Effect of alcohol on the integrity of the intestinal epithelium

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SUMMARY  The absorption of macromolecules from the small intestine of rats was studied in terms of the amount of peroxidase activity that appeared in thoracic duct lymph after a 10 mg dose of horseradish peroxidase had been injected directly into the lumen of the duodenum. When the horseradish peroxidase was injected as a solution in saline no peroxidase activity was detected in the lymph. When ethyl alcohol was included in the dose at final concentrations of 12.5–16% the flow rate of the lymph increased markedly for an hour or so and during this time peroxidase activity was detected in the lymph. An electronmicroscope study of the duodenal epithelium that had been exposed to alcoholic solutions of horseradish peroxidase showed that the enzyme had penetrated between the enterocytes. It was concluded that the presence in the intestine of substantial amounts of alcohol temporarily destabilises the intercellular junctions of the epithelium and thus promotes the absorption of materials which are normally excluded.

Absorption from the healthy adult gastrointestinal tract of immunologically significant amounts of macromolecular material is well documented and has been reviewed.1 Recent investigations of this phenomenon have used the administration of test antigens by gavage to experimental mice2 or by normal ingestion in human volunteers.3 In such situations it is not clear at which point in the gastrointestinal tract that the absorption occurs, and the role of minor traumatic lesions in the gums, oesophagus, or stomach cannot be excluded totally. In order to lessen these problems we decided to measure the absorption of antigen in rats in terms of its appearance in thoracic duct lymph, which comes principally from the small intestine4 and which is an obligatory pathway for any macromolecular material which has traversed intestinal wall.5 In spite of recent claims for substantial uptakes of protein antigens by the gut of rats6 we were unable to detect in our preliminary experiments any absorption of horseradish peroxidase after a 10 mg dose of the purified enzyme had been instilled into the lumen of the duodenum. We observed, however, that when ethyl alcohol was mixed with the horseradish peroxidase at a final concentration of about 15%, enzyme activity appeared in the lymph soon after the mixture had been instilled into the duodenum. Although the effect of alcohol on intestinal permeability to macromolecules has been mentioned as a possibility,7 we were surprised at the decisiveness of the phenomenon and undertook a series of experiments to explore it further. These experiments form the subject of this paper and are reported below.

Methods

GENERAL PLAN

Rats were prepared with a cannula in the cysterna chyli so that 'thoracic duct' lymph could be collected quantitatively. In addition, a soft vinyl cannula was inserted into the duodenum via the pylorus, through which it was introduced by making a small incision in the wall of the stomach. This incision was repaired with a purse-string suture which also served to retain the cannula. This procedure avoided obstructing the gastrointestinal tract and the rat was later able to feed and drink normally. The cannulated rats were placed in Bollman restraining cages where they recovered from the effects of anesthesia and had access to food, water ad libitum. Twenty-four hours later, those rats from which the

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lymph was still flowing spontaneously were selected for experiment and the test marker material, usually 10 mg horseradish peroxidase, was infused into the duodenum in a total volume of either 1-5 or 4-0 ml. Lymph was collected into tared, heparinised centrifuge tubes which were changed at intervals of 15 or 30 minutes until the experiment was completed. The cells were removed from the lymph by centrifugation and the supernatant plasma was assayed for the presence of marker material.

**ANIMALS AND SURGICAL PROCEDURES**

Male, Wistar rats weighing between 200–300 g were taken from our barrier-maintained animal unit as required. The details of the surgical procedures have already been described.8 9

**MARKER MATERIALS AND ASSAYS**

In most experiments horseradish peroxidase was used as the marker; this material has a molecular weight of 40 000 and has been used in previous studies.1 It can be assayed in solution and used to generate an electron dense reaction product for ultrastructural studies.

We reasoned that the appearance of unequivocal enzyme activity in the lymph would be the most direct way of showing the absorption of a functionally intact macromolecule, and would avoid the complexities of using radiolabelled proteins where the appearance of the label in the lymph might indicate only the absorption of a variety of small degradation products.

Horseradish peroxidase (grade VI) was obtained from Sigma Ltd. Stock aqueous solutions containing 25–50 mg protein/ml were prepared from which the 10 mg doses were dispensed. These doses were made up to a volume of either 1-5 ml or 4-0 ml with physiological saline and, where appropriate, included reagent grade ethyl alcohol at a final concentration of 12-5–16% v/v, as indicated in the results.

The peroxidase activity in the samples of lymph plasma was titrated by making doubling dilutions of lymph in saline buffered with 0-02M cacodylate buffer in 0-1 ml systems in plastic microtitre plates. To each well one drop of dianinobenzidine reagent10 was added and the appearance of the dark brown reaction product was judged by eye within 10 minutes against positive and negative controls. We calculated that the method detected nanogram quantities of peroxidase. Though crude, this method gave rapid results and was unaffected by the lipid turbidity which makes the more accurate methods involving quantitative spectrophotometry inapplicable to untreated intestinal lymph.

125I-polyvinylpyrrolidine (mol wt about 50 000) was obtained from the radiochemical centre, Amersham, and assayed in a γ-spectrometer.

In some experiments the dye patent blue V was used as a marker. This was supplied as a 2-5% solution for lymphography by May and Baker Ltd, Dagenham. This dye, which binds to albumin, is a sulphonated aromatic compound with a molecular weight of 1160 daltons. It was assayed in lymph by conventional spectrophotometry at 630 nanometres.

**MICROSCOPY OF RAT INTESTINE**

A small piece of the region of intestine to be studied was excised and immediately placed in a fixative of 1% glutaraldehyde in 0-2M cacodylate buffer, pH 7-4. The tissue was cut rapidly into 0-5 mm cubes, which were transferred to fresh fixative and allowed to stand at room temperature for one hour. The cubes were washed in several changes of buffer to remove excess glutaraldehyde and exposed to diaminobenzidine reagent to which hydrogen peroxide had not been added. After 30 minutes to one hour the hydrogen peroxide was added, mixed, and allowed to react for 30 minutes. The cubes were rinsed in several changes of buffer, postfixed in osmium tetroxide, dehydrated in acetone, and embedded in araldite by standard procedures. Examination in the electronmicroscope of ultra thin sections stained with lead revealed the fine structure of the intestinal epithelium and the location of peroxidase activity.

At the same time larger portions of the intestine were fixed in Bouin’s solution and sections stained with haematoxylin and eosin were prepared by conventional techniques for light microscopy.

**PROTEIN DETERMINATIONS AND CELL COUNTS**

The protein content of lymph plasma was determined by a standard dye-binding method,11 and total white cell counts of lymph were performed using a Neubauer haemocytometer.

**Results**

Lymph was collected successfully from 35 rats. Usually, these were used for only one experiment involving the administration of alcohol; however, it was convenient sometimes to give a rat a control, nonalcoholic dose of horseradish peroxidase in saline followed the next day by the test dose which contained alcohol. In all cases the dose of horseradish peroxidase administered was 10 mg.

Under basal conditions lymph flow was relatively constant but varied from 0-3 ml/h to 1-5 ml/h between individuals. The basal flow rate increased only slightly when saline solutions of 1-5–4-0 ml were given into the duodenum but increased...
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strikingly for an hour or so when alcohol was included in the dose (Figs 1 and 2). During the period of increased flow neither the white cell count nor the protein content (mean = 30 mg/ml) changed significantly. Similarly, detectable peroxidase activity never appeared in the lymph if alcohol was not included but always did so when horseradish peroxidase was administered in 16% alcohol (12 experiments). When horseradish peroxidase was administered with alcohol at a final concentration of 12.5% unequivocal enzyme activity appeared in the lymph in six out of 13 experiments. In these last experiments (Fig. 1) the horseradish peroxidase-alcohol was given in a total volume of 4 ml. It was thought that this volume was excessive and rapidly flushed the administered dose through the intestine. For this reason the volume of the dose was reduced to 1.5 ml (Fig. 2) which confined the material, initially, to the duodenum and jejunum.

Several experiments were done to investigate the duration of the effect of alcohol in increasing the permeability of the intestine. The alcohol was administered first as a 15% solution in a total volume of 1.5 ml and the 10 mg of horseradish peroxidase was administered via the same cannula at five, 30, 60, and 120 minutes afterwards in 0-2 ml volume of saline. Peroxidase activity was recovered after 15 and 30 minute intervals but not at the later times, thus emphasising the transient nature of the alcohol effect.

Individual experiments were performed to show that the alcohol and horseradish peroxidase had to impinge on the same region of the small intestine in order to promote significant absorption of the enzyme. Thus if the alcohol was injected into the duodenum and the horseradish peroxidase was injected, by another cannula, into the lower jejunum isolated from the duodenum by a ligature, no absorption occurred. Also, if the alcohol was given into the stomach and the horseradish peroxidase was given into the duodenum, the pylorus being ligated, no absorption occurred. Interestingly, when alcohol (16%) and horseradish peroxidase were placed together in the stomach no absorption of peroxidase occurred either, though in this experiment too the pylorus was ligated.

In order to exclude a role for the direct effect of alcohol on the horseradish peroxidase, alcohol was added to the enzyme to a final concentration of 16% and the mixture was dialysed overnight against distilled water. When 10 mg doses of the alcohol treated horseradish peroxidase was instilled into the duodenum of test rats no peroxidase activity appeared in the lymph.

When the general features of the alcohol effect had been established, microscopy was used to detect the lesion. No abnormality was seen by light microscopy in specimens of rat duodenum that had been obtained within five or 30 minutes of the usual dose of horseradish peroxidase and alcohol. The ultrastructural study, however, showed that when alcohol had been administered some of the intercellular junctions between enterocytes had failed, so that the horseradish peroxidase had penetrated between the cells (Fig. 3). In addition the enterocytes showed some evidence of slight general damage; the desmosomes seemed less distinct, and some of the mitochondria appeared swollen. It must be emphasised that by no means all intercellular junctions in the specimen showed these changes but several examples were seen and they were never observed in the control specimens where horse-
radish peroxidase had been given without alcohol.

In those experiments where alcohol-induced absorption of the horseradish peroxidase had occurred it was possible to calculate, from the titres of enzyme activity in measured volumes of lymph, what proportion of the administered dose had been absorbed. Obviously, such calculations can only give an approximate indication but they showed that usually about 0.01% of the dose was recovered in the lymph - that is, about 1-0 μg. It should not be concluded, however, that any macromolecule would undergo the same fate. When 125I PVP was used as a marker only barely detectable amounts were absorbed and this situation was not affected by the addition of alcohol. Similarly, alcohol did not promote the absorption of alkaline phosphatase (from bovine intestine), though in this case the enzyme assay is less sensitive than that for peroxidase and this was complicated by the presence of endogenous phosphatase in lymph collected under basal conditions.

We concluded, tentatively, that the effective diffusion diameter of horseradish peroxidase represents probably the upper limit for the size of material which can pass easily through the alcohol-induced lesion. Accordingly, we sought to repeat the experiments with a small marker molecule such as the dye patent blue V. Although this dye has a molecular weight of under 1200 it has the property of binding to serum albumin. We reasoned that if a small amount were absorbed across the intestinal epithelium it would all become bound to protein in the tissue fluid so that it would be absorbed into the lymph rather than the blood. It was found that when...
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1-0 ml of the standard 2.5% solution of dye was infused into the duodenum no dye appeared in either lymph or blood. When alcohol was included, however, with the dye at a final concentration of 12.5% the colour was easy to discern in the lymph plasma in which it appeared with the same kinetics as did horseradish peroxidase. Spectrophotometric measurement of the dye in the lymph showed that again about 0.01% of the administered material had been absorbed.

Discussion

Hitherto, it has been accepted that there are two ways in which macromolecules from the lumen may traverse the intestinal epithelium. The first is analogous to the absorption of colostral immunoglobulins by the enterocytes of neonates and involves pinocytosis and the formation of endocytic vesicles. In the adult this process may occur on a small scale but much of the small amount of material absorbed is degraded by lysosomal enzymes in the enterocytes. The second way is the apparent 'phagocytosis' of potentially antigenic material by specialised epithelial 'M' cells which overlie Peyer's patches and may be involved in antigen presentation. Two of these 'physiological' routes must be added a third, 'pathological' one – that is, an intercellular 'leak' secondary to the action of noxious agents on the epithelium. Vague speculations about such effects abound but there seems to be little hard fact. There is evidence that patients with anaphylactic hypersensitivity reactions to dietary antigens will absorb more of the antigen than healthy subjects; in this sense the gut is more 'leaky' but the route and mechanism of absorption is obscure. Where alcohol itself is concerned confusion seems to reign. Two recent reviews did not directly mention the absorption of macromolecules and one concluded that evidence about the effects of alcohol on the gut contained 'discrepancies between the postulated and proven actions, and where proved there is seldom proof of clinical relevance'.

At an experimental level, though, it is now clear that in rats the presence in the gut of substantial amounts of alcohol causes a dramatic increase in the absorption of materials that usually are excluded, and that this is apparently the result of an intercellular leak. It is also clear that the effect is confined to areas of small intestine actually exposed to the alcohol and is transient and reversible. This probably follows from the rapidity with which the local concentration of alcohol is reduced by absorption and metabolism. Routine histology showed no obvious signs of damage, and the absence of a significant increase in the protein concentration of the lymph during the periods of increased lymph flow is consistent with a transient hyperaemia that fell far short of actual inflammation and increased capillary permeability. Also, the transient damage to the epithelium caused by alcohol did not release any endogenous peroxidase activity. Alcohol given by itself – that is, without added horseradish peroxidase – never induced the appearance of peroxidase activity in the lymph. Normal lymph plasma is free of endogenous peroxidase activity unless gross haemolysis has occurred.

Both the total amounts (1.0–2.0 g/kg) and the concentrations (12.5–16%) of alcohol delivered to the gut of the experimental rats in this study are likely to exceed those achieved by all but the most enthusiastic drinkers but lesser doses might nevertheless result in significant effects. It should be remembered that we confined our experiments to easily demonstrable major effects involving relatively large molecules that entered the lymph. In intact animals these would be carried to the systemic circulation and would not impinge directly on the liver. Clinically, the importance of the lesion, at any rate in relation to alcoholic cirrhosis, would more likely relate to the direct absorption into the portal blood of a whole host of peptides, oligosaccharides etc that would be excluded normally but which would inevitably pass through the type of intercellular leak which we observed. This situation, together with a study of the effects of other alcohols and related chemicals, as well as the obvious immunological implications will be the subject of further experiments.

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