

Localization of antigen in experimental bronchoconstriction in guinea pigs

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These experiments were done to examine the position of the antigen in the tracheo-bronchial mucosa in a situation in which the antigen caused allergic bronchoconstriction. Guinea pigs were sensitized to the tracer protein, horseradish peroxidase (M.W. 40,000), and these animals were challenged with an aerosol of this antigen while lung mechanics were measured. We found that airways resistance rose significantly in sensitized animals ($p < 0.02$) while nonsensitized animals exposed to the same aerosol showed no change. The animals were sacrificed and the antigen was localized with an electron microscopic histochemical technique. Studies were also done with isolated, perfused lungs from sensitized animals challenged intravenously with the antigen. In both the in vivo studies, in which bronchoconstriction was demonstrated, and the in vitro studies, in which mediator release was demonstrated, the antigen had a superficial distribution and was not seen to penetrate the epithelial or endothelial layers. These findings suggest that the antigen-antibody reaction may occur at these sites.

In recent years there has been great interest in the junctions between cells. These junctions can be separated into two types. In one type there is fusion of the outer leaflets of adjacent cell membranes (occludens junction) and on the other, the outer leaflets of adjacent membranes are separated by a space (adherens junction).¹ Both types of junction can be further classified according to whether they completely surround the cell, partially surround the cell, or occur only at points on the cell surface.¹ Thus the term *zonula occludens* (tight junction) indicates that there is membrane-to-membrane contact at all lateral surfaces of epithelial cells near their free border. This type of tight junction is found in the respiratory and gastrointestinal tract epithelia and has been shown to be impermeable to large molecules by a number of investigators.²⁻⁴ The presence of tight junctions in the tracheobronchial tree and between alveolar cells raises the question as to how large molecular weight antigens are able to get through the epithelial layer to the interstitium of the lung where the antigen is postulated to react with mast cells, release mediators, and cause bronchoconstriction. In the present study, we investigated this problem with the tracer protein horseradish peroxidase (M.W. 40,000), used to sensitize guinea pigs. After sensitization, the guinea pigs were exposed to an aerosol of horseradish peroxidase, the bronchoconstrictive response to this antigen measured, and the

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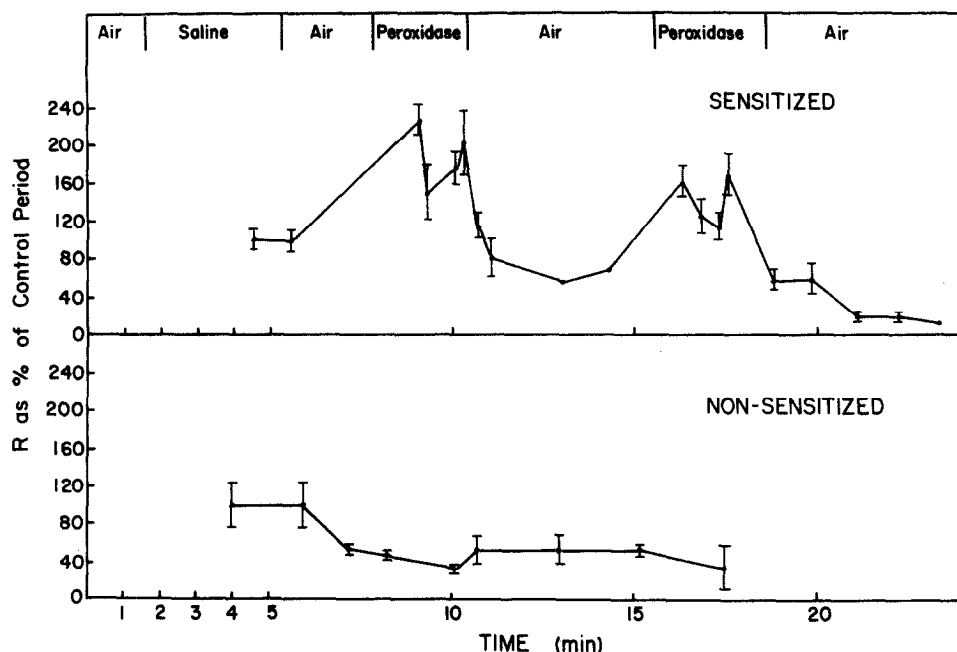


FIG. 1. Data from guinea pigs Nos. 5 and 11 (Table I) showing the time sequence of exposure to peroxidase and the marked increase in airways resistance observed in the sensitized animal. $\bar{x}$ = mean $\pm$ S. E. of 4 to 8 breaths.

TABLE I. Data from all experiments comparing airways resistance (cm. H₂O/ml./sec.) in the control period to that in the first and second peroxidase (HRP) exposures*

No.	Weight (Gm.)	Control	HRP No. 1	HRP No. 2
<i>Sensitized</i>				
1	515	0.73	0.94	1.36
2	430	0.05	0.19	0.68
3	344	0.71	1.50	1.61
4	277	0.26	0.99	1.33
5	277	0.30	0.64	0.47
6	370	0.43	0.60	0.75
Mean	368	0.41	0.80	1.03
$\pm$ S.E.	37	0.11	0.18	0.18
			p < .02	p < .01
<i>Nonsensitized</i>				
7	585	0.28	0.25	0.22
8	338	0.64	—	—
9	435	0.38	0.19	0.17
10	507	0.39	0.33	0.10
11	380	0.22	0.15	0.15
12	280	0.68	—	—
Mean	420	0.43	0.23	0.16
$\pm$ S.E.	45	0.08	0.04	0.02
			p > 0.05	p > 0.05

*p values refer to comparison of peroxidase exposure to control using t test for paired data.

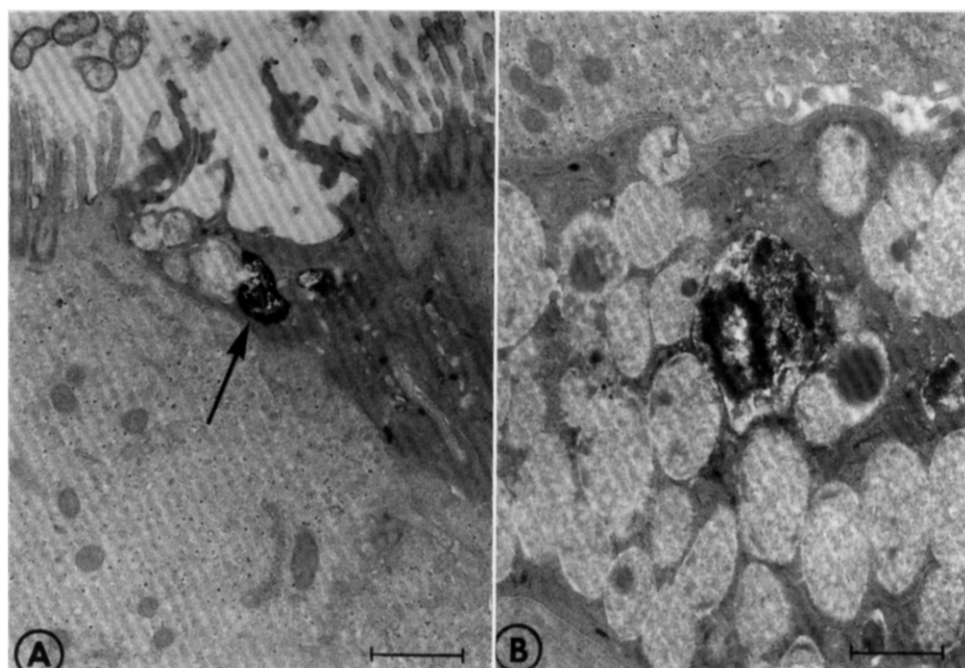


FIG. 2. **A**, Tracheal mucosa with the histochemical reaction product (arrow) within the superficial part of a goblet cell. Reaction product is also seen between the cilia and within small vesicles. **B**, Localized collection of reaction product within a goblet cell. **C**, Bronchial tissue from a guinea pig that had horseradish peroxidase put directly into the trachea. The reaction product is seen on the epithelial surface (arrow 1). Mucous droplets are present in the cell. Mast cells containing granules (arrow 2) are seen in the submucosa. **D**, Two goblet cells filled with reaction product. Reaction product is seen on the cilia but not beneath the basement membrane of the mucosa. **A** and **B** are representative of the animals exposed to the aerosol. **C** and **D** are those in which the horseradish peroxidase was put directly into the tracheobronchial tree. The markers indicate 1 μ .

position of the antigen determined by both the light and electron microscopes. Isolated guinea pig lungs, obtained from animals sensitized to peroxidase, were perfused so that the antigen-stimulated release of mediators of bronchoconstriction could be observed.⁵ These lungs were challenged with peroxidase, the release of mediators was measured, the lungs fixed, and the position of the peroxidase antigen determined.

METHODS

In vivo studies of bronchial challenge

Twelve guinea pigs of either sex, 368 ± 45 Gm., were immunized with 1.0 mg. of horseradish peroxidase (Nutritional Biochemicals Corporation, Cleveland, Ohio) administered intraperitoneally in 1.0 ml. of saline. The injection was repeated 10 days later and then, after a further 7 to 10 days, 6 of the animals were exposed to an aerosol of horseradish peroxidase generated by a DeVilbiss ultrasonic nebulizer (Model 2-100) with 20 mg. of peroxidase in 100 ml. of saline. The lung mechanics were determined in the unanesthetized state with the technique of Amdur and Mead.⁶ Six nonimmunized guinea pigs (420 ± 45 Gm.) were exposed to the aerosol under similar conditions and provided the control group. In the lung mechanics

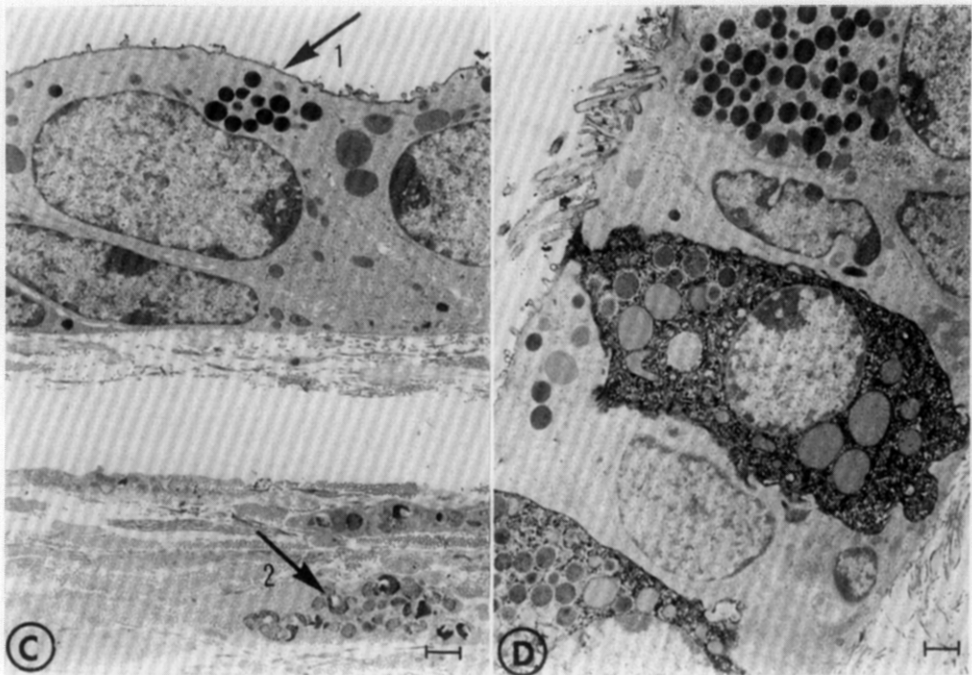


FIG. 2, C and D. For legend see opposite page.

study, control measurements were obtained during exposure to the saline aerosol. The animals were then exposed to the peroxidase aerosol for 3 to 5 minutes and then allowed to breathe air for 5 minutes before a second exposure to the peroxidase (Fig. 1). In two experiments, immunized animals were anesthetized with pentobarbital intraperitoneally (35 mg. per kilogram), a tracheostomy was performed, and the blood pressure was measured from a carotid artery. Horseradish peroxidase (3 to 5 mg.) was then blown down the trachea with a small rubber bulb and a needle. After exposure to the aerosol the animals were sacrificed.

In vitro studies of vascular challenge

In 5 experiments, lungs from immunized animals were perfused with Krebs-Hanseleit solution, and the perfusate superfused assay tissues.⁵ Ten mg. of horseradish peroxidase (Nutritional Biochemicals Corporation) or 2.5 mg. of Sigma VI (Sigma Chemical Corporation, St. Louis, Missouri) were injected into the perfusate, and, after the assay tissues showed a response to the released mediators, the perfusion was stopped and the lung fixed.

In both the in vivo and in vitro studies, tissue samples from the trachea, bronchi, and peripheral lung were fixed in 3 per cent phosphate-buffered glutaraldehyde for 3 hours and washed overnight in the same buffer. The tissue was cut into pieces averaging 5 mm. in length and one mm. in diameter and placed in a saturated solution of 3,3' diaminobenzidine in 0.05M Tris pH 7.6 for 20 minutes and then in a similar solution with the addition of H_2O_2 to a final concentration of 0.01 per cent for 30 minutes.⁷ The tissue was then placed in a 2 per cent solution of osmium tetroxide for one hour, dehydrated in alcohol, and embedded in Epon. The 3,3' diaminobenzidine is oxidized in the presence of peroxidase and H_2O_2 , and the oxidized product reacts with osmium tetroxide to form an electron-dense material. Sections were examined in both an unstained and a stained condition with a Philips 300 electron microscope.

The histochemical technique for the demonstration of the peroxidase is extremely sensitive,

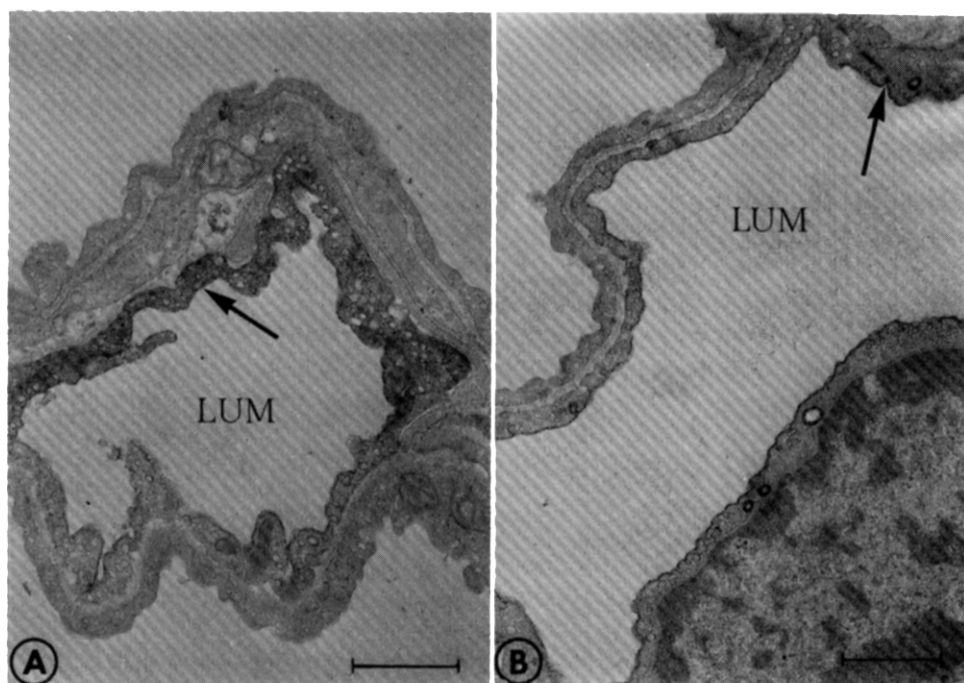


FIG. 3. Vessels from a lung perfused with purified horseradish peroxidase (Sigma VI) to accentuate the histochemical reaction. **A**, Capillary with the black reaction product localized to the endothelium (arrow). The endothelium in the lower portion of the vessel does not stain as darkly, which indicates an irregular distribution of the antigen. **B**, A capillary with black reaction product on the endothelial surface and just starting to enter the endothelial junction (arrow). **C**, A larger blood vessel with black reaction product confined to the endothelium. The arrow points to the interstitial space, which is free of reaction product. **D**, Tracings of the contraction of a rat stomach strip, **RSS**, a rat ascending colon, **RC**, and cat terminal ileum, **CTI**. The first two tissues are treated with "combined antagonists"⁵ and indicate the release of prostaglandins⁵; the terminal ileum is for histamine.⁵ The first tracing is from one half of a lung of an animal immunized against ovalbumin, and **ALB** indicates the administration of the antigen. The second tracing is of a whole lung from an animal immunized against horseradish peroxidase, and **HRP** indicates the administration of antigen.

and it has been estimated that several molecules of the enzyme would be expected to produce demonstrable reaction product.⁷

Schultz-Dale reactions were performed on ileal strips from nonsensitized guinea pigs. The strips were suspended in 10 ml. organ baths containing Krebs-Hanseleit solution at 37° C. One ml. of serum from guinea pigs sensitized to horseradish peroxidase was added to the bath, and, after an incubation period of 30 minutes, the tissue was washed and one mg. of horseradish peroxidase was added to the bath. Control experiments with no addition of serum, but with the addition of horseradish peroxidase, were also done.

The passive cutaneous reaction was performed on nonsensitized guinea pigs. The abdominal skin was shaved and 0.1 ml. of the serum was injected intradermally. After a period of 2½ hours, the animal was challenged with an intravenous injection of 10 mg. of horseradish peroxidase in 1 ml. of 0.5 per cent Evans blue dye. The injection sites were observed for the extrusion of the dye.

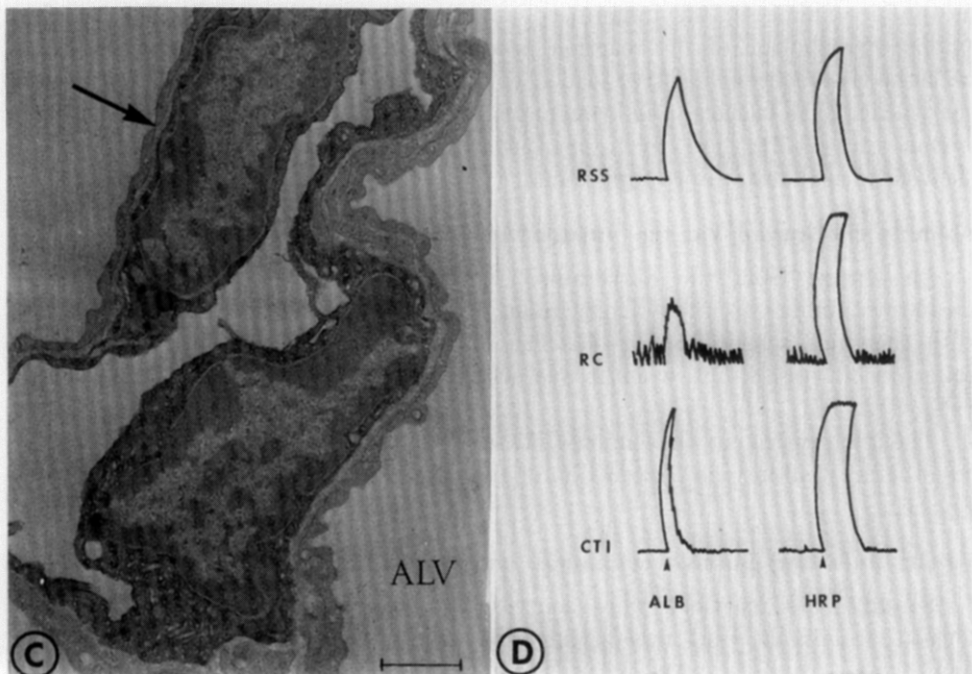


FIG. 3, C and D. For legend see opposite page.

RESULTS

Animals challenged via the airways

Exposure to the horseradish peroxidase produced a rapid increase in airways resistance in the immunized guinea pig but no increase in the nonimmunized guinea pig (Fig. 1). Mean data (Table I) showed that the immunized animals had a significant increase in airways resistance in both periods of exposure to peroxidase, $p < 0.02$ and $p < 0.01$, respectively. The two animals that had the peroxidase administered directly into the trachea had a brief period of hypertension and then died in anaphylactic shock.

Lung tissue from the animals exposed to the aerosol was fixed approximately 30 minutes after the administration of the first aerosol of peroxidase and that from the animals with the tracheostomy approximately 5 minutes after the introduction of the peroxidase. The tracheal and bronchial tissue from the guinea pigs exposed to the aerosol showed only sparse amounts of the histochemical reaction product, and this was not present on every cell. When present it was on the cilia, within small vesicles in goblet cells and glands (Fig. 2). No reaction product was found in the more distal lung parenchyma. The animals with the peroxidase administered directly into the trachea showed abundant reaction product, and this was marked in the goblet cells (Fig. 2). No reaction product was seen beneath the basement membrane of the mucosa in either the animals exposed to the aerosol or in those in which the peroxidase was administered directly into the trachea. We did not detect any difference in the distribution

of the peroxidase in the immunized and nonimmunized animals. We found a similar distribution of the reaction product in the airways of the two animals with the tracheobronchial tree loaded with peroxidase by blowing it directly into the trachea. The tracheal and bronchial mucosa of the experimental group showed edema and infiltration by numerous eosinophils while the control group showed normal respiratory epithelium.

Animals challenged via the vasculature

The lungs from the horseradish peroxidase-immunized animals released mediators similar to those from lungs of animals immunized with ovalbumin with the same schedule (Fig. 3). The histochemical reaction product was not found in all vessels, but, when it was present, it was confined to the endothelium (Fig. 3).

Though present in the endothelial vesicles and the luminal portion of the endothelial junction, no penetration of the reaction product into the interstitium was seen.

Schultz-Dale and passive cutaneous anaphylaxis reactions

Schultz-Dale reactions were consistently found with the serum from the sensitized animals, and this serum also produced positive passive cutaneous anaphylaxis reactions in the skin of nonimmunized guinea pigs.

DISCUSSION

Type of immunologic reaction

We do not have direct evidence of the type of allergic reaction in these experiments. However, the physiologic response (significant and specific increase in airways resistance when exposed to peroxidase), the pharmacologic response (release of mediators by sensitized isolated lungs), and the morphologic response (mucosal edema and infiltration with eosinophils in sensitized animals) strongly suggest that we are dealing with an antigen-antibody reaction. Furthermore, the cytophilic nature of the antibody is demonstrated by the fact that passive cutaneous anaphylaxis and Schultz-Dale reactions were easily demonstrated with serum obtained from sensitized animals. From these experiments we cannot determine whether the type of antibody found in response to the peroxidase was IgG₁ or IgE, and thus we cannot comment on the relative importance of these two antibodies in anaphylactic response of guinea pigs.

Site of antigen-antibody reaction

Examination of the airways by the light and electron microscopes showed that the antigen was always found superficial to the epithelial basement membrane when the lungs were challenged via the airways and at the endothelial surface when the lungs were challenged via the pulmonary vasculature. This evidence strongly suggests that the antigen-antibody reactions do not take place in the interstitium of the lung where the mast cells are located. Indeed, although mast cells were observed in the lung interstitium (Fig. 2, C), in no case did we find the peroxidase near them. Furthermore, we never found mast cells within

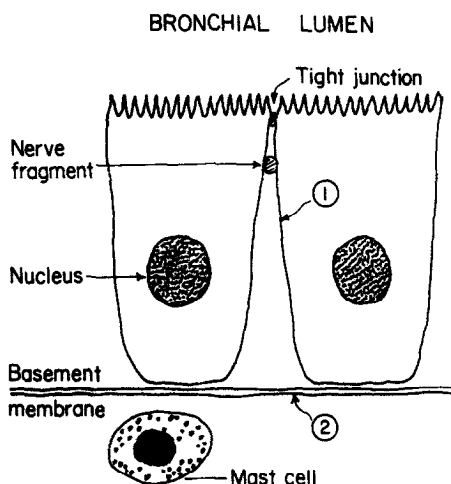


FIG. 4. Diagrammatic representation of bronchial mucosa which can be considered as three compartments (cellular, between cells, and submucosa) separated by two membranes, 1, cell membrane and, 2, basement membrane. Our studies show that the antigen is limited to the first compartment (i.e., within mucosal cells) at the time when allergic bronchoconstriction occurs. The location of nerve fragments between cells¹³ and the known relationship of mast cells to nerves¹⁴ suggest the hypothesis that mast cell degranulation may take place as part of a reflex phenomenon induced by antigen-antibody reaction occurring either at the surface or within bronchial mucosal cells.

the epithelium nor have we been able to demonstrate mast cells in lung washings from sensitized and nonsensitized guinea pigs.

These data, therefore, suggest that allergic bronchoconstriction can occur in guinea pigs without antigen-antibody reaction occurring on mast cells.

Possible sources of error

The peroxidase technique may have sources of error, and these must be examined. For example, it is possible that the portions of the molecule responsible for its antigenic and tracer properties are different. Thus the molecule might break up and the antigenic portion pass into the submucosa and fasten to cytophilic antibodies on the mast cell, while the portion of the molecule responsible for the enzymatic activity might stay at or near the mucosal surface. This seems unlikely as it has been shown that when peroxidase reacts with antibody, *in vitro*, its histochemical properties remain intact.^{8, 9} Another possible source of error is that the peroxidase might reach the interstitium of the lung in very low concentration, which would be sufficient to act as an antigen but insufficient to be detected by the histochemical technique. In short, the antigen might be deep to the epithelial basement membrane in amounts that we could not detect. This again seems unlikely, for, when horseradish peroxidase was used as a tracer to study the uptake of macromolecules in the gut, it was readily detected in the space between epithelial cells and in the submucosal capillaries when the total uptake of peroxidase in the gut was of the order of nanomole per hour.^{3, 4} Similarly, when the tracer is introduced into the vascular system, it has been

shown to pass easily through the capillaries in the submucosa of the gastrointestinal tract and enter the space between epithelial cells and stain the cytoplasm of some cells. These demonstrations between cells in the submucosa of the gastrointestinal tract epithelium were achieved by levels of peroxidase that are closely similar to those we used on the respiratory epithelium. Thus if the peroxidase had entered the interstitium of the lung when it was challenged either by the airways or by the vascular system, it is probable that it would be demonstrable. The impermeability of the pulmonary epithelium and vascular endothelium has been demonstrated.^{2, 10} It has also been shown that increased vascular pressure is necessary for peroxidase to pass through capillary endothelium and the tight junctions between alveolar cells.¹¹ This suggests that bronchoconstriction is induced in the guinea pig by antigen-antibody reactions that occur either within or at the surface of the epithelial cells, and that chemical mediators of bronchoconstriction can be released by intravascular challenge when the antigen is located at the endothelial surface.

If the interstitial mast cells cannot be shown to be the site of antigen-antibody reaction in allergic bronchoconstriction in guinea pigs, one must offer an alternative hypothesis. In doing this, it is worth pursuing the comparison of gastrointestinal and respiratory tract epithelia. Certain cells of the gastrointestinal tract epithelium pick up macromolecules and transport them into the lateral extracellular space and to the submucosal lymphatics so that the tight junctions are not traversed.³ Our studies of lung show that some cells in the respiratory tract epithelium pick up the peroxidase but they do not transport it to the lateral extracellular space or to the submucosa. In examining the absorption of substances by the gastrointestinal tract, a three-compartment model has been used¹² (Fig. 4) in which the substances entering the epithelial cells pass two membranes in series. Our studies indicate that respiratory epithelial uptake is qualitatively similar in the early stages. Since, however, the peroxidase remains within the cell, the lateral cell membrane might block further transport.

One is then left with the question as to how the antigen and antibody can produce bronchoconstriction. Only speculative answers can be offered to this question, but Mills and Widdicombe¹³ and, more recently, Gold, Kessler, and Yu¹⁴ have shown that vagotomy can markedly reduce allergic bronchoconstriction. Since many investigations have shown that nerve endings are located between bronchial epithelial cells and as distal as alveolar ducts,^{15, 16} one could postulate that the antigen-antibody reaction of allergic bronchoconstriction occurs at the cell membrane and that this reaction possibly induces a reflex bronchoconstriction via these nerve endings. Finally, it is also possible that mast cells may degranulate after a reflex has taken place as there is both abundant evidence of their relationship to nerves¹⁷ and recent evidence that mast cells can be degranulated by reflexes in the skin¹⁸ and that other mediators are released after the reaction.¹⁹ Whether this hypothesis has any validity in relation to allergic bronchoconstriction remains to be proved, but it provides an alternative working hypothesis to that which suggests that antigen must reach the mast cell to cause mediator release and bronchoconstriction.

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