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Mucosal penetration of antigen in the presence or absence of serum-derived antibody

AN *IN VITRO* STUDY OF RABBIT ORAL AND INTESTINAL MUCOSA

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Summary. Rabbit mucous membranes were mounted in diffusion chambers within 30 min after excision. The epithelial side was exposed to cell culture medium containing 'cold' and ^{125}I-labelled human albumin. The basal side was exposed to normal rabbit serum (control chambers) or to rabbit antiserum against human albumin. The chambers were incubated in a humidified atmosphere of CO_2 in air for 2–3 h at 37°. The radioactive material recovered on the basal side of the sublingual control membranes sedimented virtually like native albumin on ultracentrifugation. The amount of radioactive material recovered after penetration through antiserum-exposed sublingual mucosa was reduced by 50–80% and showed a very heterogeneous sedimentation pattern including aggregates, presumably immune complexes, as well as a considerable amount of degradation products.

In a second series of experiments the concurrent penetration of human albumin and transferrin through the sublingual mucosa of rabbits immunized parenterally with albumin was compared with that occurring through control membranes. With reference to immunochemical quantification, scintillation counting was found to overestimate the penetration of intact albumin considerably, and jeopardized evaluation of the influence of serum-derived antibody. Radial immunodiffusion showed that in controls the basal antigen concentration, expressed in percentage of the oral (30 mg/ml for both molecules), was after 2 h $0{\cdot}0032 \pm 0{\cdot}0023$ for albumin and $0{\cdot}0016 \pm 0013$ for transferrin. Penetration of immunoreactive albumin through mucosa from immunized animals was clearly reduced ($0{\cdot}0007 \pm 0{\cdot}0003$), whereas there was a significant tendency toward increased penetration of transferrin ($0{\cdot}0035 \pm 0{\cdot}0023$). These results suggest that antibody within the mucosa retards the penetration of intact homologous antigen, while immune reactions may enhance the penetration of unrelated macromolecules.

In similar experiments with colon mucosa penetration was 50–100 times increased, but the membranes did not discriminate between albumin and transferrin and there was no effect of immunization. Histological and immunohistochemical studies of the latter membranes indicated marked defects in cell viability after 2 h *in vitro*.

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INTRODUCTION

Several studies early in this century indicated that entire protein molecules may enter the blood stream directly from the gastrointestinal tract (for review

see Bazin, 1976). Attempts have been made to quantify such antigen absorption, but only approximate data are available. More recently electron microscopy and everted gut sacs have been used to study the transport mechanism and to measure the effect of immunization on the mucosal passage of macromolecules.

Mucosal exclusion of antigens is probably the major function of the secretory immune system (Heremans, 1974; Bazin, 1976). Thus, topical immunization, which stimulates the production of secretory IgA, has been shown to interfere significantly with the intestinal uptake of macromolecules (Walker, Isselbacher & Bloch, 1972; André, Lambert, Bazin & Heremans, 1974). Moreover, patients selectively lacking IgA have a relatively high incidence of serum antibodies to food and milk proteins, indicating increased penetrability for antigens (Buckley & Dees, 1969; Huntley, Robbins, Lyerly & Buckley, 1971). Prolonged parenteral immunization, which mainly gives rise to high titres of IgG antibodies, has also been reported to reduce the penetration of antigens through gut walls, although not as efficiently as topical immunization (Walker, Isselbacher & Bloch, 1973). Other experiments have indicated enhanced intestinal antigen absorption after parenteral immunization (Bockman & Winborn, 1966).

Here we report on the penetrability of rabbit mucous membranes *in vitro* and the effect of IgG antibody on the penetration of corresponding antigen. Moreover, since the influence of IgG-mediated immune reactions on the concurrent mucosal penetration of unrelated macromolecules has not been considered in previous studies, we want to emphasize this aspect of our experiments. We found that although serum-derived IgG antibody retards the penetration of corresponding antigen, it may nevertheless cause impairment of the mucosal barrier. Parts of the data have been presented in preliminary reports (Tolo & Brandtzaeg, 1977; Brandtzaeg & Tolo, 1977).

MATERIALS AND METHODS

Animals and immunization

Adult Belted Dutch rabbits were used. They were maintained on Norwegian Standard No. 3 pelleted food (National Institute of Public Health, Animal Division, Oslo, Norway). Some of them were immunized subcutaneously over a period of 2–3 months with a total of five 3-mg doses of human albumin (Behringwerke AG) emulsified in complete Freund's adjuvant (Difco Laboratories Inc.). This schedule resulted in high titres of serum antibodies, which were measured in precipitating units by a micro-double immunodiffusion technique (Brandtzaeg, 1973). The antibody activity was chiefly located in the IgG fraction, as shown by immunoelectrophoresis (Fig. 1).

Penetration experiments

Mucosal specimens devoid of a secretory immune system were obtained from the lingual frenum. This part of the oral mucosa is covered by a thin stratified squamous epithelium showing parakeratosis (see Fig. 3a). Other specimens were obtained from the colon. Each rabbit provided one oral and two to three colon membranes, which were rinsed briefly and transported to the laboratory in PBS (0·01 M phosphate buffer, pH 7·5, containing 0·15 M NaCl) at room temperature. Within 30 min they were mounted in diffusion chambers (Tolo & Jonsen, 1975). The epithelial side was exposed to 1 ml medium 199 (General Biochemicals) containing 100 IU/ml crystalline penicillin (Glaxo), 100 μg/ml streptomycin (Glaxo), 350 mg/ml sodium bicarbonate and the macromolecules to be tested (see below). The basal compartments (0·2 or 0·5 ml) were filled with medium alone, normal rabbit serum, or hyperimmune serum. The chambers were then incubated at 37° for various periods in a humidified atmosphere of CO_2 in air. Thereafter fluid from the basal compartment was withdrawn and the concentration of macromolecules was quantified immunochemically or by scintillation counting. The measurements indicated that about 10 per cent of the specimens had major membrane leaks, and the data from these chambers were excluded. Data for three membranes with apparently minor defects are included in Tables 1 and 2.

Macromolecules for penetration

Highly purified human albumin (Behringwerke AG) and human transferrin (AB Kabi) were obtained commercially as dry powders. Small portions of albumin (1–2 mg in 1 ml) were labelled electrochemically with $Na^{125}I$ in the presence of 'cold' NaI (Brandtzaeg, 1974b). On the average, 1–2 atoms of iodine were introduced per molecule. Excess of free iodine was removed by dialysis

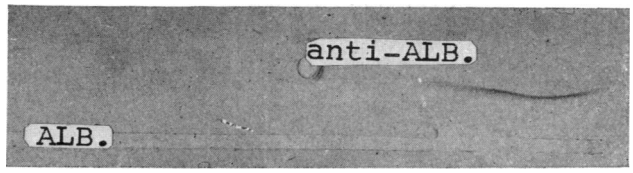

Figure 1. Immunoelectrophoresis pattern of antiserum to human albumin (anti-ALB) developed against corresponding antigen (ALB) at 0·1 mg/ml. Note single precipitin arc representing IgG antibodies. Anode to the left.

for 40 h at 4° against PBS. Radioactivity was counted in a Packard Auto-gamma spectrometer, Model 578, and the concentration of labelled albumin was determined spectrophotometrically with an $E^{1\%,\ 1cm}_{280\ nm} = 5$. The initial specific activity was about 0·5 mCi/mg. Small aliquots were stored at −70° and used within 14 days.

Quantification of penetrated material

In each experiment the macromolecule concentration in the basal compartment was quantified with reference to that in the epithelial compartment and expressed in per cent of the latter. This measurement was achieved by scintillation counting for [^{125}I]-albumin, and by single radial immunodiffusion (SRID) for the unlabelled proteins. The SRID technique was performed essentially as previously described (Brandtzaeg, Fjellanger & Gjeruldsen, 1970). To obtain sufficient sensitivity (detection limit of about 0·25 μg/ml) the specific rabbit antisera were used at final dilutions of 1 : 300 (anti-albumin) and 1 : 160 (anti-transferrin) in 1·5% agarose gel, and the 5-μl sample wells were refilled thrice. After incubation for 48 h at 37°, the plates were washed for 24 h in a 1% NaCl solution; the precipitin rings were then intensified by a second antibody (goat anti-rabbit Ig obtained from Behringwerke AG). Initially, 2 μl of undiluted antiserum was applied to each well, but—as also noted by others (Sieber & Becker, 1974)—this method was prone to give non-specific rings. A better result was obtained by overlaying the gel with filter paper soaked in a 1 : 3 dilution of the antiserum. After incubation overnight at 37°, the plates were washed and photographed. Each quantification was based on the squared ring diameters from two to four measurements.

Density gradient centrifugation

The penetrated radioactive material was characterized by centrifugation at 4° for 21 h at 39,000 rev/min on 10–35% sucrose gradients made in PBS. A Beckmann Spinco Type L-65B ultracentrifuge with an SW 56 Ti rotor was used; 100-μl fractions were collected as previously described (Brandtzaeg, 1974b).

Statistics

Because of skewed distribution of the penetration data, non-parametric tests were used for comparison of experimental groups (Siegel, 1956).

Immunohistochemistry

When the incubation fluids had been withdrawn, the compartments were rinsed with 5 ml cold PBS and then filled with cold 96% ethanol. After about 15 min the membranes were removed and small pieces were trimmed and processed for immunohistochemistry as detailed elsewhere (Brandtzaeg, 1974a). IgG was isolated from a pool of the hyperimmune rabbit sera and conjugated with tetramethylrhodamine isothiocyanate (Brandtzaeg, 1973). By performance testing on sections of directly alcohol-fixed human intestinal mucosa (Brandtzaeg, 1974a), an optimal working concentration of the purified conjugate fraction was established. It had an optical density ratio ($OD_{280\ nm}/OD_{515\ nm}$) of 7·0, an IgG concentration of 1·1 mg/ml, and contained 0·5 precipitating units/ml tested against 1 mg/ml of human albumin.

ATP determination

ATP was measured after addition of firefly extract (Sigma) to mucosal extracts (Prydz & Fröholm, 1964). Samples of oral membranes (wet weight about 10 mg) were taken before and after a 2-h incubation period. They were boiled for 15 min in 1 ml H_2O; 20 μl aliquots of the extracts were mixed in a counting vial with 1·6 ml 0·015 M Tris-HCl buffer, pH 7·4, and 200 μl firefly extract. Triplicates were prepared for each membrane. Light emission was measured in a Packard spectrophotometer, Model 3365, set on repeat 0·1 min. The time from mixing of reagents to measurement of light

emission was recorded, and the ATP content was estimated by extrapolation of the curve to zero time. Standards were prepared from a 2-fold serial dilution of a 40 μg/ml solution of ATP (Sigma) in the Tris buffer.

RESULTS

Oral mucosa

The effect of serum-derived antibody was initially tested by comparing the penetration of [^{125}I]-albumin against basal compartments (0·5 ml) filled with either normal or hyperimmune rabbit serum. Extension of the incubation time from 2 to 3 h, combined with pre-exposure of the membranes to the rabbit sera, increased the penetration markedly (Table 1). Under the latter conditions, basal concentrations of [^{125}I]-albumin appeared to be 2–5 times higher in the absence than in the presence of antibody (Table 1).

The labelled albumin used in these experiments sedimented as a homogeneous fraction (Fig. 2a). After 6 days of storage less than 5% of the radioactive material behaved as smaller molecules. This fraction was increased to 15% after 27 days. The radioactive material that had penetrated through the oral mucosa in the absence of antibody sedimented like native albumin, except for a slight increase of small molecules (Fig. 2b). Conversely, penetration in the presence of antibody resulted in a marked heterogeneity; 40–50% of the material pelleted upon centrifugation, and small molecular degradation products were observed to a varying extent (Fig. 2c).

To avoid interference with the quantifications by extensive immune complex formation in the basal compartments and by marked degradation of radioactive material, a second series of experiments was performed; here the penetration of unlabelled albumin through membranes taken from normal or hyperimmunized animals was compared and transferrin was included as a reference. The epithelial side of the mucosa was exposed to medium containing 30 mg/ml of each of these two macromolecules.

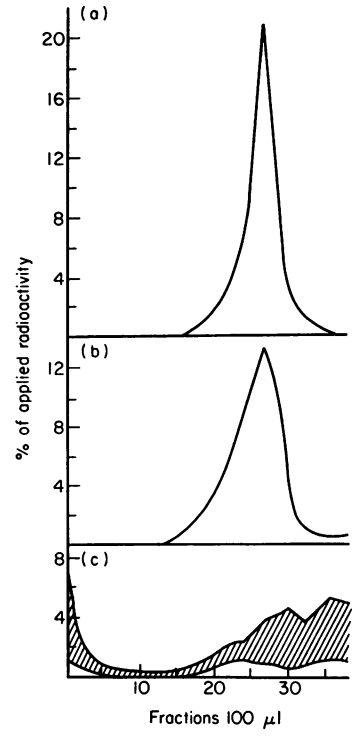

Figure 2. Distribution of radioactive material after ultracentrifugation on 10–35% sucrose gradients. Samples: ^{125}I-labelled human albumin before (a) and after *in vitro* penetration through rabbit oral mucosa in the absence (b) or presence (c) of antibody to albumin. Bottom of gradients to the left. Shaded area in (c) covers range of patterns shown by various samples, 40–65% of the applied material being pelleted.

Table 1. Penetration of [^{125}I]-albumin through oral mucosa from normal rabbits in the absence or presence of antibody*

Penetration time (h)	Oral concentration: Labelled albumin (μg/ml)	Oral concentration: Cold albumin (mg/ml)	Basal concentration in % of oral concentration: In the absence of antibody	Basal concentration in % of oral concentration: In the presence of antibody
2	60	40	0·03	0·30†
2	60	40	0·06	0·03
3	20	25	0·58‡	0·11§
3	20	50	0·39‡	0·18§
3	20	50	0·12‡	0·06§

* The basal compartments contained normal rabbit serum or antiserum to albumin during the incubation.

† Apparent membrane defect.

‡ The basal side of the membrane was pre-exposed to normal rabbit serum for 30 min, while the epithelial side was covered with medium alone.

§ The basal side was similarly pre-exposed to antiserum.

Table 2. The effect of parenteral immunization with albumin on the concurrent penetration of albumin and transferrin through rabbit oral mucosa

	Serum antibody*	Basal conc. in % of oral conc.† Albumin		Transferrin	Significance of difference‡
Control animals		(0·0215)§		(0·0155)	
		0·0061		0·0034	
		0·0054	0·0058	0·0018	
		0·0047		0·0035	
		0·0035		0·0008	
		0·0020	0·0184	0·0009	
		0·0005		0·0005	
		0·0005	0·0037	0·0005	
		N.D.¶	0·0081	N.D.	
Mean		0·0032		0·0016	$P < 0·025$
± s.d.		±0·0023		±0·0013	
Immunized animals	8	(0·0037)		(0·0131)	
	2	0·0013		0·0025	
	32	0·0010		0·0055	
	16	0·0005	0·0173	0·0076	
	32	0·0005	0·0090	0·0032	
	16	0·0005		0·0020	
	16	0·0005		0·0018	
	16	0·0005		0·0016	
	16	N.D.	0·0048	N.D.	
Mean		0·0007		0·0035	$P < 0·01$
± s.d.		±0·0003		±0·0023	
Significance of difference**		$P < 0·03$		$P < 0·05$	

* Expressed as precipitating units based on tests against albumin (1 mg/ml) in gel diffusion.

† Each figure represents average of two to four measurements by single radial immunodiffusion (detection limit 0·0008%, undetectable assigned 0·0005%), except for the right-hand column of data on albumin, which were obtained by scintillation counting.

‡ Wilcoxon matched-pairs signed-ranks one-tailed test.

§ Figures in parentheses were excluded from group calculations because of the extreme deviations, apparently reflecting a membrane defect.

¶ N.D., not determined.

** Mann-Whitney one-tailed U-test.

In some chambers the concurrent penetration of radioactive material was measured by adding 40 μg/ml of [^{125}I]-albumin. Only medium was added to the basal compartment (0·2 ml), and after incubation for 2 h the basal concentrations of immunochemically active albumin and transferrin were determined by SRID.

Penetration of radioactive material was 8–30 times lower in these experiments, but it was not further reduced for the three membranes from immunized animals (Table 2). Moreover, when the radioactivity data were compared with the immunochemically determined albumin concentrations in the same compartments, marked discrepancies were noted (Table 2). For two control membranes the penetration was overestimated 7–9 times by the radioactivity measurements, and for two membranes from immunized animals the overestimation was at least 18–35 times. When the two specimens with apparent defects were excluded, the immunochemical data indicated a significant reduction in the mucosal penetration of albumin after immunization against this antigen.

Table 2 further shows that the control membranes discriminated between albumin and transferrin according to the difference in molecular weights

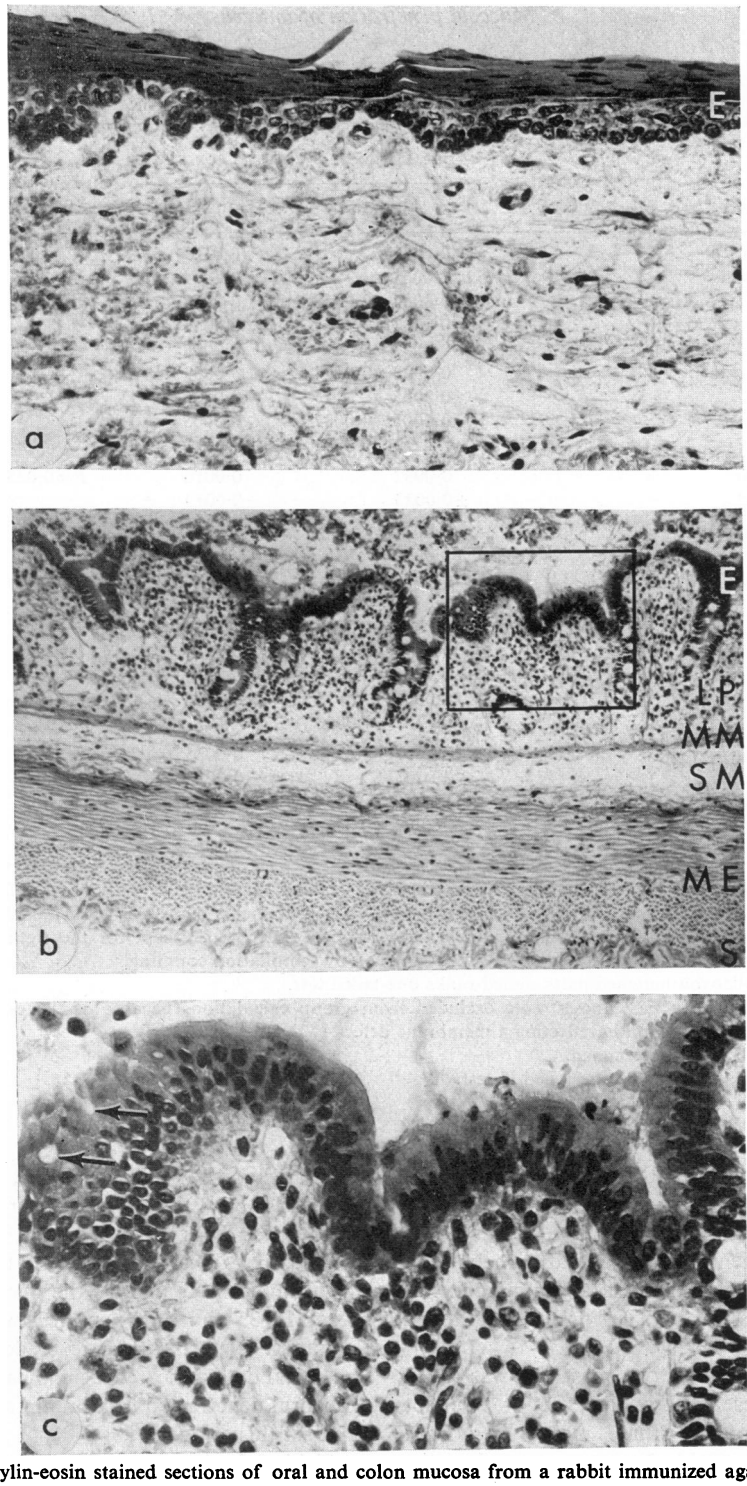

Figure 3. Haematoxylin-eosin stained sections of oral and colon mucosa from a rabbit immunized against human albumin. The membranes were incubated for 2 h *in vitro* with the epithelial side (at the top) exposed to a mixture of human albumin and transferrin, each at 30 mg/ml medium. Note parakeratosis of oral epithelium, and relative scarcity of cells in the connective tissue (a). A field with fairly good morphology of colon mucosa was selected (b). Yet, the epithelium is vacuolated (arrows) as shown at higher magnification of the framed part (c). Also, uptake of albumin by epithelial and other cells was shown in the same specimen (compare Fig. 4e). E, epithelium. LP, lamina propria. MM,muscularis mucosae. SM, submucosa. ME, muscularis externa. S, serosa. Magnification: ×270 (a), ×110 (b) and ×440 (c).

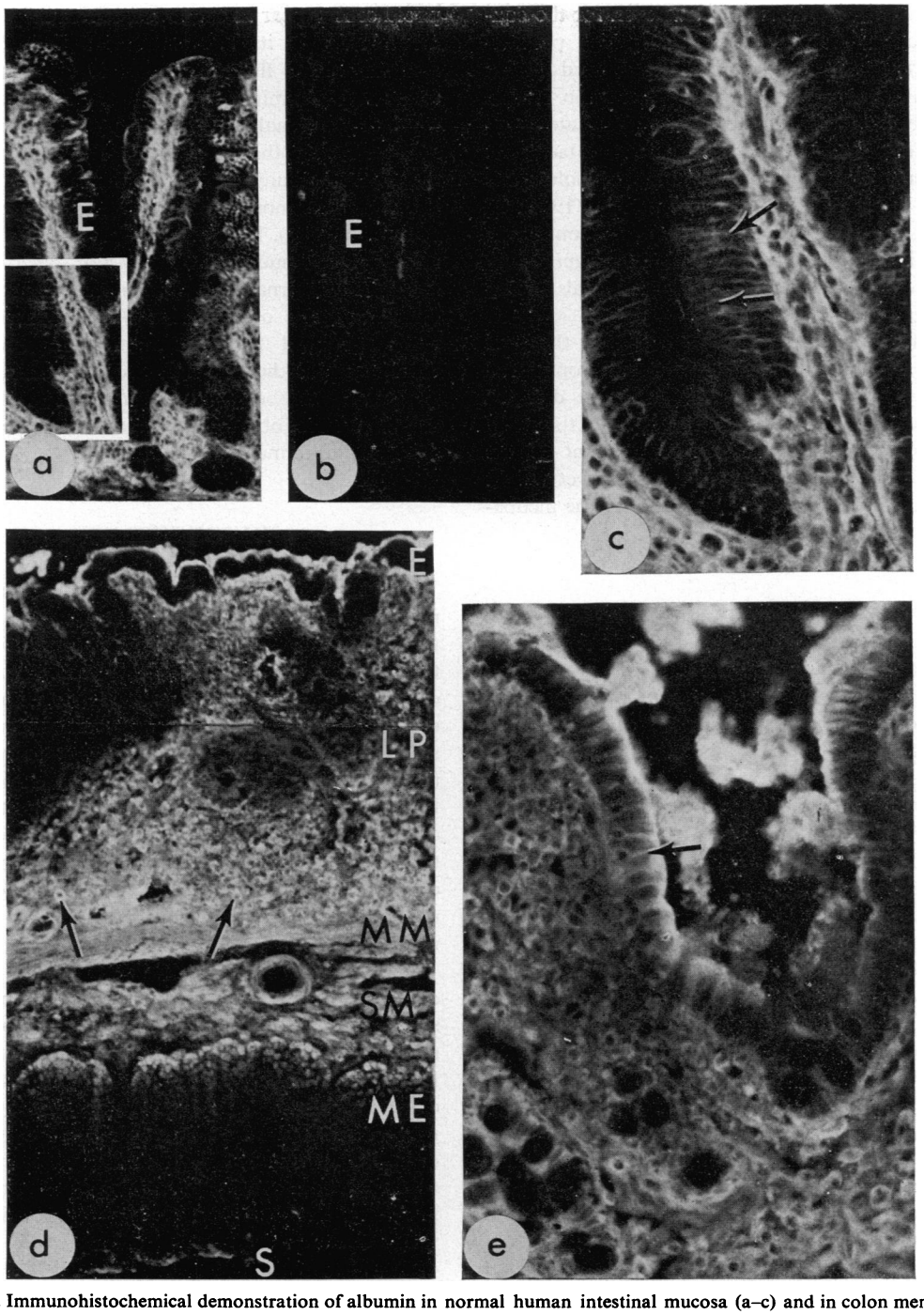

Figure 4. Immunohistochemical demonstration of albumin in normal human intestinal mucosa (a–c) and in colon membranes from an unimmunized (d) and from an immunized (e) rabbit after *in vitro* incubation as described in legend to Fig. 3. In (b) the conjugate had been absorbed with an excess of human albumin for control of staining specificity in field corresponding to (a). Note distinct extracellular distribution of albumin in human mucosa (a) with apparent restriction to the interstices (arrows) in the epithelium as shown at higher magnification of the framed part (c). By contrast, albumin has entered muscle fibres and plasma cells (arrows) in the rabbit colon membrane (d). In addition to interstitial fluorescence (arrow), there is also diffuse staining of columnar cells in the epithelium (e). High concentrations of albumin are found associated with the mucus covering the epithelium (d, e). Labels as in Fig. 3. Magnification: ×130 (a, b, d) and ×330 (c, e).

(69,000 vs 90,000). This held true even for the control specimen showing exceptionally high penetrability. Its defect was probably restricted to an epithelial damage, since recent penetration studies with rabbit intestinal mucosa have indicated that molecular selection takes place at the basement membrane whereas the epithelial layer is solely rate limiting (Kingham, Whorwell & Loehry, 1976). In contrast to the situation for albumin, the concurrent penetration of transferrin seemed to be significantly enhanced through membranes from animals immunized against albumin (Table 2).

Histologically the epithelial layer of the oral mucosa appeared intact after the incubation period (Fig. 2a). No trace of albumin could be detected in the epithelium or in the connective tissue by immunohistochemistry. The ATP content of the mucosa ranged from 0·2 to 1·0 μmol/g wet weight before and from 0·1 to 0·3 μmol/g after the incubation.

Colon mucosa

Penetration experiments with unlabelled albumin and transferrin were performed on nine colon membranes from four of the control rabbits and on ten colon membranes from six of the rabbits immunized with human albumin. The specimens were mounted in chambers and treated in parallel with the latter series of experiments on oral mucosa. Under these conditions it was found that the colon mucosa of the control animals did not discriminate between albumin and transferrin—the serosal concentrations expressed in percentage of the luminal ones being 0·180 ± 0·066 (range: 0·090–0·290) and 0·178 ± 0·078 (range: 0·058–0·280), respectively. Moreover, immunization had only a slight (non-significant) retarding effect on the penetration of albumin, and no enhancing effect on the penetration of transferrin—the respective serosal concentrations being 0·164 ± 0·037 (range: 0·120–0·250) and 0·170 ± 0·047 (range: 0·124–0·239).

After the incubation the histological appearance of the colon specimens was clearly abnormal (Fig. 3b). Vacuolization of epithelial cells (Fig. 3c) and areas of epithelial denudation were regularly observed, and immunohistochemistry showed that human albumin had leaked into several cell types. This is illustrated in Fig. 4 by comparison with the albumin distribution in a normal specimen of human intestinal mucosa. In the lamina propria the lymphoid cells appear as shadows because they exclude albumin despite its high concentrations in the surrounding tissue fluid (Fig. 4a); in the epithelium traces of albumin are found in the interstices indicating external diffusion (Fig. 4c). In the rabbit colon mucosa there was similarly indication of diffusion of albumin between epithelial cells, but at the same time most of the cells had taken up some protein (Fig. 4e). In the lamina propria, muscularis mucosae, submucosa and adjacent layer of the muscularis externa there was likewise a prominent cellular uptake of albumin; only the muscularis externa seemed to have served as an efficient barrier against the penetration of albumin towards the serosal side of the membrane (Fig. 4d). No difference was observed between specimens from controls and immunized rabbits.

DISCUSSION

This study shows that serum-derived IgG antibody retards the penetration of corresponding antigen through a mucous membrane, but as a side effect the concurrent penetration of other antigens may be enhanced. These results were obtained with rabbit oral mucosa that during the *in vitro* incubation seemed to retain sufficient viability to warrant reliable conclusions. Thus, all of the control membranes discriminated between the two test molecules—human albumin and transferrin—according to their different molecular sizes. A similar molecular selection has been observed for biological membranes *in vivo* (Nencioni, Brambati & Crosignani, 1970).

Another viability criterion was the tissue content of ATP, which for the oral mucosa was found to be similar to that of the basal layers of human epidermis (Hammar, 1973) and rat jejunal mucosa (Bronk & Leese, 1973). After *in vitro* incubation the ATP content of the oral mucosa was decreased by 30–50 per cent, and had reached the level considered critical for the viability of kidney transplants (Calman, Quin & Bell, 1973). Apparently, the oral mucosa is in this respect comparable to epidermis, which showed a 38 per cent drop of ATP content during 30 min with anoxia (Hammar, 1973). A more rapid loss has been observed for rat jejunal mucosa, which after 4 min *in vitro* contained only 14 per cent of the original ATP (Bronk & Leese, 1973). We did not measure the ATP content of rabbit colon

mucosa, but the histological features and the demonstration of albumin leakage into various cell types indicated severe damage after 2 h *in vitro*. Moreover, the colon membranes did not discriminate between albumin and transferrin. According to recent experiments with ischaemia and salicylate exposure in the rabbit intestine (Kingham *et al.*, 1976) such lack of molecular selectivity indicates that not only is the epithelial cell layer defective, but the basement membrane barrier function has been altered. Other data likewise attest to the vulnerability of intestinal mucosa (Rhodes & Karnovsky, 1971).

Most previous studies on mucosal penetrability were performed with everted sacs of the small intestine, and the *in vitro* incubation periods varied from 1–3 h (Walker, Isselbacher & Bloch, 1973; Walker, Wu, Isselbacher & Bloch, 1975a; Walker, Abel, Wu & Bloch, 1976). Viability criteria were based on histology, active transport of L-histidine, and cell exclusion of trypan blue. After 1 h some areas of epithelial denudation and the appearance of numerous large vacuoles in the epithelial cells were noted (Walker, Cornell, Davenport & Isselbacher, 1972), and after 3 h there was some uptake of dye into the cells (Walker *et al.*, 1975a). Persistence of molecular selectivity in these membranes was apparently not tested. Our results indicated that such selectivity is much better retained in the oral than in the intestinal mucosa. One reason may be the high connective tissue cellularity in the latter, increasing the possibility of endogenous enzyme release from neutrophils and macrophages. It is well known that lysosomal enzymes can affect the ground substance and basement membrane zones (Goldstein, 1976; Hensom, 1976).

Penetration of immunochemically intact albumin through the oral mucosa from control animals was in the order of 0·003 per cent or 0·5 $\mu g \cdot cm^{-2} \cdot h^{-1}$. However, since for practical reasons dose dependency could not be evaluated with this membrane, it is not known how meaningful our figure is. We chose an epithelial concentration of 30 mg/ml in order to obtain penetration measurable by single radial immunodiffusion. Radioimmunoassay was not attempted since André *et al.* (1974) reported that technique to be highly unreliable in similar experiments.

Bovine serum albumin labelled with ^{125}I has been used in most previous studies on mucosal penetrability (Walker *et al.*, 1972b, 1973, 1975a, b, 1976); interpretation of the data obtained depends on the stability of the labelled protein, which is influenced by several factors. It has been shown that the number of radioactive iodine atoms incorporated into the molecule is important for its immunochemical stability (Ceska, Berglund, Lundkvist & Grossmüller, 1972), but the specific activity of [^{125}I]-albumin used has not been reported in previous penetration studies. We used less than two atoms of iodine per albumin molecule, and only a fraction of the material was radioactive. Yet, in control membranes of oral mucosa radioactivity measurements gave an 8-fold overestimation compared with parallel immunochemical quantification of penetrated albumin. This was either due to degradation of the protein molecule or release of the label—a problem that may be even more pronounced in the gut wall where neutrophils and macrophages are more abundant and where deiodinases apparently are present (Parkins, Dimitriadou & Booth, 1960). The presence of antibodies to albumin increased the degradation of [^{125}I]-albumin markedly and the overestimation of penetrated material was at least 25-fold compared with the immunochemical data. The most likely explanation for this increase is that immune complex formation enhances the release of lysosomal enzymes from phagocytes—a process which may be induced by extremely low antigen concentrations (Cardella, Davies & Allison, 1974).

Walker *et al.* (1972b, 1973, 1975a, b, 1976) dialyzed the penetrated material to remove small molecules before scintillation counting; this might have jeopardized comparisons between penetration in the absence or presence of antibody, giving a *relative* underestimation in the latter situation due to more extensive degradation of [^{125}I]-albumin. Conversely, in our study without dialysis, the retarding effect of antibody present in the oral mucosa was masked by such degradation; only in the first series of experiments with high concentrations of antibody on the basal side of the membranes was the penetration of radioactive material reduced. However, the immunochemical analyses showed significantly retarded penetration of antigenically intact albumin when oral mucosa from immunized animals was tested without the presence of antiserum on the basal side. This result was achieved with an oral antigen concentration of 30 mg/ml, whereas Walker *et al.* (1975a) observed retardation of [^{125}I]-albumin only at concentrations below 1 μg/ml in similar experiments with small intestinal mucosa from the rat. This pronounced discrepancy may reflect a dif-

ference in the mechanical barrier function of the two membranes as well as a difference in their resistance to *in vitro* alterations. The epithelium of the small intestine actively takes up protein molecules by pinocytosis (Walker, 1975), whereas the passage through squamous epithelium is mainly restricted to the interstices (McDougall, 1972, 1974).

So far the morphological level at which serum-derived antibody retards mucosal antigen penetration has not been clearly defined. The concentration of IgG in interstitial fluid of human skin is about 6·5 mg/ml (Lönsmann Poulsen, 1974), and immunohistochemical studies have demonstrated that the lamina propria of mucous membranes likewise contains an abundance of serum-derived IgG—traces of which diffuse into the epithelial interstices (Brandtzaeg, 1975; Brandtzaeg & Baklien, 1976). In accordance with this observation, immune complex formation has been indicated ultrastructurally in epithelial interstices after application of antigen on the oral mucosa of parenterally immunized animals (McDougall, 1972, 1974). For the small intestinal mucosa immunological binding of antigen to the surface mucus has been suggested (Walker *et al.*, 1975a, 1976), although enhanced leakage of antigen and small immune complexes into damaged epithelial cells was not excluded. Indeed, an early ultrastructural study indicated increased antigen uptake by intestinal epithelial cells after parenteral immunization (Bockman & Winborn, 1966).

Retardation of mucosal antigen penetration may also be due to immune complex formation in the connective tissue. This was shown by our first series of experiments in which only the basal side of the membrane was exposed to antibody. Moreover, antigen application on epithelial surfaces of sensitized animals results in a rapid local emigration of neutrophils (Bellamy & Nielsen, 1974; McDougall, 1974); this is indicative of a chemotactic effect induced by immune complexes in the connective tissue or basement membrane zone. Phagocytosis and protein degradation may hence contribute to an apparently retarded antigen absorption. In addition, release of lysosomal enzymes probably explains the most important finding in our study, namely the enhanced penetration of an unrelated macromolecule. As discussed above these enzymes can in various ways increase mucosal penetrability. This effect is probably more important *in vivo* than what was shown in our *in vitro* system where there could be only a very limited diapedesis of phagocytes.

Altered mucosal penetrability for food constituents and microbial components apparently underlies the pathogenesis of a variety of diseases occurring in mucous membranes and distant sites such as liver and joints (Walker, 1975). In addition, there are several possible hazards of circulating antibodies resulting from vaccination (Craighead, 1975). Our results point to one way in which the formation of IgG antibodies may jeopardize mucosal integrity since undue penetration of new antigens in turn may stimulate a broader IgG response and thus set up a vicious circle. The merit of secretory IgA and IgM antibodies is most likely to limit such a development. This must be taken into account when immunoprophylaxis is attempted by vaccines and when the value of breast feeding is debated. In the neonatal period antigens are absorbed into the circulation in sufficient amounts to evoke a systemic IgG response in a great proportion of individuals, especially in those being artificially nourished (Editorial, 1976). This may predispose to untoward sensitization of the child. In addition our results substantiate one pathogenetic potential of local IgG-cell responses in mucosal disease (Brandtzaeg, 1975; Brandtzaeg & Baklien, 1976).

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