated monoclonal anti-Thy 1.2 antibody were obtained from Becton Dickinson FACS Systems, Sunnyvale, California.

Tissue Preparation

Cutaneous specimens from normal mice, rats, and guinea pigs were removed with a surgical blade after general anesthesia; fixed in 10% buffered formalin overnight; washed, and embedded in paraffin. Four micron-thick sections were prepared from all specimens, and deparaffinized with xylene and graded alcohols. Whole mounts of dermis were obtained from surgically excised specimens by removing the underlying fat with blunt dissection, and incubating in 20 mM EDTA as reported previously [5]. After 4 h, the epidermis was separated mechanically from the dermis and discarded. The dermal specimens were fixed in acetone for 30 min at 23°C, divided into 4 × 4 mm squares, and washed 3 times with 6.7 mM phosphate-buffered saline (PBS) pH 7.4.

Immunization and Skin Testing of Guinea Pigs for Cutaneous Basophil Hypersensity (CBH)

A guinea pig was immunized for CHB with ovalbumin (OA), by the injection of 0.1 ml of a 1:1 saline emulsion of OA (250 μg/ml) and IFA into each footpad. One week later, the animal was skin tested by the intradermal injection into flank skin of 0.05 ml saline containing 50 μg OA. A similarly challenged nonimmune guinea pig served as the negative control for the CBH reaction. Skin responses were measured 24 h later, and, as expected, the CBH site exhibited a characteristic reaction of erythema (16 mm in diameter) with minimal induration [6,7] while the negative control site had a barely perceptible, nonindurated, red macule (5 mm).

Six-millimeter punch biopsies were obtained from the center of each skin test site and were divided in half. One portion was fixed in 10% neutral buffered formalin; the other in Helly's solution (Zenker-formol, pH 4.7) according to methods of Askenase et al [8]. The fixed specimens were then embedded in paraffin, and 4-μm sections prepared. To identify infiltrating basophils optimally, the Helly's-fixed tissue sec-

tions were stained with Giemsa according to the previously described procedures [8,9]. Examination of these sections confirmed the expected basophil-rich nature of the infiltrate in the CBH reaction site [6–8], when compared with the negative control site.

Rat Peritoneal Mast Cell Preparations

Purified peritoneal rat mast cells were obtained according to the methods of Sullivan et al [10]. Cell suspensions were counted in a hemocytometer; diluted in PBS to a final concentration of 2.5×10^5 cells/ml, and 1×10^5 cells were cytocentrifuged onto acetone-cleaned glass slides. Adherent cells were fixed in acetone for 30 min at 23°C and washed 3 times in PBS.

Avidin Staining

Fixed tissue sections and cell preparations were washed with PBS and incubated with varying concentrations of FITC-avidin, or TRITC-avidin for 60 min at 23°C in a humidified chamber. Initial experiments indicated that dermal mast cell tissue specimens stained optimally with a 1:100 dilution of fluorochrome-labeled avidin. Therefore, this concentration was routinely employed for staining mast cells in tissue sections. Isolated rat mast cell preparations were readily identified at lower avidin concentrations (1:800 dilution) with optimal staining occurring at a 1:200 dilution.

Following incubation with conjugated avidin, specimens were washed 3 times for 10 min with 10 ml of PBS; covered with 90% glycerol in PBS and a glass coverslip, and examined by fluorescence microscopy with an Orthoplan Fluorescence microscope equipped for epiillumination (E. Leitz, Inc., Rockleigh, New Jersey).

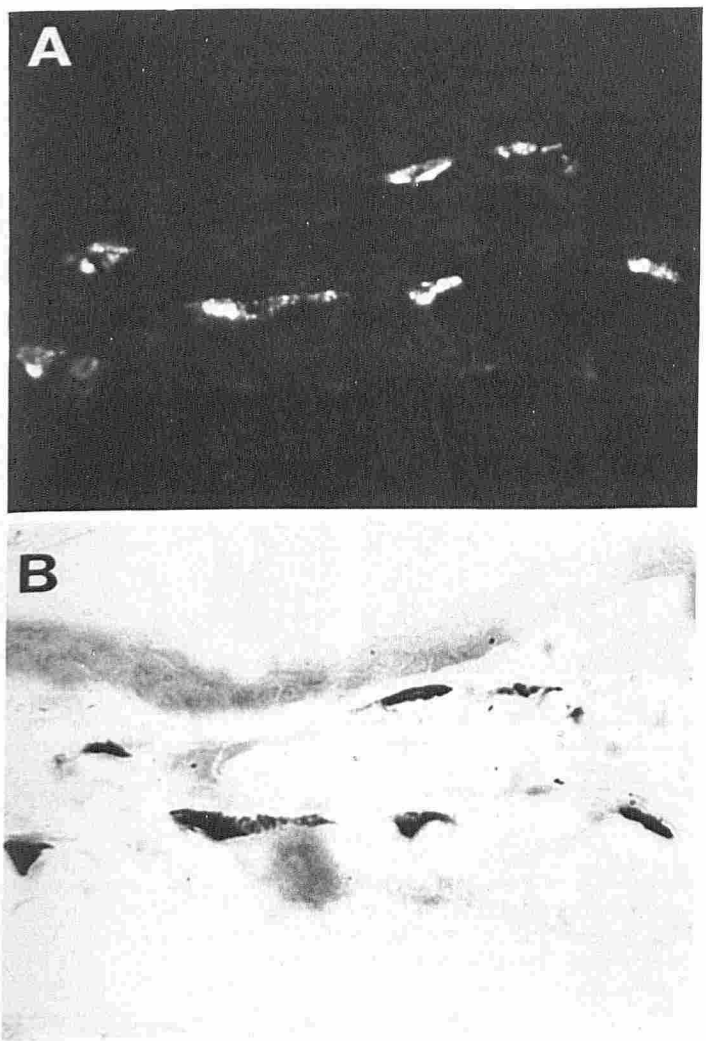

FIG 1. Fluorescein-labeled avidin identification of mast cells in 4 μm-thick formalin-fixed sections of murine ear skin. *A*, Brightly fluorescent granular cells in the dermis (× 65). *B*, Giemsa stain of the same specimen identifies the fluorescent granular cells as metachromatic staining mast cells (× 65).

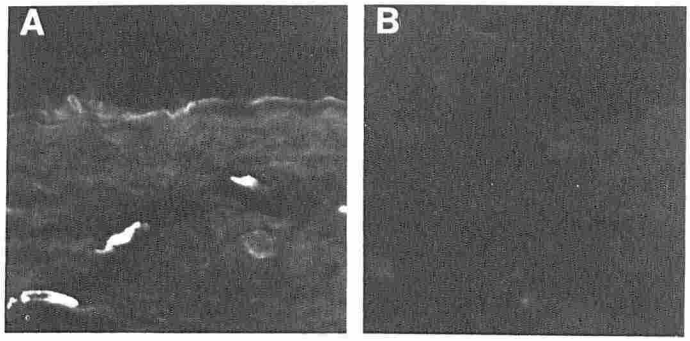

FIG 2. Fluorescein-labeled avidin identifies cells in ear skin of mast cell-replete (+/+), but not mast cell-deficient (W/W^v) mice. *A*, Avidin-positive granular cells in the dermis of +/+ mice (× 110). *B*, W/W^v mice do not have avidin-positive cells in the dermis (× 110).

RESULTS

Avidin Identifies Murine Cutaneous Mast Cells

Four micron-thick, deparaffinized, formalin-fixed murine ear specimens were incubated with FITC-avidin for 60 min at 23°C, and then washed with PBS. Under fluorescence microscopy, brightly stained, granular-appearing cells were observed in the upper to middermis (Fig 1*A*). These intensely fluorescent cells closely resembled dermal mast cells in their granular appearance and their characteristic clustering around blood vessels and pilosebaceous structures [11]. To confirm their identity, murine ear specimens that had been stained with FITC-avidin and examined and photographed by fluorescence microscopy were washed free of glycerol, and stained with Giemsa. In multiple sections from several normal murine ear specimens, FITC-avidin-positive granular cells also stained metachromatically with Giemsa, identifying them as mast cells (Fig 1*B*). Thus, FITC-avidin-positive cells in the dermis appeared to be mast cells by their anatomic location, their morphology, and most importantly, by their numerous Giemsa-positive metachromatic granules.

To determine whether FITC-avidin would bind to cells in the dermis other than mast cells, ear specimens from 4-month-old mast cell-deficient mice (W/W^v) [12,13] and their mast cell-replete +/+ littermates were stained in parallel with FITC-avidin. In each of 3 specimens, brightly fluorescent, granular cells were observed in the dermis of the +/+ animals (Fig 2*A*). By contrast, none of the specimens from the W/W^v mice contained FITC-avidin-positive cells (Fig 2*B*). We concluded from these observations that mast cells in the dermis possess a unique affinity for either FITC-avidin or for unconjugated FITC alone.

Experiments to define the active binding component of the FITC-avidin complex were performed using murine ear sections incubated with FITC-avidin, TRITC-avidin, or FITC alone. Both FITC-avidin and TRITC-avidin stained dermal mast cells with equal intensity while FITC alone failed to identify these granular cells in the dermis. These results indicated that avidin is the active component in the staining process and that this molecule binds to one or more structures associated with mast cell granules.

Avidin Identifies Unique Distributions of Mast Cells in Murine Dermis

Acetone-fixed specimens of murine dermis from abdominal wall, ear, and tail were examined as whole mounts after staining with FITC-avidin. When stained specimens of ear dermis were examined at low power, the number and vascular orientation of the dermal mast cells in the tissues were striking (Fig 3*A*).

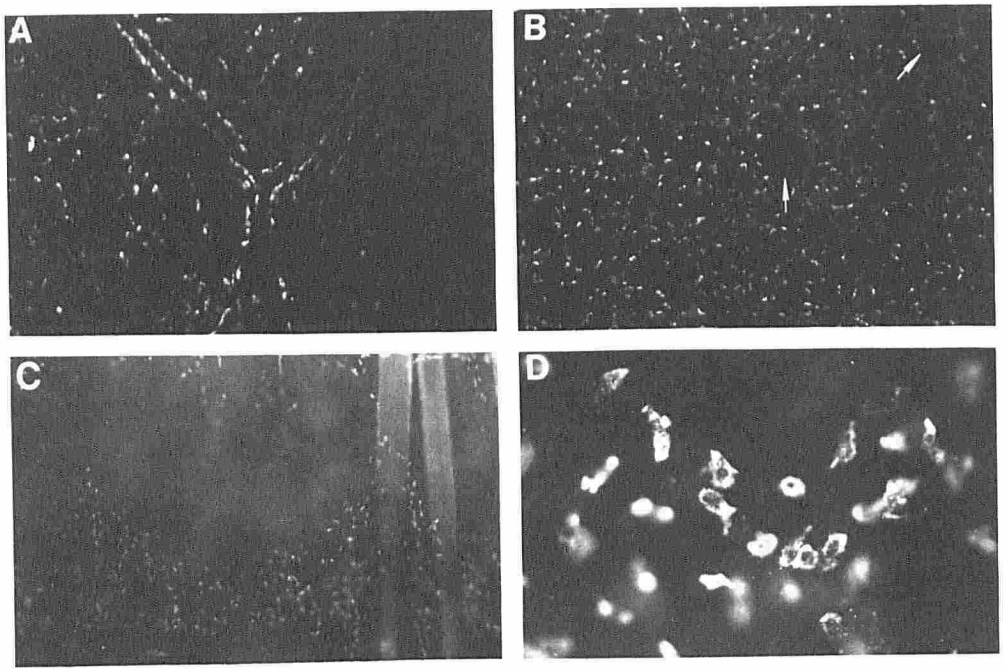

FIG 3. Fluorescein-labeled avidin identifies unique distributions of mast cells in whole mounts of murine dermis. *A*, At 10×, mast cells in ear skin are seen outlining dermal vessels. *B*, At 10×, ear skin mast cells are absent in the superficial dermis in areas penetrated by hair follicles (*arrows*). *C*, Tail skin dermis possesses mast cells primarily in interscale regions (× 10). Fluorescent cells at the bottom and lateral edges identify interscale regions while the large central area of scale is devoid of fluorescent cells. *D*, Abdominal wall dermis demonstrates a relatively uniform distribution of mast cells (× 65).

Interestingly, in these same specimens, mast cells were clustered around hair follicles in the mid to lower dermis; yet, were absent at sites where hair follicles penetrated the superficial dermis (Fig 3*B*). Specimens of tail dermis also demonstrated unique distributions of dermal mast cells that closely resembled the arrangement of Langerhans cells in the overlying epidermis (Fig 3*C*) [5]. Dermal mast cells were seen in great numbers beneath interscale regions, and only rarely were identified directly beneath each scale. As in the ear specimens, however, mast cells were abundant around hair follicles in the mid and lower dermis of tail skin. Whole-mount preparations of dermis from abdominal wall skin, perhaps because of their thickness, demonstrated a regular distribution of mast cells without obvious patterning (Fig 3*D*). An interesting and common feature to all of these tissue whole mounts was the striking dendritic morphology of many of the dermal mast cells.

Avidin Stains Rodent and Human Dermal Mast Cells

To examine the capacity of avidin to bind to mast cells from different species, cutaneous specimens from rat, guinea pig, and human were stained with FITC-avidin or TRITC-avidin. Fig 4*A* demonstrates brightly fluorescent, slightly dendritic, granular mast cells stained with FITC-avidin in the dermis of rat abdominal wall skin. Dermal mast cells that were identical in morphology also were observed in rat ear and tail skin, and no differences in cell staining were noted between FITC-avidin and TRITC-avidin. Avidin's ability to identify guinea pig dermal mast cells was examined using formalin-fixed specimens of ear and abdominal wall skin. In contrast to murine and rat cutaneous mast cells, guinea pig dermal mast cells appeared less granular, stained less intensely, and were fewer in number (Fig 4*B*). Nonetheless, these cells were readily stained with both FITC-avidin and TRITC-avidin in all 6 of the cutaneous specimens examined. We concluded from these studies that conjugated avidin identifies mast cells in tissues from several different rodent species.

In view of the recognized differences between rodent and human mast cells [14], it was unclear whether labeled avidin also would stain mast cells in human tissue. Therefore, formalin-fixed specimens of normal skin from the back and normal human foreskin were stained with FITC-avidin and TRITC-avidin. As was observed in the rodent species, brightly staining, granular mast cells were readily identifiable in the dermis of both specimens (Fig 4*C*). Interestingly, as judged by cell morphology and intensity of staining, human dermal mast cells most closely resembled rat cutaneous mast cells. To confirm that avidin was staining human mast cells, a cutaneous specimen from a patient with mastocytosis also was examined for the presence of FITC-avidin-positive cells. As seen in Fig 4*D*, numerous granular cells that were Giemsa-positive stained brightly with FITC-avidin. These observations indicated that avidin binds to both rodent and human cutaneous mast cells

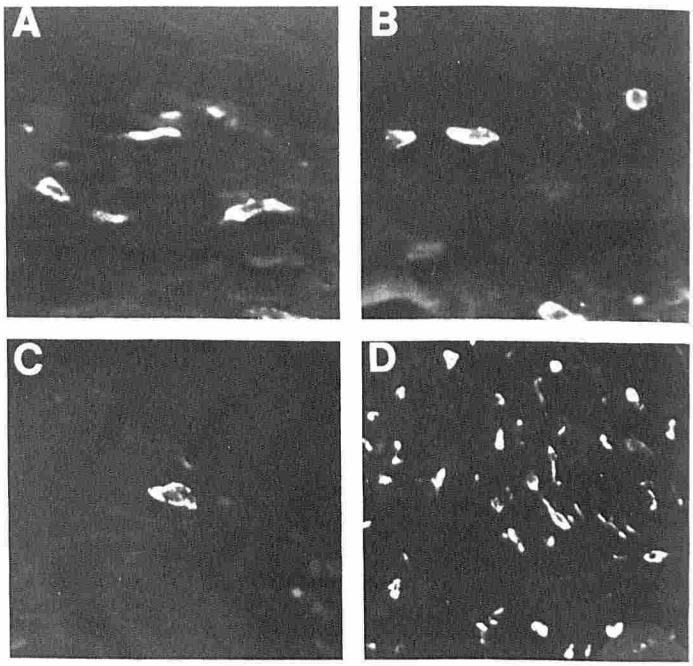

FIG 4. Rat, guinea pig, and human mast cells also bind FITC-avidin. *A*, Rat mast cells in abdominal wall skin (× 110). *B*, Guinea pig mast cells in abdominal wall skin (× 110). *C*, Human mast cell in normal skin from the back (× 100). *D*, Avidin-positive dermal mast cells in a patient with mastocytosis (× 60).

and that this molecule also identifies mast cells from a patient with mastocytosis.

Rat Peritoneal Cavity Mast Cells Bind Avidin

To determine whether extracutaneous mast cells exhibited avidin binding comparable to that of dermal mast cells, crude and purified preparations of rat peritoneal mast cells were obtained. Approximately 5% of the total cell population that is normally recovered from the rat peritoneal cavity is comprised of mast cells. Purified rat mast cell preparations of greater than 95% homogeneity can be obtained, however, through the use of bovine serum albumin gradients [10]. In two different experiments using either an unpurified rat peritoneal cavity preparation or a mast cell-enriched population, 5% and 95% of the cells, respectively, possessed numerous brightly fluorescent granules that stained with FITC-avidin (Fig 5*A*). When these same cell preparations were stained with Giemsa, all fluorescent cells recovered from the peritoneal cavity were also Giemsa-positive (Fig 5*A*,*B*). In subsequent experiments, it was demonstrated that TRITC-avidin bound to mast cell granules equally well, indicating that avidin rather than its fluorochrome conjugate represented the relevant binding molecule.

Avidin Does Not Bind to Other Granular Cells in Skin Infiltrates

Previous studies in our laboratories have indicated that mice passively sensitized with a murine monoclonal IgE anti-dinitrophenyl (DNP) antibody and challenged epicutaneously with 2,4-dinitrofluorobenzene (DNFB) develop an infiltrate relatively rich in eosinophils [15]. To determine whether avidin also identified eosinophils, ear sections from these reaction sites were stained with FITC-avidin. Only occasional FITC-avidin-positive cells were seen in this eosinophil-rich infiltrate. These fluorescent cells had the typical morphology and distribution of dermal mast cells (data not shown). To insure that this observation was not species-limited, a formalin-fixed skin biopsy specimen from a patient with Churg-Strauss disease containing a dense infiltrate of eosinophils and fewer, but readily detectable numbers of polymorphonuclear cells (PMNs) was stained with FITC-avidin. Only occasional brightly fluorescent, granular cells typical of dermal mast cells were observed, while none of the smaller eosinophils, or PMNs infiltrating the dermis were FITC-avidin-positive.

To test the ability of avidin to identify basophils, CBH was elicited in a guinea pig by the intradermal injection of OA 1 week after immunization with this antigen in IFA. The characteristic and striking basophil-rich nature of the cellular infiltrate of the CBH reaction site [6–8] was confirmed by examination of tissue sections with a modified Giemsa staining technique [8,9]. Serial sections from the same CBH reaction site tissue block were stained with FITC-avidin. Within the dense, basophil-rich cellular infiltrate, only occasional, brightly staining, large granular cells with unilobular nuclei, typical of mast cells, were observed.

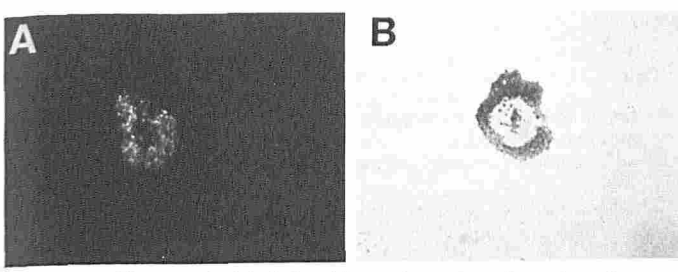

FIG 5. Fluorescein-labeled avidin stains rat peritoneal cavity mast cells. *A*, An avidin-positive rat peritoneal cavity mast cell (× 130). *B*, A rat mast cell with numerous metachromatic granules stained with Giemsa from the same cell preparation as (*A*) (× 130).

Avidin Binding to Mast Cells is not Biotin Related

Because of its great affinity for biotin, we postulated that avidin binding to mast cell granules might be biotin related. Since one avidin molecule has 4 potential biotin binding sites [1], a 1/100 dilution of FITC-avidin was preincubated with a 40-fold and a 400-fold molar excess of unlabeled biotin for 15 min at 23°C. Dermal specimens from murine ears were stained either with these "blocked" reagents or with untreated FITC-avidin. Even at a 400-fold excess, biotin failed to block avidin staining of tissue mast cells. In parallel experiments, acetone-fixed whole mounts of murine epidermis known to contain Thy 1.2^+ cells [16,17] were exposed to biotin-conjugated monoclonal anti-Thy 1.2 antibodies. After washing with PBS, these specimens were exposed to either FITC-avidin or 1 of the 2 "blocked" preparations containing FITC-avidin plus unlabeled biotin. Both concentrations of unlabeled biotin blocked the binding of FITC-avidin to the Thy 1.2^+ epidermal cells. We concluded from these experiments that unconjugated biotin does not inhibit the binding of avidin to mast cell granules, suggesting that the mast cell granule recognition site for avidin is not biotin or a biotin-like molecule.

DISCUSSION

In the reported studies we have demonstrated that fluorochrome-conjugated avidin binds specifically to mast cell granules in both rodent and human skin. In addition, rat mast cells isolated from the peritoneal cavity are readily stained with similar low concentrations of conjugated avidin, indicating that this molecule is capable of identifying extracutaneous mast cells as well. Our studies demonstrate that both FITC-avidin and TRITC-avidin stain mast cells with equal intensity, while similar concentrations of FITC alone fail to identify these cells, suggesting that avidin rather than its conjugate specifically binds to mast cell granules. Moreover, pretreatment of mast cells with excess unlabeled avidin results in nearly complete blocking of subsequent mast cell staining with either FITC- or TRITC-labeled avidin.

Studies in both rodent and human specimens indicate that avidin does not bind to other resident cells in normal skin nor does it bind to the granules of eosinophils, basophils, or PMNs in inflammatory skin lesions. In sections from murine and human skin replete with eosinophils and PMNs, avidin selectively stained only dermal mast cells. Moreover, avidin identified only the normal complement of dermal mast cells in basophil-rich cutaneous specimens of CBH. Thus, the specificity of avidin binding provides a powerful tool for the differentiation of mast cells from other granular cells infiltrating tissues.

Previous work has demonstrated that avidin binds to a variety of compounds, the majority of which are structurally related to biotin [1]. Wood and Warnke [18] have shown that conjugated avidin binding occurs in tissues rich in biotin, such as human liver and kidney. Their work suggested to us that conjugated avidin might identify biotin or biotin-like substances in or on mast cell granules. We, therefore, performed two experiments to address this issue directly. When FITC-avidin was preincubated with up to a 400-fold excess of unlabeled biotin, no appreciable diminution in mast cell staining was observed. In a control experiment, these same avidin-biotin preparations failed to stain Thy 1.2^+ epidermal cells labeled with biotinylated monoclonal anti-Thy 1.2 antibodies [16,17]. These studies indicate that avidin's association with mast cell granules is independent of the biotin molecule. Since under special circumstances conjugated avidin will bind to tissues as a result of electrostatic interactions and/or hydrophobic prop-

erties of the conjugate complex [18], it is possible that avidin binds to mast cell granules as a result of similar charge and/or hydrophobic interactions. Studies to clarify the chemical nature of this binding process are currently in progress.

To date, the identification of mast cells in tissue specimens has depended primarily on special stains that demonstrate characteristic metachromatic granules. However, these special stains are beset by potential pitfalls. Variation in tissue fixation, dehydration, and mounting may affect cell staining and morphology [19,20]. Stain pH and duration of tissue exposure to the stain are critical for demonstrating granule metachromasia; yet, these important parameters vary considerably depending upon the type and concentration of dye used [20,21]. Although mast cell morphology is well delineated with 1 μm-thick tissue sections, this technique requires special embedding and cutting procedures. By contrast, the staining of tissue mast cells with conjugated avidin is not limited by any of these factors. Although tissue fixation is required for avidin staining, the type of fixative appears to be relatively unimportant. No appreciable differences in staining were observed among tissue mast cells fixed in acetone, formalin, or Helly's fixative. In fact, mast cells in frozen tissue sections can be rapidly identified with conjugated avidin after fixation in acetone for only 1 h, indicating an additional advantage of this technique over conventional fixation and staining methods. Preliminary studies also indicate that conjugated avidin binding to tissue mast cells is unaffected by small changes in pH, an observation that would be predicted from the known stability of this molecule over a broad pH range [1]. Finally, mast cell morphology is readily delineated with conjugated avidin in routine, paraffin-embedded, 4 μm-thick tissue sections, making special embedding and cutting procedures unnecessary.

The use of conjugated avidin has several important investigative and clinical applications. Preliminary studies indicate that conjugated avidin identifies connective tissue mast cells in a number of rodent and human organs. Brightly fluorescent, avidin-positive mast cells have been observed in the lamina propia but not in the epithelium of murine intestine, indicating that avidin differentiates connective tissue mast cells from granular mucosal cells [22,23]. Avidin also may prove useful in discriminating connective tissue mast cells from other subpopulations of mast cells, including those derived from mouse bone marrow [24] and from human umbilical cord blood [25]. Because of its remarkable ability to stain individual mast cell granules, the conjugated avidin technique should be an excellent method in experimental systems for correlating agonist-induced histologic changes in mast cell morphology with the release of mediators. Clinically, conjugated avidin already has provided us with a reliable method for accurately enumerating mast cells in normal and pathologic tissues, and has permitted ready differentiation of mast cells from eosinophils and neutrophils infiltrating the skin. In summary, the conjugated avidin staining technique is a mast cell-specific and species-independent method that is simple and reliable, one that should prove useful both as an investigative and a clinical tool.

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