Intestinal Absorption of Dinitrophenyl-Lysine and Effect of Immunization with Dinitrophenylated Bovine Serum Albumin

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Summary The intestinal absorption of dinitrophenyl-lysine (DNP-lys) was studied with a special interest on the role of the immune system in the absorption of small molecules which are recognized as nonself. [3H]-DNP-lys was rapidly absorbed by ligated intestinal loops in situ via a saturable and unique route. When [3H]-DNP-Iys was preincubated with the immune serum obtained from rats immunized with dinitrophenylated bovine serum albumin (DNP-BSA), the [3H]-DNP-lys absorption was depressed. The absorption of [3H]-DNP-lys in DNP-BSA-immunized rats was depressed compared to the control. The results obtained suggest that the immune system play a role in avoiding the absorption of small molecules with antigenicity.

Key Words intestinal absorption, dinitrophenyl-lysine absorption, immunization, dinitrophenylated bovine serum albumin

Evidence has suggested that the immune system is involved in refusing intestinal uptake of macromolecules (1-3). For example, it was demonstrated that the uptake of horse radish peroxidase or bovine serum albumin by everted gut sacs was depressed in the rat previously immunized with the same antigen (1, 2). Antibodies present in intestinal secretions may interfere with the absorption of antigenic macromolecules (1-3). One possible mechanism of this interference has been thought to be that the antibodies complex with these macromolecules so as to prevent the adsorption of the macromolecules to microvillus membranes (4).

In general, the intestinal absorption of small molecules seems to proceed more rapidly than that of macromolecules. Thus, it is of interest to investigate whether the immune system is involved in refusing the intestinal absorption of small molecules which can be recognized as nonself. Haptens are small, chemically defined substances which are not immunogenic but react with antibodies. Dinitrophenyl-
lysine (DNP-lys) is one of well characterized haptens (5) and antibodies against this molecule is induced by immunization with appropriate forms of the antigen such as dinitrophenylated protein conjugates (5). It is possible that antibodies against DNP-lys affect the intestinal absorption of this hapten molecule. The present study is concerned with the effect of immunization with dinitrophenylated bovine serum albumin (DNP-BSA) on the absorption of DNP-lys. Some general characteristics of the absorption of DNP-lys are also described.

**MATERIALS AND METHODS**

*Animals.* Male Sprague-Dawley rats weighing 190–200 g were obtained from Charles River Japan Co. and fed by a rat chow (Oriental Yeast Co., # MF). DNP-BSA-immunized rats were dosed 0.2 ml of DNP-BSA emulsified with Freund’s incomplete adjuvant (FIA) intraperitoneally six times at ten day intervals. Control rats were dosed 0.2 ml of FIA-emulsified saline on the same schedule as for the DNP-BSA-immunized rats. DNP-BSA (20 mol/l mol BSA) was prepared by coupling dinitrofluorobenzene with bovine serum albumin (6), and 1 vol of DNP-BSA (20 mg/ml in saline) was emulsified with 2 vol of FIA.

At the end of immunization, there was no significant difference of the body weight between the two groups. The rats were fasted for 18–20 h before the DNP-lys absorption experiment.

*DNP-lys absorption experiment.* The intestinal DNP-lys absorption was studied using $\varepsilon$-[phenyl-3,5-3H(N)]-dinitrophenyl-L-lysine ($[^3H]$-DNP-lys, 5.04 Ci/ mmol (New England Nuclear)) by an in situ loop technique (7) with a little modification. The rats were anesthetized with sodium pentobarbital (2.5 mg/100 g body weight, dosed intraperitoneally), and four loops of the jejunum measuring 5–7 cm were isolated and ligated. Five hundred μl of a dose solution was injected into the loops using a syringe with 26 G needle. The dose solution was 100 nM [3H]-DNP-lys (5.5×10^5 dpm/ml)/0.1% glucose/Krebs-Ringer phosphate buffer (KRPB), pH 7.4. Thirty minutes after the dose, the ligated loop was exercised and its outside was rinsed with ice-cold KRPB. The sac was opened, drained over a counting vial, and 2 ml of ice-cold KRPB was flushed through the sac. The flushing was repeated once and the washing was collected in another vial. The sac was slit open, and rinsed in three 5 ml ice-cold KRPB and then three 5 ml ice-cold 1 M propyonic acid with shaking for 30 sec per each rinse. The rinsed intestinal tissue was homogenized in 5 ml KRPB using a Polytron homogenizer (Kinematica, Switzerland). After adding 18 ml of a liquid scintillator (Omnifluor (New England Nuclear): Triton X-100: toluene at 12 g: 1 liter: 2 liters) to each rinsing or tissue homogenate, the radioactivity was counted in a Packard Tricarb 3380 liquid scintillation spectrometer (counting efficiency, 10–35%).

The absorbed $[^3H]$-DNP-lys was calculated by subtracting the unabsorbed $[^3H]$-DNP-lys from the dosed $[^3H]$-DNP-lys. The unabsorbed $[^3H]$-DNP-lys represented the total radioactivity in the rinsings. The absorption ratio (% of absorbed
INTESTINAL ABSORPTION OF DINITROPHENYL-LYSINE 565

[3H]-DNP-lys/dosed [3H]-DNP-lys) was corrected for 500mg of wet intestinal tissue.

Miscellaneous methods. Protein was determined by the method of Lowry et al. (8).

Binding activity to [3H]-DNP-lys in serum was determined as described previously (9).

RESULTS

Some characteristics of dinitrophenyl-lysine absorption by rat intestine

After a dose of [3H]-DNP-lys into a loop of the jejunum, the radioactivity rapidly appeared in the serum (Fig. 1), showing a rapid movement of DNP-lys from the intestine to the blood stream. The complete absorption of the dosed [3H]-DNP-lys does not account for the apparent saturation of the radioactivity concentration in the serum. Because ca. 75\% of the dosed [3H]-DNP-lys was recovered in the washing of the jejunal segment at 30 min after the dose (Fig. 2). The radioactivity recovered in the jejunal tissue was less than 10\% of the dosed [3H]-DNP-lys (Fig. 2). When [3H]-DNP-lys was dosed into jejunal segments separated from the body and the segments were immediately washed by the standard procedure as described in the METHODS section, more than 95\% of the dosed [3H]-DNP-lys was recovered in the washings (data not shown). The standard washing procedure was efficient enough to estimate the unabsorbed [3H]-DNP-lys. Therefore, it is reasonable to calculate the absorbed [3H]-DNP-lys by subtracting the unabsorbed [3H]-DNP-lys from the dosed [3H]-DNP-lys. Figure 3 shows that [3H]-DNP-lys was continuously absorbed up to 60 min with a slight decrease in the absorption rate.

[3H]-DNP-lys was absorbed faster in the upper intestine than the lower intestine (Fig. 4). In routine experiments, the [3H]-DNP-lys absorption was

![Fig. 1. Time course of the serum level of the radioactivity after a dose of [3H]-DNP-lys into a ligated loop of the intestine. A ligated intestinal loop measuring 60 cm in length was made in normal rats and 5 ml of a dose solution (1 \mu M [3H]-DNP-lys, 1.1 \times 10^7 dpm/ml) was injected. Blood was collected from the caudal vein and the radioactivity in 0.1 ml serum was counted. Values are means ± SE of three rats.](image)

Vol. 29, No. 5, 1983
Fig. 2. Unabsorbed [³H]-DNP-lys recovered after washing the intestinal segment. 500 μl of a dose solution containing 100 nm [³H]-DNP-lys was injected into ligated jejunal loops made in normal rats. 30 min after the dose, the unabsorbed [³H]-DNP-lys was recovered by the standard procedure described in the text. The recovery (% of dosed [³H]-DNP-lys in each washing and the tissue homogenate) is shown in the figure. Values are means ±SE of five determinations.

Fig. 3. Time course of the [³H]-DNP-lys absorption in normal rats. All procedures and calculations were exactly as in the text except that the jejunal loops were exercised and washed at varied time after the dose of [³H]-DNP-lys. Values are means ± SE of five determinations.

determined by using four loops per rat made in the jejunum in order to minimize the technical variations.

In the presence of unlabeled DNP-lys, the [³H]-DNP-lys absorption was depressed, whereas no effect of unlabeled lysine was observed (Fig. 5). This result suggests that DNP-lys is absorbed via a saturable and specific route which is distinguishable from a route for lysine.

J. Nutr. Sci. Vitaminol.
Fig. 4. Absorption of $[^3\text{H}]-\text{DNP-lys}$ by various parts of the intestine. Ligated loops of the duodenum, jejunum (made four loops from the proximal side), and ileum (made one loop at the distal end) were made in normal rats. The absorption of $[^3\text{H}]-\text{DNP-lys}$ was determined as in the text. Values are means ± SE of nine rats. *Significantly different from the values for the duodenum and the jejunum ($p<0.05$).

Fig. 5. Effect of unlabeled DNP-lys on the $[^3\text{H}]-\text{DNP-lys}$ absorption. The absorption of $[^3\text{H}]-\text{DNP-lys}$ was determined in the absence (●) or presence (○, DNP-lys; △, lysine) of competing unlabeled amino acids. All procedures were as in the text. Each point is a mean of seven determinations.

Effect of immunization with dinitrophenylated bovine serum albumin on dinitrophenyl lysine absorption

The effect of immune serum obtained from rats immunized with DNP-BSA on the $[^3\text{H}]-\text{DNP-lys}$ absorption was shown in Table 1. Specific binding activity to DNP-lys was detected as 86 pmol/ml in the immune serum, whereas undetectable (<200 fmol/ml) in the control serum. $[^3\text{H}]-\text{DNP-lys}$ was first incubated with these sera, and then the $[^3\text{H}]-\text{DNP-lys}$ absorption was determined as in legend (Table 1). In the presence of the immune serum 8.5% of the dosed $[^3\text{H}]-\text{DNP-lys}$ was
Table 1. Effect on the [3H]-DNP-lys absorption of the immune serum obtained from DNP-BSA-immunized rats.

| Donor rat          | DNP-lys binding activity in serum | % of dosed [3H]-DNP-lys |
|--------------------|-----------------------------------|------------------------|
| Control            | <200 fmol/ml                      | 25.5 ± 3.4             |
| DNP-BSA immunized  | 86 pmol/ml                        | 8.5 ± 2.5              |

Pooled sera (diluted to 1:1,000 with KRBP) from DNP-BSA-immunized rats were first incubated with 100 nm [3H]-DNP-lys at 0°C for 15 h, and then injected into ligated loops of the jejunum made in normal rats. Control sera were obtained from rats which received FIA-emulsified saline. Values are means ± SE of nine determinations. Binding activities to [3H]-DNP-lys in the sera were determined as described (9).

Table 2. Effect of immunization with DNP-BSA on the [3H]-DNP-lys absorption.

| Rat                | % of dosed [3H]-DNP-lys |
|--------------------|-------------------------|
|                    | Absorbed    | Tissue       | Transferred into blood |
| Control (33)       | 28.5 ± 2.1 | 8.6 ± 0.9    | 20.0 ± 2.0             |
| DNP-BSA immunized  | 17.6 ± 1.8*| 3.5 ± 0.4*   | 14.1 ± 1.5**           |

Ligated jejunal loops were made in control and DNP-BSA-immunized rats and the absorption of [3H]-DNP-lys was studied by the standard procedure. Values are means ± SE. Numbers of the loops studied are in parentheses. * ** Significantly different at p < 0.001 (*) and p < 0.05 (**).

absorbed. This value was significantly lower than the value of 25.5% determined on the control serum. This result suggests the immune serum has a potency to depress the DNP-lys absorption in some limited conditions.

In the rats immunized with DNP-BSA, 17.6% of [3H]-DNP-lys was absorbed by jejunal loops in situ (Table 2). In the control rats, the absorption ratio of [3H]-DNP-lys was 28.5% (significantly higher than the DNP-BSA-immunized rats at p < 0.001). Thus, the intraperitoneal immunization with DNP-BSA resulted in the depression of the DNP-lys absorption. Overall evidence described suggest that the absorption of DNP-lys by the intestine can be interfered with by the immune system which is switched on against this hapten molecule.

DISCUSSION

In the present paper, the absorption of DNP-lys was studied as a model system.
to investigate the involvement of the immune system in the absorption of small molecules which are recognized as nonself. DNP-lys was rapidly absorbed by ligated intestinal loops in situ. The upper part of the intestine had a higher potency to absorb DNP-lys than the lower part. DNP-lys was absorbed via a saturable and unique route.

In rats previously immunized with DNP-BSA, the DNP-lys absorption was depressed. The depression of the DNP-lys absorption was supposed to be mediated by the presence of anti-DNP antibodies in luminal fluid or the mucosal surface. An experimental evidence for this speculation is that the preincubation of DNP-lys with anti-DNP antiserum resulted in the depression of the DNP-lys absorption. It is possible that the formation of DNP-lys-antibody complexes diminishes the diffusion rate of DNP-lys, resulting in the depression of its absorption rate.

Ingested proteins are subjected to digestion by proteolytic enzymes in the gastrointestinal tract, but not completely. A small amount of antigenically intact proteins is absorbed by the intestine and gains to access to the systemic circulation (10, 11). It is unlikely that the intestinal absorptive epithelia is by its nature a rigid barrier against ingested proteins. The penetration through the intestinal mucosa of xenogenic proteins with biological activities (ex. enzymes) will evoke adverse reactions in animals due to their own biological activities. On the other hand, evidence suggests that the intestinal uptake of xenogenic proteins is depressed in the animals in which the immune system is switched on against the same proteins (1–3). Unless the penetration of antigenically intact proteins is restrained at the mucosal surface in sensitized animals, the proteins readily reach the submucosa and the systemic circulation wherein they react with their antibodies and sensitized cells. In consequence, adverse reactions to the ingested proteins will be evoked due to hypersensitivity (12). Thus, the intestinal immune system may secure the absorptive surface to avoid the uptake of ingested proteins with adverse effects due to the particular biological activity or the hypersensitivity.

Haptens are small, chemically defined substances which are not immunogenic but react with antibodies. It is possible that haptenic determinants are introduced onto food proteins during cooking, processing, or chemical modification of the proteins (13). Hapten molecules released from the food proteins by proteolysis in the gastrointestinal tract probably evoke adverse reactions in the case that these xenobiotics penetrate the intestinal mucosa. The present study suggests that the intestinal immune system plays a role in avoiding the intestinal absorption of xenogenic small molecules like haptens in sensitized animals.

Although the preincubation of DNP-lys with anti-DNP antiserum resulted in the depression of the DNP-lys absorption, the mechanism which depressed the absorption of DNP-lys in DNP-BSA-immunized rats is unclear. The gut-associated lymphoid tissue (GALT) is one of the predominant sites secreting antibodies into luminal fluid (4). The liver is also involved in supplying antibodies into luminal fluid (14, 15). The antibodies secreted from GALT and the liver, especially secretory IgA (sIgA) antibodies adsorbed to the mucosal surface, are currently considered to
function as an anti-septic paint in avoiding the penetration of pathogenic microorganisms or the absorption of antigenically intact macromolecules (4). sIgA antibodies may be involved in blocking the absorption of xenogenic small molecules with antigenicity.

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