Purification of Histamine Receptor Proteins from Detergent-Solubilized Human Peripheral Blood Mononuclear Cells†

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ABSTRACT: Histamine is released from mast cells and basophils by either immunological or nonimmunological mechanisms. Histamine, which is the most potent short acting mediator released from these cells, exerts its diverse biological actions by binding to cell surface histamine receptors. We report the affinity purification of histamine receptor proteins from Triton X-100 solubilized peripheral human blood mononuclear cells which include lymphocytes and monocytes. Three different designs of histamine affinity columns were constructed; all three resulted in the same material being eluted. This consisted of bands which on SDS–PAGE after boiling and reduction had the following molecular weights: 193K, 84K, 58K, 48K, 37K, and 16K. The most abundant bands were of molecular weights 193K, 48K, and 16K, and these were disulfide bonded together to form a high molecular weight complex. (The 58K band was present in lower amounts than the others, and in only a few fractions. It had the same molecular weight as the dimeric form of histamine methyltransferase which is present in small amounts in mononuclear cells and may therefore have copurified.) The histamine binding proteins described in this report were purified by conventional affinity chromatography, rather than by an expression cloning approach which obviates the use of any protein chemistry. Consequently, we had the advantage of being able to verify the histamine binding specificity of our purified proteins directly and with several independent assays as follows. The histamine binding specificity of all three columns was established by specific elution with histamine, by preabsorption of crude cell extract with excess free histamine prior to column application, and by comparison with control columns. Independent determination of the binding specificity, using a radioreceptor dot blot assay, of the eluate containing only the 193K, 48K, and 16K disulfide-linked subunits confirmed that the purified material bound specifically to [^{3}H]histamine and that a 300–500-fold degree of purification from tissue extract had been obtained. Following cell surface radioreceptor cross-linking of radiolabeled histamine to intact mononuclear cells, the 16K band was detected, indicating it to be the ligand-binding subunit for histamine. These same three proteins were purified from T lymphocyte and monocytoid cell lines, indicating that both lymphocyte and monocyte subsets of mononuclear cells express these proteins. The trimolecular structure, consisting of 193K, 48K, and 16K subunits appears to be a novel histamine cell surface receptor protein complex as the molecular weights of the 193K, 48K, and 16K bands bore no relation to the predicted molecular weights of the recently expression cloned H1 receptor gene derived from bovine adrenal and H2 receptor gene derived from human parietal cells; this may reflect receptor heterogeneity within a tissue, between tissues, and/or between species.

Histamine release occurs from mast cells and basophils, and this can be triggered by a variety of mechanisms, including specific allergen binding to the antigen-binding region of cell surface IgE. In large amounts, histamine increases vascular permeability, resulting in the egress of inflammatory cells and plasma proteins into tissues (Orange & Austen, 1971; Coombs & Gell, 1975). Histamine exerts a broad range of biological effects in many tissues, sometimes multiple even in the same tissue, along with variation in these responses with different species. For example, in most mammals, histamine results in vasodilation and increased permeability in capillaries/venules, whereas its application to large arteries and arterioles results in strong vasoconstriction in rodents, vasodilation in dogs, and weak vasoconstriction in cats and humans. Other described effects include HCl secretion by human and bovine parietal cells, increased cAMP in cerebral tissues of most species, catecholamine release from all mammalian adrenal tissues, etc. (Douglass, 1970; Chand & Eyre, 1975). The effects of histamine on the human immune system have been extensively examined; at concentrations greater than 10^{-5} M there is a general reduction in both humoral and cell-mediated responses, whereas at concentrations less than 10^{-5} M, general enhancement of these two arms of the immune system occurs (Schnitzler et al., 1982; Plaut & Lichtenstein, 1982; Beer et al., 1984). These effects have been assumed to be mediated through histamine-binding cell surface receptors. Heterogeneity of histamine receptors has been demonstrated using different groups of drugs which were synthesized according to various parts of histamine's structure; these have antagonized various histamine-induced biological actions in vitro and in vivo. H1 antagonists incorporate the ethylamine group of histamine and antagonize such action as vascular response (Melville, 1973), H2 antagonists are based on the imidazole ring and antagonize other actions, e.g., gastric acid secretion (Black et al., 1972),

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and a third group of antagonists (H3), based on an alkylamine structure, were recently found to antagonize still other actions of histamine (Schwartz et al., 1986; Arrang et al., 1987; Ishikawa & Sperelakis, 1987).

Human peripheral blood mononuclear cells (which consist of 85% lymphocytes and 15% monocytes) display a spectrum of immunological activities when incubated with histamine, thereby indicating that this vasoactive amine exerts an immunomodulatory role (Schnitzler et al., 1982; Beer et al., 1984; Plaut & Lichtenstein, 1982). These cells bind avidly and specifically to histamine immobilized to large macromolecular supports such as beads (Weinstein et al., 1973; Ballet & Merler, 1976) and red blood cells (Kedar & Bonavida, 1974; Saxon et al., 1977), resulting in a variety of biological responses. This indicates that there are binding molecules, located on the surface of mononuclear cells, which specifically bind to histamine and that these mediate histamine's biological activities. We have previously demonstrated that these histamine-binding molecules could be solubilized using Triton X-100, and we went on to determine the optimal conditions required for specific histamine binding and dissociation (Warlow et al., 1986; Warlow & Bernard, 1987). These parameters were utilized in this study to purify histamine-binding proteins from human mononuclear cells by affinity chromatography.

MATERIALS AND METHODS

Reagents. Phenylmethanesulfone fluoride (PMSF[1]), Tris–HCl, Tris–base, sodium borohydride ($NaBH_4$), histamine dihydrochloride, riboflavin, ethylene glycol bis(succinimidyl succinate) (EGS), and Tween 20 were purchased from Sigma Chemical Co., St. Louis, MO. SDS, APS, TEMED, acrylamide, Bis, Coomassie Blue R250, high molecular weight markers, Bromophenol Blue, nitrocellulose (0.45 μm), Zetaprobe, cellulose membrane, and the dot blot manifold were purchased from BioRad Inc. Formaldehyde, boric acid, $CaCl_2 \cdot 2H_2O$, 2-mercaptoethanol, 2-propanol, monoethanolamine, Na_2CO_3, NaOH, acetic anhydride, sodium acetate, phenol, glycine, glycerol, and $NaH_2PO_4 \cdot 2H_2O$ were purchased from BDH Chemicals, Australia. Methanol, ethanol, $AgNO_3$, NaCl, 14.8 M NH_4OH, HCl, acetic acid, $Na_2HPO_4 \cdot 12H_2O$, and acetone were from Ajax Chemicals. Agarose was from FMC, Rockland, ME. Low molecular weight markers, Ficoll-Hypaque, Sepharose 6B, AH-Sepharose 4B, and fast-flow AH-Sepharose were from Pharmacia Fine Chemicals, Uppsala, Sweden. Triton X-100 was from Packard, Illinois. Bovine serum albumin was from Boehringer Mannheim, GmbH. Diglycidyl ether was from Aldrich Chemical Co., Wisconsin. Fmoc-L-histidine (Fmoc-OH) was from Cambridge Research Biochemicals Ltd., England. *N,N*-Diisopropylethylamine (DIPEA) and *N,N*-dimethylformamide (DMF) were from Applied Biosystems, CA. 1,3-Dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) were from Protein Research Foundation, Japan. Piperidine was from Merck Chemicals, Germany. Radioactively ^{125}I labeled histamine dihydrochloride (specific activity 2200 Ci/mmol) and radioactively tritium (^3H) labeled histamine dihydrochloride (specific activity 50 Ci/mmol) were purchased from Amersham, England. Enhance spray was from Dupont, Massachusetts. DNase I was from Boehringer Mannheim, GmbH. Bovine hemoglobin was from Calbiochem, Los Angeles, CA. Whatman chromatography paper no. 1 was from Whatman. Kodak X-Omat AR film was from Kodak, Rochester, NY. Dimethyl adipimidate dihydrochloride (DMA), dimethyl pimelimidate dihydrochloride, and disuccinimidyl suberate (DSS) were from Pierce, Rockford, IL. Acetonitrile and $MgSO_4$ were from Mallinckrodt, Australia. $MgCl_2 \cdot 6H_2O$ was from Merck. Human γ-globulin (Cohn Fraction II) and sodium heparin were from Commonwealth Serum Laboratories, Melbourne.

Preparation of Triton X-100 Extract from Mononuclear Cells. Human peripheral blood mononuclear cells were purified from 500-mL packs of fresh blood from patients with hemochromatosis by Ficoll-Hypaque fractionation. These patients formed a small core group who were being regularly therapeutically venesected. None of these subjects had a history of allergic diatheses or were taking antihistamines prior to or during the venesection. The blood was collected and gently mixed in 500-mL blood collection bags which had been preloaded with 10 000 units of preservative free heparin diluted with 20 mL of 0.15 M NaCl. The blood was then loaded into 50-mL tubes and spun at 200*g* for 20 min and the platelet-rich plasma supernatant was discarded. The blood was diluted 1:1 with 0.15 M NaCl, and 35 mL of this mixture was layered atop 15 mL of Ficoll-Hypaque (specific gravity 1.077) in 50-mL plastic tubes. The tubes were immediately centrifuged at 200*g* for 30 min at room temperature in a swingout rotor centrifuge, resulting in a distinct band at the interface between the Ficoll and the plasma. This band was collected, resuspended, and washed three times in 0.15 M NaCl. Cell suspensions were counted and morphology was assessed in a counting chamber by microscopy. In general 500 mL of blood yielded a total of (480–550) × 10^6 cells, consisting of 80–90% lymphocytes and 10–20% monocytes as assessed by morphology. The cells were pelleted, the supernatant was aspirated, and the cells were placed on ice at 4 °C. To each 1 × 10^6 cells were added 10 μL of cold 0.15 M NaCl, 0.5% Triton X-100, and 1 mM PMSF followed by gentle mixing with a Pasteur pipet for 3 min. Solubilization proceeded for 30 min at 4 °C, and then centrifugation was performed at 500*g* for 5 min at 4 °C. (The PMSF was always prepared fresh as a 100 mM solution in 2-propanol which was then diluted to 1 mM in the freshly prepared solubilization buffer.) The clear supernatant collected had a protein concentration of 4.4–5.3 mg/mL as measured by the method of Lowry et al. (1951) using bovine serum albumin standards. This solubilized cell fraction was centrifuged at 100000*g* for 60 min at 4 °C in a Beckman L8-M ultracentrifuge, the clear supernatant was diluted 1:4 in a solution of 0.1 M NaCl, 25 mM Tris, pH 8.0, and 2.5 mM $CaCl_2$ and applied directly to the affinity matrix. Unless otherwise stated, the above procedure, including application of the extract to the column, was done on the same day.

Preparation of the Histamine Sepharose Affinity Matrix. Histamine was coupled through its terminal amine group to epoxy-activated Sepharose 6B by synthesizing a bifunctional

[1] Abbreviations: 2-ME, 2-mercaptoethanol; APS, ammonium persulfate; Bis, bisacrylamide; BSA, bovine serum albumin; DCC, 1,3-dicyclohexylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; DMA, dimethyl adipimidate dihydrochloride; DMF, *N,N*-dimethylformamide; DMP, dimethyl pimelimidate dihydrochloride; DSS, disuccinimidyl suberate; EDTA, ethylenediaminetetraacetic acid; EGS, ethylene glycol bis(succinimidyl succinate); HOBT, 1-hydroxybenzotriazole; $NaBH_4$, sodium borohydride; PAGE, polyacrylamide gel electrophoresis; PBS–Ca, phosphate-buffered saline, pH 7.4, with 2 mM calcium chloride; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene fluoride); SDS, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; TBS–Ca, Tris–HCl-buffered saline, pH 8.0, with 2 mM calcium chloride; TBS–TX–Ca, Tris–HCl-buffered saline, pH 8.0, with 2 mM calcium chloride and 0.1% Triton X-100; Tris, tris(hydroxymethyl)-aminomethane.

oxirane group on the Sepharose and then coupling the amine under alkaline conditions according to the method of Sundberg and Porath (1974). Briefly, 1 g of suction-dried Sepharose 6B was washed on a glass filter funnel with DDW and then mixed with 1 mL of diglycidyl ether and 1 mL of 0.6 M NaOH solution containing 2 mg of sodium borohydride ($NaBH_4$)/mL. The suspension was mixed overnight in a plastic tube at 25 °C (without a stirring bar), and the reaction was stopped by washing the gel on a glass filter funnel with large volumes of DDW (>500 mL). Histamine dihydrochloride (100 mg) was dissolved in 10 mL of 0.5 M sodium carbonate buffer, pH 10, and mixed with 5 g of suction-dried oxirane–Sepharose 6B for 24 h at 25 °C. Unbound oxirane groups on the matrix were blocked with 1 M monoethanolamine for 24 h. The coupled matrix was then washed on a glass filter funnel with the following solutions: 0.5 M Na_2CO_3, pH 10, 1 M NaCl, DDW, 0.1 M glycine, 1 M NaCl. The gel was poured into a minicolumn and washed and stored at 4 °C in the following buffer until ready for use: 0.1% Triton X-100, 0.1 M NaCl, 2 mM $CaCl_2$, 20 mM Tris–HCl, pH 8.0.

Preparation of the Histidine AH-Sepharose Affinity Matrix. Fmoc-protected L-histidine was coupled through its carboxyl group to the amine groups attached to the six-carbon spacers on the affinity matrix AH-Sepharose 4B using Fmoc synthetic peptide chemistry. The coupling protocol was as follows: 3 g of AH-Sepharose was washed twice in 10% *N*,*N*-diisopropylethylamine (DIPEA) in *N*,*N*-dimethylformamide (DMF), and then the gel was allowed to swell in DMF. The DMF had been degassed in N_2 for 30 min prior to use to remove any free bases which could potentially interfere with coupling. In a separate flask, 0.36 g of Fmoc-L-histidine (Fmoc-OH) was dissolved in 2–3 mL of DMF, with gentle mixing and warming at 37 °C. In another flask 2–3 mL of DMF was added to 0.12 g of 1,3-dicyclohexylcarbodiimide (DCC) which was allowed to dissolve, and then the above L-histidine solution was added to this mixture. Then 0.08 g of 1-hydroxybenzotriazole (HOBT) was added to the histidine–DCC mixture, and when all constituents were thoroughly dissolved, this mixture was added to the drained but moist Sepharose. This mixture was gently agitated and allowed to incubate for 60 min at 25 °C. The solution was drained from the gel, and the Sepharose was washed with 20 mL of DMF four times, then with 20 mL of 10% DIPEA in DMF twice, and finally with 20 mL of DMF four times. Unbound amine sites on the gel were blocked with 3% acetic anhydride in DMF for 10 min, the gel was washed again with 80 mL of DMF, and the Fmoc protective groups were removed by the addition of 20% piperidine in DMF. This deprotective step resulted in considerable gel shrinkage and contraction. The gel was washed in 20% methanol in DMF which was gradually increased to 100% methanol in DMF, then 90% DMF in distilled water was added, and the concentration of DMF was progressively reduced to 0% so that the gel was left in water alone. Although the gel was contracted, it still retained acceptable flow characteristics.

Preparation of the Histidine Fast-Flow AH-Sepharose Affinity Matrix. Because of the contracting effect that the piperidine exerted on the agarose chains of the Sepharose matrix which resulted in reduced flow characteristics and surface area availability, it was decided that a more alkali-resistant matrix should be used for the coupling. Because no such matrix was commercially available, Pharmacia Fine Chemicals was commissioned to custom-make an alkali-resistant affinity matrix with a six-carbon spacer, at the end of which projected an amino group which was available for coupling to the carboxyl group of L-histidine. They utilized a matrix called fast-flow Sepharose which did not undergo contraction when exposed to piperidine, or to any other reagents during the conjugation procedure. In addition, it displayed excellent flow characteristics after conjugation. The same coupling protocol was used as shown above for the AH-Sepharose except that the starting gel weight used was about 15 g. The amounts of reagents were increased accordingly, and the methanol equilibration step after deprotection was omitted.

Column Wash and Elution Protocols. Prior to use, the affinity matrix to be used was always washed with several void volumes of the following regeneration buffers to ensure that it did not contain unspecifically bound material retained from previous procedures: 0.5 M Na_2CO_3, pH 10, 1 M NaCl, DDW, 0.1 M glycine, and then 1 M NaCl. The column was washed and equilibrated with several volumes of column wash buffer which contained 100 mM NaCl, 0.1% Triton X-100, 2 mM $CaCl_2$, and 20 mM Tris–HCl, pH 8.0 (TBS–TX–Ca). The Triton cell extract was applied to the gel and allowed to permeate it, and the column was sealed, rotated end over end several times so as to ensure maximal gel surface exposure to the extract, and left overnight at 25 °C. The next morning the unbound material was drained off, and then the gel was washed with at least 20 void volumes of the TBS–TX–Ca wash buffer. Bound material was eluted with either 50 mM histamine in TBS–TX–Ca or 0.1 M NaCl–acetate–acetic acid buffer adjusted to pH 3.0. Fractions of 1 mL were collected in 200 μL of 40% glycerol in 1 M Tris–base, pH 9.0, using a Gilson fraction collector and were stored at −70 °C.

Dot Blot Radioreceptor Assay. This method involved the binding of crude protein extracts and purified proteins to a membrane, followed by incubation with radiolabeled histamine, and then washing the membrane and performing autoradiography so as to detect ligand binding. The details of this novel radioreceptor assay are as follows: the BioRad dot blot manifold which had been modified by inserting rubber O-rings into the underside of each well was soaked in distilled water overnight and cleaned in 70% ethanol prior to use. All components were dried, and the apparatus was assembled according to the manufacturer's instructions, except that the rubber gasket was omitted. Also included was a rectangular piece of precut PVDF, prewetted in methanol for 5 min and then equilibrated in 0.1 M NaCl, 20 mM Tris–HCl, pH 8.0, and 2 mM $CaCl_2$ (TBS–Ca). Suction from a water pump was applied so as to dry the membrane, then the manifold was disconnected from the suction, and the outflow valve was closed. After this, samples were applied to the wells as follows: to wells 1, 2, 9, and 10 was added 200 μL of 1 mg/mL bovine hemoglobin, to wells 3, 4, 11, and 12 was added 200 μL of acid-eluted material from mononuclear cells purified using the L-histidine fast-flow AH-Sepharose gel but without prior histamine elution (this eluate contained the 193K, 48K, and 16K proteins, but not the 58K band), to wells 5, 6, 13, and 14 was added 200 μL of TBS–Ca buffer, and to wells 7, 8, 15, and 16 was added 200 μL of Triton X-100 solubilized mononuclear cell extract (which had been prepared as described previously in this section). The samples were left to adsorb onto the membrane for 2 h at 25 °C. Thereafter, the outflow valve was opened, the suction applied, and the remaining liquid in the wells drained. The outflow valve was closed and the suction disconnected. (The sequential steps of well application followed by incubation with the outflow valve closed, suction drainage, and valve closure were performed for each stage of the procedure as they significantly prevented

cross-well contamination during suction and maximized the amount of material adsorbed to the membrane.) The wells were incubated with 0.05% Tween in TBS–Ca (Tween–TBS–Ca) for 60 min. After drainage, the wells were loaded with 1×10^{-8} M [^{3}H]histamine made up in Tween–TBS–Ca. In wells 9–16 inclusive, 50 mM unlabeled histamine was also included in the buffer as a control for nonspecific binding. The radiolabeled ligand was incubated in the wells for 16 h; during this period, the outflow valve had been closed and the manifold covered by parafilm. The wells were drained and washed three times with Tween–TBS–Ca. The manifold was dismantled, and the PVDF membrane was removed, allowed to air dry, sprayed lightly three times with Enhance spray (NEN), air dried, attached to Whatman chromatography paper, and autoradiographed with preflashed Kodak X-Omat AR film for 21–35 days.

Surface Affinity Cross-Linking of [^{3}H]Histamine to Whole Mononuclear Cells. Intact human peripheral blood mononuclear cells were purified as described above. The intact cells were aliquotted into multiple microfuge tubes such that each tube contained 10^7 cells. These were pelleted by a 15-s spin in a microfuge at 250*g* and gently resuspended in 100 μL of 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.4, and 2 mM $CaCl_2$ (PBS–Ca). To this was added ^{3}H-labeled histamine at a final concentration of 5×10^{-8} M. In a duplicate set of tubes the same amount of radiolabeled histamine was added which included 50 mM histamine as a nonspecific binding control. The tubes were incubated at 25 °C for 2 h, the cells were pelleted as previously, the unbound radioactive supernatant was aspirated, and the cells were gently resuspended in 990 μL of cold PBS–Ca and placed on ice at 4 °C. The cross-linking protocol used was based on that used by Nicola and Peteresen (1986). To each tube was added microliter amounts of one of the following freshly prepared cross-linkers, 100 mM DSS, 100 mM DMA, 100 mM DMP, or 100 mM EGS, such that the final concentration was approximately 1/1000 of the stock. The DSS and EGS were dissolved in acetonitrile while the DMA and DMP were dissolved in DDW. The cells were gently but thoroughly mixed and incubated at 4 °C for 15 min. The cells were pelleted as before, and the supernatant was aspirated and the pellet stored at −70 °C until required for gel electrophoresis. Immediately after thawing the frozen pellets, 20 μL of 10 mM Tris–HCl, pH 7.4, was added. The pellets were freeze–thawed three times in ethanol/dry ice and hot water baths, and after this, 2 μL of 2 mM $MgCl_2$ and 2 μL of 4000 units of DNase I were added, and the tubes were vortexed and incubated for 60 min at 4 °C. Then 2 μL of 50 mM $MgSO_4$ and 2 μL of 1 mM PMSF were added and the tubes vortexed. The final cell mixture volume was about 30–40 μL, and an equal volume of sample buffer was added for the SDS gel electrophoresis.

SDS–Polyacrylamide Gradient Gel Electrophoresis. Samples were run in a gradient electrophoretic gel system which was composed of a 1.5-mm, 3–15% linear polyacrylamide gradient with a fixed percentage of Bis (%C = 0.25), a 0.625–2.5% glycerol gradient, 0.38 M Tris–HCl, pH 8.4, and 0.1% SDS utilizing a Protean II cell (BioRad). The gels were poured with a Pharmacia gradient maker and peristaltic pump after sealing the bases of the glass plates with 0.7% agarose, and were photopolymerized by including the following catalysts: 0.05% TEMED, 0.011% APS, and 0.0004% riboflavin. Samples of 50 μL were dissolved 1:1 in sample buffer which was composed of 0.05 M Tris–HCl, pH 6.8, 50% glycerol, 2% SDS, 0.05% 2-mercaptoethanol, and 0.001% Bromophenol Blue. The samples were boiled at 100 °C for 5 min prior to application to wells and were subjected to running buffer containing 0.025 M Tris–base, 0.20 M glycine, pH 8.3, and 0.1% SDS running buffer at 40 mA/gel with cooling at 6–8 °C until the dye front was 1–2 cm from the gel bottom. Molecular weight markers were included with each gel. Where indicated, gels were silver stained according to the protocol of Wray et al. (1981) or by colloidal Coomassie Blue according to the method of Neuhoff et al. (1985).

Gel Drying and Autoradiography. After electrophoresis, the gels were fixed in 30% methanol and 10% acetic acid for 60 min, and two-step fluorography was performed using Entensify which is commercially available from Dupont. After fixation, the gels were gently agitated for 45 min in the precipitating agent poly(ethylamine imine) and, after its removal, in the aqueous fluorographic reagent [a combination of 4-(5-phenyloxazolyl)benzenesulfonic acid ammonium salt and 2-naphthalenesulfonic acid ammonium salt] for 90 min. The gels were placed on a piece of cellulose membrane, covered with a piece of Glad wrap, and dried under suction at 60 °C for 2 h using a BioRad 1125 gel dried and liquid nitrogen cooled vapor traps. After drying, the Glad wrap was gently peeled away, any moist areas on the gel surface were allowed to air dry, and the dried gel was placed in an autoradiography cassette and autoradiographed with preflashed Kodak X-Omat AR film for 2–12 weeks.

RESULTS

Amino Acid Analysis of Conjugated Affinity Matrices. The amino acid content of the conjugated affinity matrices was determined by amino acid analysis in order to determine the coupling efficiency of the conjugation protocols used. Samples of coupled Sepharose were hydrolyzed in 6 N HCl in the presence of phenol and heated at 110 °C for 24 h in vacuo. The amino acid content was then determined using a Beckman 6300 amino acid analyzer. The amino acids detected and their amounts were as follows: *histidine fast-flow AH-Sepharose*, histidine, 52.1 μmol/(g of Sepharose), trace amounts of glycine; *histidine AH-Sepharose*, histidine, 79.9 μmol/(g of Sepharose), no glycine detected.

Analysis of Eluates from the Histamine Sepharose Affinity Matrix. After the mononuclear cell extract had been on the column for 16 h, the column was washed extensively with TBS–TX–Ca and bound material was eluted with 50 mM histamine dissolved in TBS–TX–Ca. The results of this specific elution are seen in Figure 1, lanes 1–5. The most prominent bands had molecular weights of 193K, 84K, 58K, 48K, 37K, and 16K. The most abundant bands, which were also seen in all these eluted fractions, are the 48K and 16K bands. The column was then washed with the 0.1 M NaCl–acetate–acetic acid buffer (lanes 6–10). The same bands were seen as in the histamine-specific eluate (except the 58K band), indicating that, during the specific elution, histamine had removed most but not all of the specifically bound material which was subsequently acid eluted. A cluster of low molecular weight bands ranging from 15K to 21K were also present in both eluates.

Analysis of Eluates from the Histidine AH-Sepharose Affinity Matrix. After the cell extract had been left on the column overnight, the column was washed extensively and bound material was eluted with 50 mM histamine in TBS–TX–Ca. The results of this specific elution are seen in Figure 2, lanes 1–5. The most prominent bands which had molecular weights of 193K, 84K, 58K, 48K, 37K, and 16K were identical to those eluted from the histamine Sepharose matrix. The column was then eluted with the 0.1 M NaCl–acetate–acetic

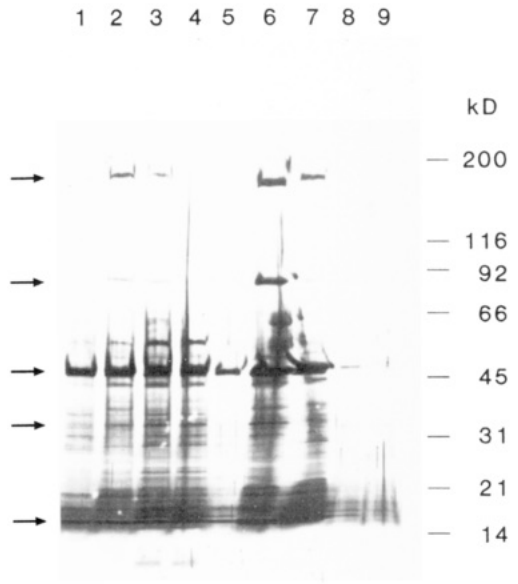

FIGURE 1: Progressive 1-mL fractions collected from the histamine Sepharose affinity column after Triton X-100 solubilized mononuclear cell extract in TBS–TX–Ca derived from 200 × 10^6 cells had been incubated on the column. Samples were boiled and reduced with 50 mM 2-ME prior to loading onto a 3–15% SDS gradient gel which was subsequently stained with ammoniacal silver. Lanes 1–5 contain specifically bound material eluted with 50 mM histamine dissolved in TBS–TX–Ca. Lanes 6–10 contain fractions eluted with 0.1 M NaCl–acetate–acetic acid buffer, pH 3, immediately after histamine-specific elution.

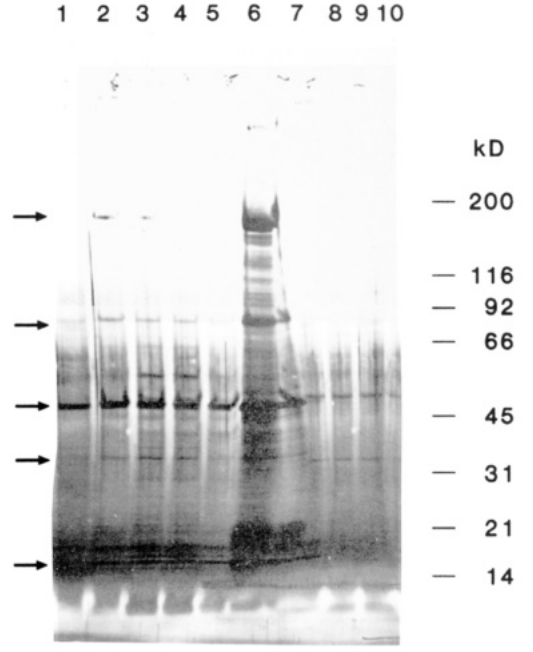

FIGURE 2: Progressive 1-mL fractions collected from the histidine AH-Sepharose affinity column after Triton X-100 solubilized mononuclear cell extract in TBS–TX–Ca derived from 200 × 10^6 cells had been incubated on the column. Samples were boiled and reduced with 50 mM 2-ME prior to loading onto a 3–15% SDS gradient gel which was subsequently stained with ammoniacal silver. Lanes 1–5 contain specifically bound material eluted with 50 mM histamine dissolved in TBS–TX–Ca. Lanes 6–10 contain fractions eluted with 0.1 M NaCl–acetate–acetic acid buffer, pH 3, immediately after histamine-specific elution.

acid buffer (lanes 6–9). The bands obtained were the same as those seen in the histamine-specific eluate except for the absence of the 58K band. During specific elution, histamine removed most but not all of the specifically bound material; the acid removed the remainder, most of which eluted in a single fraction (lane 6). The gel shown in Figure 2 had been silver stained more intensely than in Figure 1, producing a higher background and some minor bands which included a 15–21K cluster.

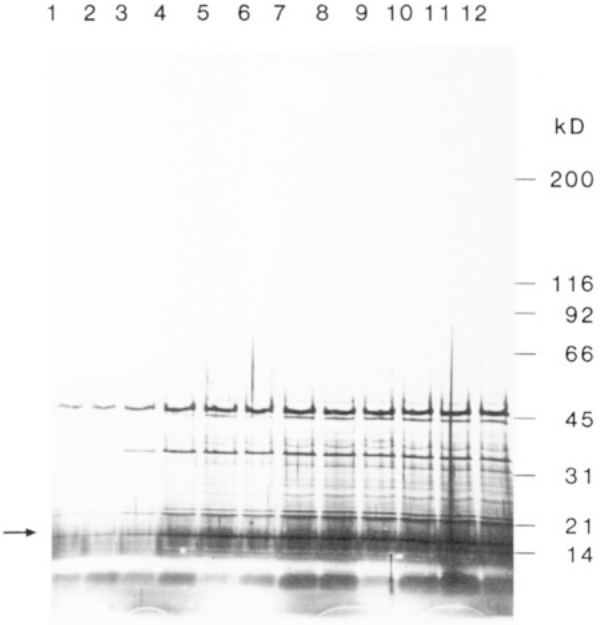

FIGURE 3: Progressive 1-mL fractions collected from the fast-flow AH-Sepharose affinity column after Triton X-100 solubilized mononuclear cell extract in TBS–TX–Ca derived from 200 × 10^6 cells had been incubated on the column. Samples were boiled and reduced with 50 mM 2-ME prior to loading onto a 3–15% SDS gradient gel which was subsequently stained with ammoniacal silver. Lanes 1–12 contain 1-mL fractions specifically eluted with 50 mM histamine dissolved in TBS–TX–Ca. Lane 1 contains the first fraction while lane 12 contains the last fraction.

Analysis of Eluates from the Fast-Flow AH-Sepharose Affinity Matrix. After the cell extract had been incubated with the matrix overnight, it was washed extensively and bound material was eluted with 50 mM histamine in TBS–TX–Ca. The results of this specific elution are seen in Figure 3. The most prominent bands which had a molecular weight of 48K, 37K, and 16K were identical to three of the bands seen in the eluates of the above two columns. The 48K band appeared as a doublet (as in all the eluates), the higher molecular weight component having the greater staining intensity. A minor intensity doublet around 19–21K was better seen here than in the above two column eluates. In the low molecular weight region, there were fewer bands clustering around the 16K band than in the other purifications. The column was then washed with the 0.1 M NaCl–acetate–acetic acid buffer. The most prominent bands eluted had molecular weights of 193K, 84K, 48K, and 37K which were identical to those seen in the previous two column eluates (Figure 4). The 58K and 16K bands were barely detectable.

An additional specificity experiment performed with this column was the preincubation of 5 mL of a fresh Triton X-100 extract derived from 500 × 10^6 mononuclear cells with unlabeled excess histamine so as to preabsorb out all the histamine-binding sites. This was performed for 2 h at 25 °C in a solution with a final concentration of the following: 0.1 M NaCl, 20 mM Tris–HCl, pH 8.0, 2 mM $CaCl_2$, and 50 mM histamine. This mixture was placed on the column overnight, and after extensive gel washing with TBS–TX–Ca the next morning, the column was eluted with NaCl–acetate–acetic acid, pH 3, buffer. A total of 40 1-mL fractions were collected and analyzed by SDS gel electrophoresis and

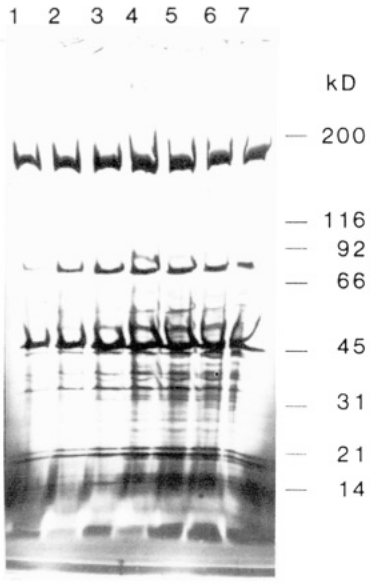

FIGURE 4: Progressive 1-mL fractions collected from the fast-flow AH-Sepharose affinity column after Triton X-100 solubilized mononuclear cell extract in TBS–TX–Ca derived from 200×10^6 cells had been incubated on the column. Samples were boiled and reduced with 50 mM 2-ME prior to loading onto a 3–15% SDS gradient gel which was subsequently stained with ammoniacal silver. Lanes 1–7 contain 1-mL fractions eluted with 0.1 M NaCl–acetate–acetic acid buffer, pH 3, immediately after histamine-specific elution shown in Figure 3. Lane 1 contains the first fraction while lane 7 contains the last fraction.

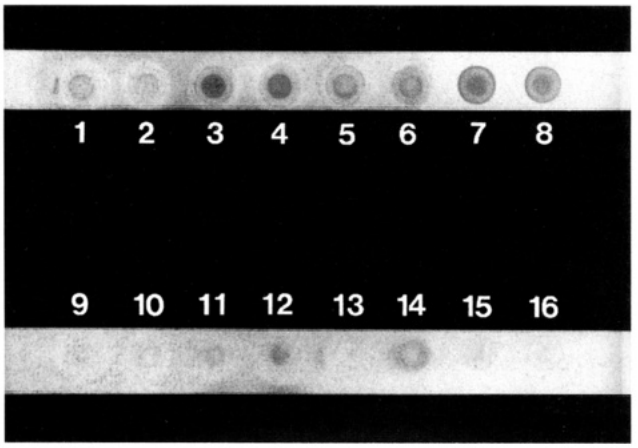

FIGURE 5: Autoradiogram of a dot blot radioreceptor assay using PVDF as the adsorbent matrix. The following materials were applied to each well: 50 μg of bovine hemoglobin to wells 1, 2, 9, and 10, 0.2–0.4 μg of purified protein to wells 3, 4, 11, and 12, 110–130 μg of mononuclear cell extract to wells 7, 8, 15, and 16. After washing with Tween–TBS–Ca, all wells were incubated with 1×10^{-8} M [^{3}H]histamine in Tween–TBS–Ca for 16 h; wells 9–16 inclusive also contained 50 mM unlabeled histamine in this incubation solution as a control for nonspecific binding. The wells were drained and washed three times with Tween–TBS–Ca, and the membrane was dried, sprayed three times with Enhance spray, dried again, and autoradiographed for 30 days.

ammoniacal silver staining. No material could be detected in any of these fractions (gel not shown).

Dot Blot Radioreceptor Assay. Figure 5 shows the result of the dot blot radioreceptor assay performed with [^{3}H]-histamine. In wells 3 and 4, the purified histamine-binding proteins derived by histamine affinity chromatography gave an intense signal, indicating binding by [^{3}H]histamine. A positive, though weaker, signal was also obtained for the whole cell extract (wells 7 and 8), whereas no signal could be seen in the blank wells (5 and 6), or those containing bovine

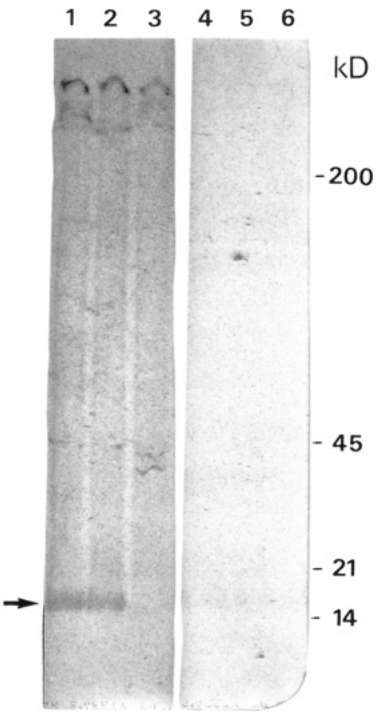

FIGURE 6: Autoradiogram of radioreceptor cross-linking of [^{3}H]-histamine to purified intact human peripheral blood mononuclear cells using DSS. About 10^7 cells per tube were incubated for 2 h in PBS–Ca with 5×10^{-8} M [^{3}H]histamine alone (lanes 1–3) or with addition of 50 mM histamine (lanes 4–6). The following final concentrations of DSS were added after the cells had been pelleted, the unbound histamine aspirated, and the cells resuspended in PBS–Ca: 0 mM (lanes 3 and 6); 0.0625 mM (lanes 2 and 5); 0.125 mM (lanes 1 and 4). After cross-linking, the cells were disrupted according to the regime stated in the Materials and Methods, solubilized with SDS, reduced with 50 mM 2-ME, boiled, and subjected to SDS–PAGE using a 3–15% gradient gel. The gel was impregnated in a fluorographic reagent, dried, and autoradiographed for 1 month. The arrow indicates the 16K band detected.

hemoglobin (1 and 2). These results indicate that both the crude tissue lysate and the purified material contained histamine-binding proteins, and that the [^{3}H]histamine bound neither to an irrelevent protein (bovine hemoglobin) nor to the PVDF membrane alone. The strongest signal seen with the purified proteins was consistent with a concentration effect. A further verification of the specificity of histamine binding was the abrogation of the positive signals obtained for the purified material and crude cell extract when excess, unlabeled histamine was included during the [^{3}H]histamine incubation step.

In devising this radioreceptor assay, several adsorbent matrices were tested, with PVDF giving the clearest discrimination between positive and negative signals. Nitrocellulose also gave satisfactory results, but with slightly higher nonspecific binding of [^{3}H]histamine, whereas nylon membranes were entirely unsuitable due to the high nonspecific binding of the [^{3}H]histamine to the membrane, in spite of the addition of various combinations of detergents.

Surface Affinity Cross-Linking of Whole Mononuclear Cells. In Figure 6, surface radioreceptor cross-linking with [^{3}H]histamine using DSS is shown. A 16K band was clearly and unequivocally identified (lanes 1 and 2). A broad high molecular weight band just below the application well was also detected in these lanes. Where DSS had been omitted, no bands could be detected, even after 2 months of autoradiographic exposure (lane 3). The inclusion of excess unlabeled histamine in the incubations led to the disappearance of the

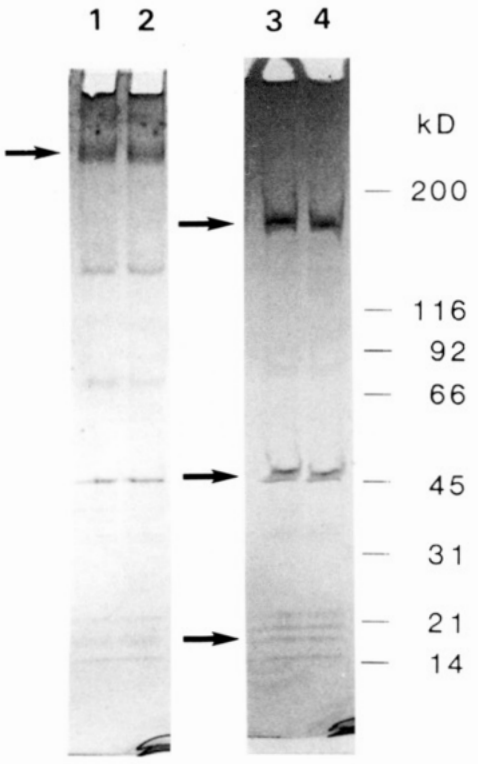

FIGURE 7: Analysis by SDS–PAGE of the structural variation of unreduced and reduced histamine-binding proteins purified from human blood mononuclear cells using the histidine fast-flow AH-Sepharose affinity column. Duplicate samples of acid-eluted purified proteins were electrophoresed by SDS–PAGE using a 3–15% gradient gel, which was subsequently stained with colloidal Coomassie Blue. Proteins in lanes 3 and 4 were boiled in sample buffer at 100 °C for 5 min in the presence of 50 mM 2-ME, whereas in lanes 1 and 2, 2-ME was absent from the sample buffer and the samples were left unboiled. In lanes 1 and 2 containing the unboiled, unreduced proteins, the arrow indicates the high molecular weight complex. In lanes 3 and 4, which contains the boiled, reduced purified proteins, the top arrow indicates the 193K band, the middle arrow the 48K band, and the bottom arrow the 16K band.

high molecular weight band and the 16K band (lanes 4 and 5). The 16K band was also detected when EGS was used as a cross-linker, but this band could not be detected when DMA and DMP were used (data not shown). This 16K band migrated to the same position as the prominent 16K band seen on SDS–PAGE of boiled, reduced histamine-binding proteins purified from Triton X-100 extracts of human mononuclear cells.

Subunit Structure of Histamine-Binding Proteins Purified from Blood Mononuclear Cells. A comparison was made of the purified material in SDS–PAGE before and after sample reduction with 50 mM 2-ME (Figure 7). In the presence of the reducing agent 2-ME (lanes 3 and 4), the 193K, 48K, and 16K bands were clearly seen and are indicated with arrows. In the absence of reducing agent, the 193K could not be detected and the 48K and 16K band intensities were almost completely abrogated, indicating that these three bands are subunits which are disulfide bonded together. Their parent high molecular weight molecule could be visualized effectively under nonreducing conditions using a sensitive colloidal Coomassie Blue stain (though not by silver stain), and it is indicated by an arrow as shown in Figure 7 (lanes 1 and 2). In this figure, an additional 150K band can be identified; it only became apparent when the purified material was in both an unreduced and unboiled state.

Tissue Specificity of Purified Histamine-Binding Proteins. In order to determine whether the purified histamine-binding proteins were isolated from the lymphocyte or monocyte subset of the mononuclear cell extract, we also purified histamine-binding proteins from the lysates extracted from two cell lines using the same method as described for the mononuclear cells: U937 which has monocyte-like characteristics and is derived from a human histiocytic lymphoma (Sundstrom & Nilsson, 1974) and also Jurkat which is of T lymphocyte lineage (Weiss et al., 1984). As can be seen in Figure 8, the 193K, 48K, and 16K proteins could be detected in the acid eluates of both the U937 (lanes 1 and 2) and Jurkat (lanes 3 and 4) cells following histamine affinity chromatography. This indicates that both lymphocytes and monocytes express these proteins. We also purified human neutrophil extracts by histamine affinity chromatography, and obtained the 16K protein in the acid eluate, as well as 12K, 20K, 30K, and 50K proteins, but could not detect the 48K or 193K protein (details of this work will be published in a separate report). It therefore appears that the 193K and 48K bands are specific to mononuclear cells, whereas the 16K band, which is the ligand-binding subunit to histamine is present on monocytes, lymphocytes, and neutrophils.

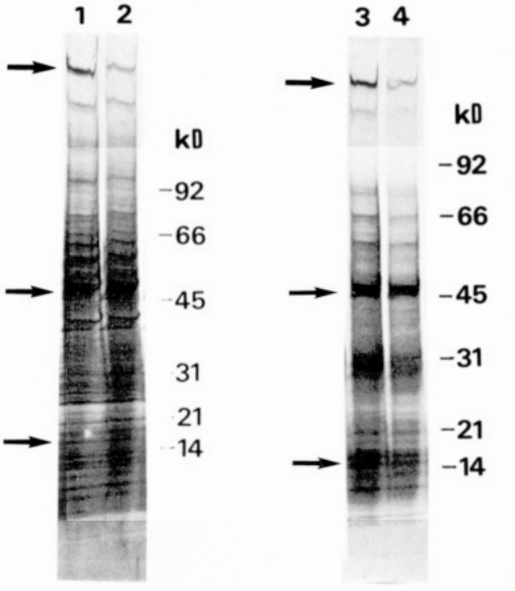

FIGURE 8: Analysis by SDS–PAGE of reduced, boiled histamine-binding proteins purified from U937 (lanes 1 and 2) and Jurkat (lanes 3 and 4) cells using the histidine fast-flow AH-Sepharose affinity column. Duplicate lanes of acid-eluted purified proteins were electrophoresed by SDS–PAGE using a 3–15% gradient gel. The sample buffer contained 50 mM 2-ME, and the samples were boiled for 5 min prior to well application. The gel was silver stained as described in the Materials and Methods. The top arrow indicates the 193K band, the middle arrow the 48K band, and the bottom arrow the 16K band.

DISCUSSION

Characteristics of the Three Conjugated Sepharose Matrices. On amino acid analysis, the histidine AH-Sepharose had a slightly higher coupling efficiency [79.9 μmol/(g of Sepharose)] than the fast-flow AH-Sepharose matrix [52.1 μmol/(g of Sepharose)]. Both these figures represented excellent levels of coupling efficiency, given that 200 μmol of histidine was available per gram of gel. In the case of the commercially available AH-Sepharose, the above figure is actually better than the stated coupling efficiency which is 24–40 μmol/(g of gel). The fact that a composition determination could be made indicated that the contractile effects of the piperidine on the agarose component of the Sepharose did not denature or detach the coupled histidine. Although

the results are expressed as amounts of histidine bound to Sepharose, it can be assumed that the moiety being detected was histamine since the carboxyl group of the histidine, which was used as the coupler to Sepharose, would assume the configuration of the seventh carbon spacer projecting from the matrix. The histidine would in effect be converted to histamine, just as occurs during its *in vivo* synthesis, except that it is now immobilized to Sepharose through the converted carboxyl group. An amino acid analysis could not be performed for the histamine Sepharose column as the analyzer was unable to detect histamine *per se*. The known coupling efficiency for this type of conjugation is generally about 90% according to the literature, however (Sundberg & Porath, 1974), i.e., 100 μmol/(g of gel).

Selection of Purification Conditions. The buffer, electrolyte, and detergent compositions and concentrations selected for specific histamine binding to the affinity matrix and for subsequent specific elution were determined by examining the data derived from the ligand-binding experiments previously reported (Warlow et al., 1986; Warlow & Bernard, 1987). The extraction buffer which had been shown to give the highest ligand binding, particularly of the moderate and high-affinity sites on human peripheral blood mononuclear cells, was 0.10 M NaCl, 0.5% Triton X-100. Pilot purifications had shown that a greater yield of purified material was obtained when the protease inhibitor PMSF was included in this extraction buffer. Another factor which considerably improved the yield was to perform the extraction, centrifugation, and affinity purification on one day. If the extract was frozen at −70 °C prior to purification, on rethawing, precipitable material appeared in the extract, regardless of whether it had been centrifuged previously at 100000*g*, and this resulted in a considerable decrease in the yield of purified material. When the whole procedure, including centrifugation, was performed in continuum, hardly any material appeared as precipitate after ultracentrifugation, indicating that protein precipitation was a direct result of freeze–thawing. The extract was reconstituted in a calcium-containing buffer as this divalent cation had been shown to enhance the binding of histamine to its binding sites. For this reason, EDTA was not included as a protease inhibitor as the reconstitution of the optimal calcium concentration of 2 mM would have been difficult to control. Tris was shown as the ambient buffer as phosphate-containing buffers resulted in precipitation of solubilized histamine-binding proteins, and pH 8.0 was selected as this was the optimal pH for binding, particularly of the moderate- and high-affinity sites.

We had previously shown that millimolar concentrations of histamine were required to completely inhibit binding to both high- and low-affinity binding sites on human mononuclear cells (Warlow et al., 1986; Warlow & Bernard, 1987). Initial pilot purification experiments revealed that the concentration of histamine required for the complete competitive displacement of binding proteins to immobilized histamine was actually greater than that required in the free state, indicating that histamine-binding proteins adhered more avidly to column-coupled than to native histamine in the fluid phase. This may have been due to an alteration in the steric structure of histamine, resulting in an increase in the affinity of the ligand for its binding site or because histamine was coupled via a spacer, thereby allowing the binding molecule to wrap itself around this large, inflexible immobilized molecule. Alternatively, the spacer and/or Sepharose may have exerted nonspecific binding forces on the bound material, rendering displacement more difficult. All these phenomena have previously been reported to retard the elution of specifically bound proteins, frequently necessitating higher concentrations of ligand for competitive elution (Jacobs & Cuatracasas, 1981). Therefore, millimolar amounts of unlabeled histamine were generally used for specific elution studies as this gave greater recovery and therefore easier detection of specifically bound material. When lower concentrations of histamine were used for elution, identical bands in smaller amounts were always obtained. The sodium acetate, pH 3.0, used for acid elution was the same buffer that effectively resulted in the dissociation of histamine following binding to moderate- and high-affinity binding sites. Acetic acid was selected as it is the mildest and least denaturing of acids to proteins. The collection buffer, which gave a final concentration of 0.17 M Tris, restored the acid eluate to a pH of 8.0 while the 6.7% glycerol was shown on pilot purifications to considerably inhibit the structural degradation of the eluted proteins due to freezing and thawing.

Histamine-Binding Specificity of Proteins Purified from the Three Affinity Matrices. The proteins derived from the histamine affinity matrices by specific histamine elution followed by acid elution were identical. This indicated that no unspecifically bound material remained adherent to the column following the postincubation washes. Both histamine Sepharose and histidine AH-Sepharose matrices recognized the same binding proteins even though the column designs and the matrices were different. No proteins could be detected by the silver staining of subsequent acid eluates. As an additional control for column design, cell extract was incubated with an unconjugated, inert Sepharose column; histamine-binding proteins did not adhere to this column and simply eluted in the void volume. As a test of the steric binding specificity of the histamine affinity column, a Sepharose column conjugated with ethanolamine was incubated with cell extract; after extensive washing, the same proteins (193K, 48K, and 16K) eluted off with acid, indicating that histamine's ethylamine side chain (and not the imidazole ring) was the predominant epitope recognized by the 16K ligand-binding subunit (data not shown). It is therefore most unlikely that other proteins which bind to protides possessing an imidazole ring, e.g., histidine and serotonin, have been copurified, and also indicates the ethylamine-restricted histamine specificity of the proteins isolated by us.

Selective elution of only the 48K, 37K, 21K dimer, and 16K bands was seen in the histamine-specific eluate from the fast-flow Sepharose affinity column. These were also the most abundant bands obtained in the histamine-specific eluates from the other two affinity columns. It is probably that they were more intimately involved in ligand binding to coupled histamine than the high molecular weight bands (rather than because they represented low-affinity histamine-binding sites which eluted more easily). Therefore, selective elution can best be explained on the basis that the fast-flow Sepharose exerted greater nonspecific binding forces than Sepharose on the higher molecular weight subunits, resulting in selective subunit retention and dissociation. This deduction is supported by the observation that this dissociation phenomenon did not occur with the other two Sepharose-based matrices. As an additional evaluation of the specificity of the fast-flow AH-Sepharose, histamine was preincubated with a mononuclear cell extract and was then introduced onto the gel. This preincubation resulted in complete binding inhibition of proteins to the affinity matrix.

The presence of the four bands in the low molecular weight group is difficult to reconcile with the idea of one binding site unless they are separate subunits in close association with the

ligand-binding subunit. One ligand-binding subunit only could be identified on human mononuclear cells by surface affinity cross-linking, and that was the 16K band. In all of our purification work, only the 48K, 37K, and 16K bands have ever appeared reproducibly and abundantly in all eluate fractions. The 21K band often stained at low intensities and in only a few fractions. Therefore, it is most likely that the 48K and 37K subunits copurified with the 16K ligand-binding subunit and they bound together more intimately than to the higher molecular weight subunits. The 21K band along with the other low molecular weight, low-intensity bands seen in silver-stained gels most likely represented small amounts of denatured protein fragments stripped off larger molecules by the SDS and were detected because of the nanogram detection sensitivity of the ammoniacal silver stain (Wray et al., 1981). The protein from which this fragment would most likely be derived is either the 48K or the 37K band as there are no higher molecular weight bands present in this eluate. Another possible explanation is that some of the eluted proteins represented either copurified regulatory proteins bound to the receptor or nonspecifically bound contaminants. The latter explanation is unlikely because preabsorption with excess free histamine resulted in the complete abrogation of protein binding to the affinity gel and because specific histamine elution, followed by acid elution, revealed identical band patterns. Also, the binding of the same nonspecifically bound proteins to two different compositions of Sepharose would be a remarkable and unlikely coincidence.

Radioreceptor Dot Blot Estimates of Histamine-Binding Specificity and Degree of Purification. The radioreceptor dot blot results shown in Figure 5 clearly demonstrate that histamine-specific binding proteins have been successfully affinity purified. The significance of this result in terms of the specificity of the proteins can be further appreciated by calculating their concentrations in each well: bovine hemoglobin, 50 μg per well (1, 2, 9, and 10); mononuclear cell extract, 110–130 μg per well (7, 8, 15, and 16); and purified histamine-binding protein, 0.2–0.4 μg per well (3, 4, 11, and 12) (the latter figure was derived from densitometry analysis of Coomassie Blue stained gels of the purified proteins). From this it can be seen that the hemoglobin and cell extract containing wells contained 120–350 times the amount of protein present in wells filled with purified material. Both the whole cell extract and the affinity-purified proteins specifically bound radiolabeled histamine, and because the signal strengths were higher in the purified protein wells, it can be inferred from the above figures that in excess of a 300–500-fold purification had been achieved. The specificity of histamine binding was unequivocally established by the demonstration that the binding of radiolabeled histamine could be abrogated by excess unlabeled histamine and that pure unrelated protein which was present in 100-fold greater amounts did not bind the radiolabeled histamine under the same incubation conditions. Also, wells containing unrelated protein, i.e., bovine hemoglobin, gave the same low signal levels as the blank wells, and those which were filled with either cell extract or purified receptor proteins and then co-incubated with excess unlabeled histamine gave low signals which were indicative of nonspecific binding. Because the purified receptor fraction selected for dot blotting did not contain the 58K band (a possible dimer of histamine methyltransferase), the specific histamine binding detected in this fraction indicated that novel histamine-binding proteins from human mononuclear cells had been purified.

The dot blot assay described here was the most convenient method for detecting the presence of receptors in eluted fractions. It was chosen over other methods because of its rapidity and ease of performance. Up to 96 samples could be applied per tray, nonspecific binding could be minimized by testing various blocking agents and nonionic detergents in a checkerboard format, and ambient buffer conditions were easily altered without any dilutional effects, i.e., the rapid removal of glycerol and acetic acid and their replacement with TBS–Ca.

PVDF membrane in combination with 0.05% Tween 20 gave the highest signal to noise ratio. The next best result was obtained with nitrocellulose in combination with the blocker 3% BSA and a detergent (either Tween 20 or Triton X-100). Similar combinations of reagents have been reported as optimal for the immunodetection of electroblotted antigens (Batteiger et al., 1982). Nylon membranes and nitrocellulose with Tween alone gave unacceptably high backgrounds as did nitrocellulose with BSA alone, probably because histamine is somewhat hydrophobic (Verburg & Henry, 1984). The addition of the detergent along with 3% BSA markedly reduced the nonspecific binding of the radiolabeled histamine to nitrocellulose, probably by inhibiting its hydrophobic binding to the matrix. In addition to reducing the nonspecific binding of [^{3}H]histamine to the membrane, the detergent may have assisted ligand binding to the receptor by renaturation effects as has been observed for other receptor systems (Haeuptle et al., 1983; Flanagan & Yost, 1984).

Surface Affinity Cross-Linking of [^{3}H]Histamine to Whole Mononuclear Cells. The detection, after affinity cross-linking, of the same 16K band under reducing conditions as that seen in the histamine-specific eluate from all three affinity columns indicates this to be the ligand-binding subunit of a histamine-binding protein present on the surface of peripheral blood mononuclear cells. The inclusion of a covalent cross-linker was essential since without it, neither the 16K nor any other band could be detected. This indicated that histamine binding to the 16K band occurred at the cell surface, it was a specific and not a hydrophobic process, and SDS disrupted the ligand–receptor complex if cross-linker was omitted. This band was one of the most abundant obtained by histamine elution and was one of only four bands that specifically eluted from the fast-flow AH-Sepharose affinity gel, further indicating it to be a ligand-binding subunit. Both DSS and EGS were successful in covalently bonding the radiolabeled histamine to the 16K band, whereas both DMA and DMP failed to do so. Perhaps this was because their chain lengths were at least twice those of DMA and DMP or because their succinimide reactive groups were more appropriate for binding histamine to its receptor than the imidoester groups on DMA and DMP. The appearance of an ill-defined high molecular weight band occurring in parallel with the 16K band suggested that the 16K band is a constituent subunit of a partially degraded high molecular weight parent molecule.

Subunit Structure of the Novel Histamine-Binding Protein. The 16K band was shown to be the ligand-binding subunit by affinity cross-linking. In mononuclear cells, the 16K band is covalently linked through disulfide bonds to the 193K and the 48K bands such that all three subunits are constituents of a high molecular weight unreduced band. These three bands could constitute the entire receptor complex, giving a cumulative molecular weight of 257K. Alternatively, if all of the major bands are detected by SDS–PAGE under reduced conditions (which in addition include the 84K and 37K bands which are not disulfide bonded to the other three bands) the

cumulative molecular weight of the intact receptor would be 378K. Alternatively, the 84K and 37K bands may simply have copurified because they are weakly bonded structural or regulatory proteins. The 150K band which is detected in SDS gels only in the unboiled, unreduced state is most likely to be a metastable intermediate breakdown product of the intact molecule. This is because a protein is formally designated as a subunit on the basis that its identification has been made by SDS–PAGE after the sample has been both boiled and reduced. The boiling and reduction disrupt both naturally occurring and artifactually formed polymers and intermediate variants, breaking them all down into subunits (Hames, 1981). Further subunit studies are currently in progress to ascertain the precise subunit composition of the high molecular weight histamine-binding protein.

Molecular Weight Comparisons of Histamine-Binding Proteins. Earlier studies have attempted to identify the structure of histamine-binding proteins on the surface of cells by protein chemistry methodologies. The first was devoted to the partial purification of histamine receptors from the smooth muscle of cat intensities and was accomplished by a combination of adsorptions with H_1 antihistamines, sucrose density gradient, and gel filtration. The material obtained was shown bind to tritiated histamine by ligand binding, and the molecular weight was estimated to be about 100K (Uchida, 1978). In the second report, crude purification of histamine receptors from calf thymocytes was attempted using single-step ion-exchange chromatography. The researchers reported that DEAE cellulose selectively eluted H_1 binding sites whereas phosphocellulose selectively eluted H_2 binding sites. Histamine binding *per se* was characterized using tritiated histamine. The molecular weight of H_1 binding sites was estimated as 50K, and that of H_2 sites was estimated as 40K (Osband & McCaffrey, 1979). In neither of these reports was the purified material examined by SDS gel electrophoresis, and thus determinations of purity and subunit structure were not made. Both studies reported molecular weights by gel extrusion chromatography in the presence of Triton X-100. The values derived must therefore be viewed with caution, given that receptors are often asymmetric molecules, the size and shape of which may become artifactually altered by detergents, resulting in physicochemical characteristics different from those of the globular protein standards used (Nozaki et al., 1976). A subsequent report has described irreversible photoaffinity labeling of a radiolabeled H_1 antagonist to guinea pig brain membranes (Ruat et al., 1988). In that study a 350–400K band was detected which after 2-ME treatment produced 56K and 47K bands. An inherent assumption in these studies was that antihistamines bound preferentially to histamine receptors *per se*.

We considered the possibility that some of the purified proteins described in this paper may represent histamine-binding proteins inside mononuclear cells which are not present on the cell surface but may have been coincidentally and partially extracted during Triton X-100 detergent solubilization of the cell membranes. The most likely candidates are the cytoplasmic catalytic enzymes histamine methyltransferase and diamine oxidase which are known to bind specifically to histamine. The published molecular weight of the monomeric form of histamine methyltransferase derived from rat and mouse brain is 29K, with dimers of 58K and tetramers of 116K (Axelrod & Vessell, 1970; Sellinger et al., 1978). The dimer has the same molecular weight as a 58K band detected in a few of the histamine-specific eluates and could be that enzyme, particularly as it is known to be present in low amounts in human monocytes [10.08 (nmol/h)/(mg of protein)] and in lymphocytes to an even lesser extent [0.77 (nmol/h)/(mg of protein)] (Zeiger et al., 1976). It was less abundant than the other major bands detected, which would be consistent with the partial extraction of a cytoplasmic protein from Triton X-100 treatment of intact cells, and displayed unusual elution characteristics as compared with the other bands, suggesting it to be unique and unrelated. The acid eluate fractions selected for the dot blotting assay did not contain this 58K band. Therefore, the histamine binding detected in the eluate was not due to this enzyme. The reported molecular structure of diamine oxidase is as a 170–172K disulfide-bonded dimer in hog kidney and bovine plasma, with subunits of 85–91K (Kluetz & Schmidt, 1977; Achee et al., 1969). In human placenta, monomers of 90K have been characterized to date (Bardsley et al., 1968). Because there were no bands in the gel eluates that had the same molecular weights as either the monomeric or polymeric forms of this enzyme, it is unlikely that it constitutes any of the histamine-binding proteins purified. This is unsurprising, considering that its enzymatic activity cannot be detected in either human monocytes or lymphocytes (Zeiger et al., 1976; Korpela et al., 1981). The other enzyme which might conceivably bind to histamine if only with a low affinity is the synthetic enzyme histidine decarboxylase. This enzyme as derived from rat liver has a reported molecular weight of 210K and consists of a 145K and a 66K subunit (Tran & Snyder, 1981). Again these bear no correlation to the molecular weights of any of the purified bands. Therefore, it must be concluded that a previously undescribed histamine-binding protein has been purified from human blood mononuclear cells. Because the majority of proteins present in the Triton X-100 solubilized extracts were derived from the cell surface and the 16K subunit was detected by surface affinity cross-linking, it seems likely that the novel high molecular weight complex histamine-binding protein represents a histamine receptor.

Two groups have recently detailed the gene cloning and DNA sequencing of H_1 receptors (from bovine adrenal cells) and H_2 receptors (from canine and human parietal cells) using molecular biology techniques without prior protein purification and amino acid sequencing. The cloning of the H_1 receptor was achieved by expression cloning in xenopus oocytes with subsequent detection of biological response to histamine by the specifically transfected clone using a voltage clamp electrophysiological assay (Yamashita et al., 1991a). Expression of the cloned gene in COS cells revealed high-affinity (nanomolar K_d) binding to mepyramine, and this could be competitively displaced by histamine. The gene codes for a single polypeptide chain with seven predicted transmembrane domains includes a sequence for G protein binding and has a predicted molecular weight of 56K, which is consistent with the values obtained by photoaffinity labeling with an H_1 ligand to bovine adrenal (53–58K) (Yamashita et al., 1991b) and guinea pig tissues (56–57K) (Ruat et al., 1990). The cloning of the H_2 receptor was achieved by polymerase chain reaction amplification of a parietal cell cDNA library using degenerate oligonucleotide primers homologous to the guanine nucleotide binding domains of already cloned G protein-linked receptors. Verification of the H_2 specificity was demonstrated by gene transfection into L cells and then measuring histamine stimulated cAMP production with and without cimetidine inhibition and also by specific binding to radiolabeled tiotidine. The predicted molecular weight of the single gene product obtained is 36K (Gantz et al., 1991a; Gantz et al., 1991b).

We chose to use conventional protein chemistry techniques to characterize histamine-binding proteins in order to isolate all associated subunits, and in conjunction with native histamine as we were concerned that synthetically produced ligands may not preferentially bind to histamine's ligand binding site *per se* (though still capable of acting as a competitive inhibitor to histamine), or could possess hydrophobic properties not present in native histamine, resulting in nonspecific binding of proteins. It is noteworthy that H_1, H_2, and H_3 antagonists are so designated by their ability to antagonize various biological actions of histamine; that they also preferentially bind to histamine's ligand-binding site on its receptor with high affinity, rather than to other regions of the receptor or even to other molecules is yet to be determined. In this paper, we have shown that the 193K/48K/16K complex purified by histamine affinity chromatography from human mononuclear cells, in its intact form, binds specifically to histamine in a radioreceptor assay, and also that the same mononuclear cells affinity cross-linked native histamine through a 16K protein at the cell surface, this having the same molecular weight as the 16K protein purified by histamine affinity chromatography. Therefore, two independent assays which both utilize native histamine in specific binding techniques have demonstrated a 16K protein to be involved in histamine binding, and because this same protein cross-linked with histamine at the cell surface, it must be the ligand-binding subunit of the purified trimolecular receptor complex. The relationship that this 16K band and its disulfide-linked 193K and 48K subunits have to the high- and low-affinity histamine-binding sites on the Triton X-100 solubilized extracts of human blood mononuclear cells described previously by us in a radioreceptor assay (Warlow et al., 1986; Warlow & Bernard, 1987) and to H_1, H_2, and H_3 pharmacologically defined histamine receptors awaits elucidation. We have also shown that the 193K/48K/16K complex could be purified from lymphocyte and monocytoid cell lines, indicating that these proteins are expressed in both of these subsets in mononuclear cells.

The differences in molecular weights between the cloned adrenal H_1 and parietal cell H_2 receptors and the histamine-binding 16K subunit disulfide bound to 48K and 193K subunits on human mononuclear cells as reported by us may be a reflection of species or tissue variation. An alternative and increasingly likely explanation is that there may be greater genetic heterogeneity of histamine receptors than has hitherto been suspected on the basis of pharmacological classification. This has certainly proved to be the case for other receptors; to date, six genetically distinct subtypes of dopamine receptors (Hayes & Shine, 1992), five subtypes of the rat muscarinic receptors, and three subtypes of serotonergic receptors have been identified at a DNA level (Caron, 1989). Multiple receptor variants would certainly explain the diverse range of biological actions observed for hormones even in a single tissue, with each receptor subtype mediating a specific physiological function. In the case of histamine receptors, this could include such disparate actions as smooth muscle contraction, gastric acid secretion, cardiac chronotropism, cAMP production, and T cell cytotoxicity. Such putative variants may be more or less susceptible to H_1, H_2, and/or H_3 antagonists, and indeed may be specifically bound by some of these. Preliminary evidence for this at the DNA level suggesting that there are multiple variants of histamine receptors within a species derives from the group that cloned the H_1 receptor gene. They could not detect H_1 receptor mRNA in cardiac atria or liver, with only very low levels of expression in lymphocytes and cerebral cortex, even though all these tissues are known to exhibit considerable H_1 receptor pharmacological responses. It will be interesting to compare the DNA sequence that we obtain for the histamine receptor subunits purified from human mononuclear cells (which include lymphocytes) described in this report to the already cloned H_1 and H_2 receptor genes to determine whether homologies exist. We are currently performing sequencing studies on these purified proteins. In addition, in studies to be reported, we were able to purify the 16K protein from human neutrophils by histamine affinity chromatography, but the 193K and 48K proteins were not present in any of the acid eluates, adding further evidence to the concept of molecular heterogeneity of histamine receptors because of the tissue-specific differences thus identified.

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