Effect of Protein Inhibitors on Protein and Amino Acids in Mesenteric Lymph* (33637)

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Lipoprotein synthesis by the intestine was investigated by various workers (1, 2). There is evidence that protein formation in the intestine is necessary for transport of long chain fatty acids from the intestinal lumen. Using protein inhibitors, Isselbacher (3) has shown the inability of the mucosal cell to transport re-esterified lipid out of the cell into the lymphatic system.

Much interest has been generated in the use of protein inhibitors to block the transport of lipids out of the mucosal cells in animals since it mimics the human familial syndrome, β-lipoproteinemia (4). In this hereditary disease, alimentary lipemia results with no chylomicrons being formed by the mucosal cells after ingestion of a lipid-rich diet. Also, no β-lipoprotein is produced by the liver (3).

We traced a labeled dietary amino acid into protein and free amino acid fractions found in the mesenteric lymph of rats. The effect of protein inhibitors on the protein and amino acid concentrations were also observed.

Methods and Materials. Male rats of Sprague-Dawley origin weighing between 250–400 g fed a diet of Purina laboratory chow were used in these studies. The cannulation of the mesenteric lymph duct, postoperative care of the animal, collection of lymph, protein-amino acid separation, and chemical and radioactivity measurements were as described previously (5). Experiments with the cannulated rats were undertaken at least 20 hr after surgery; the rats were taken off food 6–8 hr before each experiment.

Cycloheximide (Acti-dione, Mann Research Laboratories, New York, N. Y.) dissolved in saline was injected intraperitoneally at a dosage of 1 mg/kg of body weight 2 hr before feeding the radioactive test meal.

Puromycin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in phosphate buffer as described by Sabesin *et al.* (4) , and injected at a dosage of 75 mg/kg of body weight 2 hr before feeding the radioactive test meal. Radioactive test meals were prepared by mixing 20 μCi of L-leucine-^{14}C, (random label, New England Nuclear Corp., Boston, Mass.) with 1 ml of cream (12.5% butterfat). This preparation was fed by stomach tube to the rats after the protein inhibitor had been injected.

Mesenteric lymph protein was separated by disc electrophoresis. The method of Clarke (6) was employed, using 7.5% polyacrylamide gel. Between 75–150 μg of protein, diluted with 5% sucrose, was placed on each gel, and separated at 4 mA per tube for 40 min. After staining with 0.1% Naphthol Blue Black (Amidoschwartz) in 7% acetic acid for 1 hr, the gels were cleared with 7.5% acetic acid and scanned for density gradients and integrated on a recording densitometer (Densicord, model 542, Photovolt Corp., New York, N. Y.), equipped with a special carriage, a 0.1 $\times$ 5 mm light slit and 595 mμ filter for gel scanning (recordings were made on a D-2 Response, a quasi-logarithmic response).

The electrophoresed protein was measured for radioactivity by cutting the gel in 2.0-mm slices and disbursing each in an all-glass homogenizer with 1.0 ml of NCS reagent (Nuclear Chicago Corp., Des Plaines, Ill.) to be solubilized for liquid scintillation counting.

Rat plasma, obtained by heart puncture, was also separated and measured by disc electrophoresis for comparison to lymph proteins.

Results and Discussion. Protein levels. The

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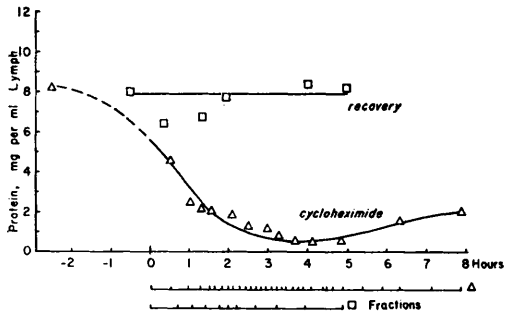

FIG. 1. Protein levels in mesenteric lymph after cycloheximide (injected at —2 hr). The test meal was fed at zero-time. The "recovery" data were obtained 3 days after cycloheximide injection (the same animal). Fraction points indicate sample collection frequency (0.5 ml of lymph/fraction), indicating lymph flow rate.

effects of the antagonist cycloheximide and puromycin upon the protein level in mesenteric lymph are shown, respectively, in Figs. 1 and 2. Protein levels after recovery from cycloheximide intoxication (3 days after cycloheximide, Fig. 1) are also shown. The lymph protein level was markedly affected by systemic cycloheximide. The total protein content of the lymph had dropped rapidly after cycloheximide injection, and continued to drop to the fourth and fifth hour after feeding, at which time there was a steady rise in the protein content of the lymph. The protein in the lymph returned to normal level after

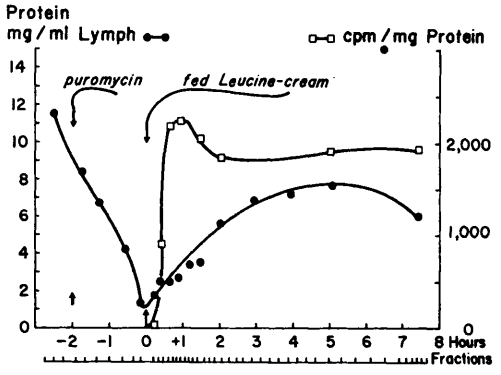

FIG. 2. Protein levels and specific activity of lymph protein in mesenteric lymph after puromycin (injected at —2 hr). The test meal (with leucine-^{14}C) was fed at zero-time. Lymph flow is indicated by the frequency of 0.5-ml fractions. These and all radioactivity data are corrected for background.

recovery from the effects of the antagonist (within 3 days).

Labeled protein. Figure 3 reveals that the incorporation of labeled leucine into the lymph protein of the cycloheximide-treated animal was nearly zero. Also, lymph collected during this time had an opalescent watery appearance. The lymph became milky about 6 hr after feeding the test meal coinciding with a slight rise in protein level.

Puromycin injection depressed the protein level in the lymph for the first 2 hr, but under the influence of the cream meal, it continued to rise until back to a normal concentration range (Fig. 2). However, the ability of the animal to detoxify the inhibitor is

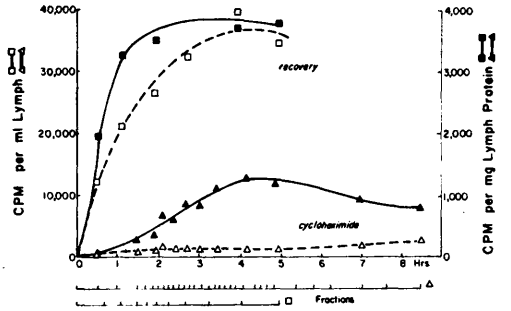

FIG. 3. Radioactivity in lymph protein after feeding leucine-^{14}C. Radioactivity in lymph per volume and specific activity of the protein is shown as influenced by cycloheximide and 3 days after recovery.

pertinent since Sabesin and Isselbacher (4) and others (7) gave repeated injections to the animals every 2 hr. In these experiments a single dose at 75 mg/kg of body weight was administered. The fed amino acid was incorporated into the protein in the puromycin-treated animal, and there was transport of lipid, since the lymph was milky in appearance. Total lipid or its nature was not investigated in these experiments.

The specific activity of protein found in the lymph of the rat treated with cycloheximide (Fig. 3) showed a much lower incorporation of leucine into protein than with puromycin. Also, the puromycin-treated animal showed an uptake approaching that of a normal animal, whereas the cycloheximide caused a very slow incorporation of the ami-

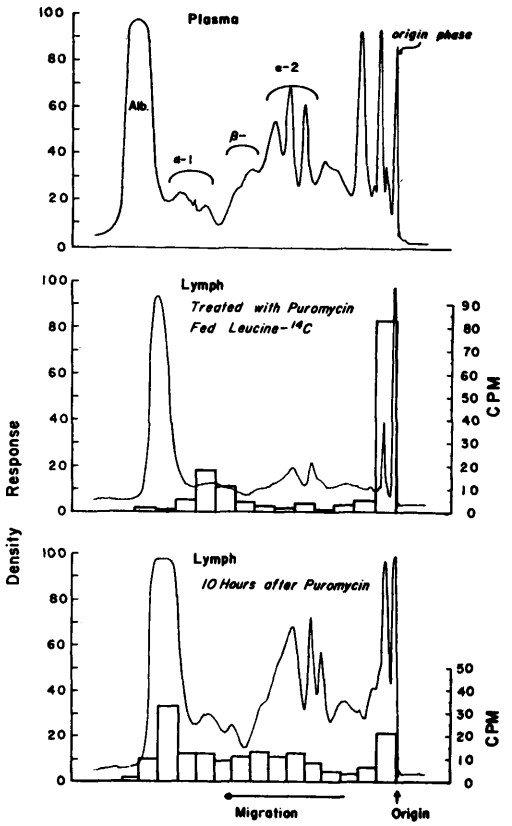

FIG. 4. Disc electrophoresis patterns of normal rat plasma, lymph after puromycin and after a 10-hr recovery from the antagonist. Radioactivity of the fraction is indicated by superimposed bar-graphs. Chylomicrons accumulate on the surface at the origin on the gel. See text for details.

no acid into lymph protein. However, 3 days later, after recovery from cycloheximide, this animal incorporated leucine-^{14}C normally, as revealed by the specific activity[2] of the protein released into the lymph.

Electrophoretic distribution. Mesenteric lymph and plasma protein separated by disc electrophoresis had similar distribution profiles (Fig. 4); Borgstrom and Laurell (8) reported a similar observation using paper electrophoresis. However, the largest difference was in the portion of the gel near the origin, which Clarke (6) located as the α-2 proteins. The plasma contained several more peaks which were quite small or did not appear in the lymph (Fig. 4).

The effect of puromycin on lymph collected 144–151 min after injections not only lowered the total protein concentration, but decreased the percentage of α- and β-proteins, with respect to the albumin fraction. Lymph from normal rats, and rats recovered from puromycin injection, contained about 33% of the protein as albumin. Plasma from cannulated rats revealed about the same value. Lymph collected 144 min after puromycin injection contained about 60% of the protein as albumin. The data reveal a lowering of globulin proteins in the lymph after puromycin injection.

Electrophoresed protein, of the lymph collected 24–31 min after feeding leucine-^{14}C[3] showed most of the radioactivity at the origin, predominantly in the chylomicron fraction, with activity also in the α-1 and β-2 proteins. Lymph collected 8 hr after feeding showed most of the radioactivity in the albumin fraction. However, the chylomicrons still contained a sizable portion of the radioactivity.

Labeled leucine in lymph. The distribution of radioactivity (leucine-^{14}C) in the amino acid fraction after cycloheximide treatment is shown in Fig. 5. In the normal animal there

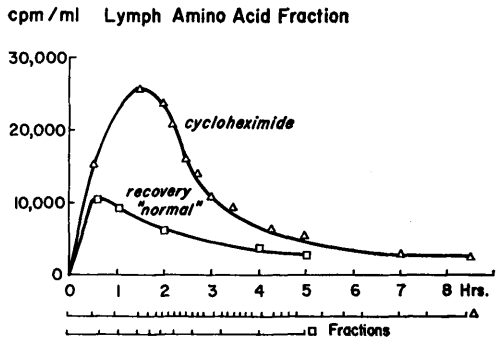

FIG. 5. Radioactivity in the lymph amino acid fraction (nonprotein) as influenced by cycloheximide and after recovery from the antagonist.

was a rapid rise in radioactivity in the lymph which dropped to a low point after 4–5 hr. The peak of radioactivity occurred within the

[2] This specific activity curve was superimposable over that obtained with the normal animal.

[3] 144–151 min after puromycin injection.

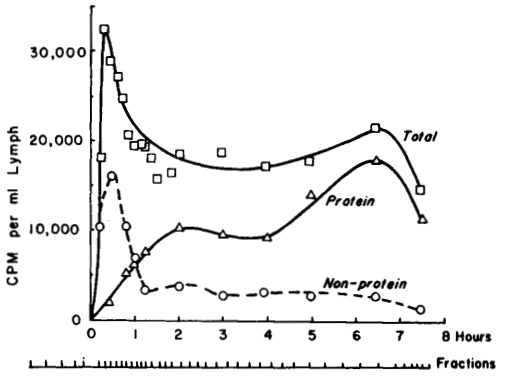

FIG. 6. Distribution of radioactivity after feeding leucine-^{14}C to a rat having been treated with puromycin 2 hr before feeding. Leucine (20 μCi) was fed at time zero. The total lymph radioactivity (□), protein fraction (△), and the non protein (free amino acid) fraction (○) are plotted, as is the frequency of 0.5-ml lymph fractions.

first hour after feeding, corresponding to the active phase of amino acid absorption from the gut. After treatment with cycloheximide it might be expected that the level of the fed radioactive amino acid should appear to be higher in the amino acid fraction than in the protein fraction, since protein synthesis was inhibited. This was apparent in the cycloheximide-treated animal which showed a definite increase in the free radioactive amino acid which had been fed.

In the puromycin-treated animal, however, the radioactive leucine showed a distribution curve similar to that formed in the normal rat since it appeared that protein synthesis was not completely blocked by the antagonist as revealed by the distribution of radioactivity under these experimental conditions (Fig. 6).

Lymph amino acid levels. Lymph collected during an 8-hr period after feeding normal rats and those injected with cycloheximide or puromycin was analyzed by column chromatography for free amino acids. With normal rats the amino acid distribution was lowered to some extent for most of the measured amino acids during the active phase of absorption. On the other hand, there was a marked disturbance induced by cycloheximide in the distribution pattern of a variety of amino acids as well as an increase in the total amount released into the lymph. For example, during the active absorption phase in the normal animal the amino acids were released into the lymph at a concentration of 173 μmoles %; under similar conditions with cycloheximide the neutral and acidic amino acids were at a concentration of 260 μmoles %, an increase of 51% above the normal. After recovery from cycloheximide the concentration of lymph amino acids was 171 μmoles %, showing a return to the normal quantities and distribution. The lymph from animals treated with puromycin had an increase in amino acid concentration to the extent of 22%, however, the increase in tyrosine concentration was 147%, the greatest increase of any amino acid measured.

The large increase in tyrosine may be related to the structural similarity of puromycin to the end group of s-RNA-tyrosine. The antibiotic, puromycin, has a chemical structure resembling amino acyl-sRNA as first noted by Yarmolinsky and De la Haba (10). The end group of this molecule has the same structure as tyrosine but with its hydroxyl group methylated. The precise mode of puromycin action is not known, but it appears to inhibit protein synthesis at a late stage, namely the premature release of the unfinished peptide from the ribosomes (11). Studies using puromycin-^{14}C revealed that the antibiotic was convalently bound to the soluble polypeptide released from the ribosomes in reticulocytes (12). Apparently one residue of puromycin is bound for each polypeptide chain. It is interesting to note that while puromycin increased total amino acid concentration by 22%, it increased the tyrosine concentration greater than any other amino acid. In the rat treated with cycloheximide, which inhibits peptide elongation, there was 51% increase in total amino acid concentration but only a small increase in the tyrosine concentration. Since the large increase in tyrosine occurs with a protein inhibitor which is a structural analog of the end of the sRNA-tyrosine molecule, it is interesting to speculate that this may be the point of binding of puromycin to the peptide chain. Also, it is noted that protein inhibitors

acting in different ways result in different distributions of amino acids found in the lymph.

These findings lead one to speculate that the *amino acid pool-protein* relationship varies in regard to the nature of the protein or peptide moieties which are undergoing biosynthetic reactions. The size and profile of the amino acid pool could be influenced by antagonists to the synthetic reactions at either the sRNA-amino acid stage or during peptide lengthening, as would be influenced by puromycin or cycloheximide, respectively. This pool then could release amino acids from the cell to the lymphatic system, as was observed in these experiments; however, one might expect that this release to the circulation (i.e., blood, intestinal lumen, or the lymph) would reflect the amino acid distribution in the cell under the influence of either of these antagonists.

There is an indirect relationship between synthesized protein and amino acid transported into the lymph, as seen when leucine-^{14}C is fed with or without an antagonist; less synthesis, more free leucine-^{14}C in the lymph. What is of some concern is the level of tyrosine released into lymph of the puromycin-treated system. This change in amino acid distribution, with relatively high tyrosine leads one to postulate that there are specific steps involving particular amino acids in the synthesis of protein in the intestine, in this case, tyrosine, as influenced by puromycin for the formation of protein which the intestinal cells can transport into the lymph.

Both antagonists interfere with the transport of lipoprotein and triglyceride from the mucosal cell, as was shown by Isselbacher (13). Previous work (14) showed a dynamic bidirectional flux of amino acids across the gut from the lumen to the circulation. Our findings indicate that the antagonistic effect upon lipid transport, as seen by others, is related to protein synthesis and its relationship to the amino acid pool as reflected by the appearance and distribution of both proteins and free amino acids in the mesenteric lymph. These relationships indicate a possible mode or mechanism of action, in that

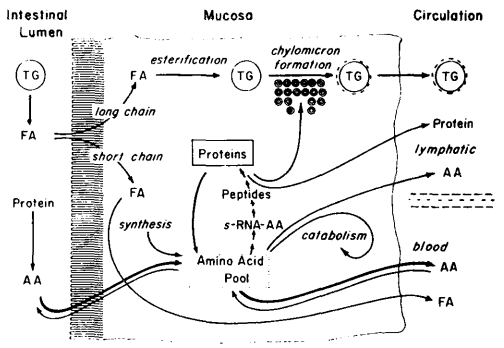

FIG. 7. Relationships between dietary lipid and amino acids of the diet and amino acid pools of the mucosal cell and circulation: TG represents triglycerides; FA, fatty acids; TG encircled with double ring, the chylomicron; ⊙, protein used to form the chylomicron; AA, amino acids. This simplified diagram does not take into account the role played by phospholipid, bile salts, or carbohydrate metabolism. It points out where the puromycin may act at the sRNA-AA stage and cycloheximide at the peptide stage in the synthesis of protein required for lipid transport as related to amino acids in the cell pool and released into lymph.

puromycin influences the synthesis of protein released into lymph at the sRNA-AA stage and the cycloheximide at the peptide stage. Since the amino acid pool seems to be different with these two antagonists it may be that there are different amino acid requirements for the final stages of synthesis or other pathways to be realized in the final synthesis of the lipoprotein fractions found in lymph. It is also apparent that there is a possible direct relationship between amino acid and lipid transport from the intestinal lumen to the lymphatic system. This has been summarized in Fig. 7.

Summary. The effects of the protein antagonists cycloheximide and puromycin upon the transport of amino acids and newly synthesized protein into the mesenteric lymph of the intact rat was investigated. Upon feeding a fatty meal (bovine cream) fortified with leucine-^{14}C these antagonists depressed the formation of lymph protein with a concurrent increase of amino acids, as measured chemically and by tracing radioactivity of these fractions. The influence of cycloheximide was more lasting than puromycin. These two an-

tagonists affect protein synthesis differently as evidenced by the rates of new protein synthesis and the nature of the amino acids transported by the lymph leaving the upper small intestine.

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Susceptibility of Human Lymphoblasts (RPMI 7466) to Viral Infections *in Vitro* (33638)

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Since the reports of Pulvertaft (1) and Epstein and Barr (2) on the successful serial culture of lymphoblasts from Burkitt's human lymphoma, several other lymphoblast cell strains derived from Burkitt's lymphoma (3–5) and from normal (6, 7) or leukemic (8–11) peripheral blood have been described. These cell strains were of particular interest because of their infection with a herpes-type virus (12) and because of the possible association of this virus with the human malignancies from which the cells were derived.

Little is known of the general viral susceptibility of human cells of this type. Attempts have been made in this laboratory to isolate viruses from sera of persons with infectious hepatitis, using a strain of lymphoblasts (RPMI 7466) derived from normal human peripheral blood (6). In these studies, this strain of lymphoblasts was found resistant to the cytopathic effect (CPE) of some viruses which normally replicate in human cells in tissue culture. These cells were capable however, of supporting persistent viral infections in most instances, and an investigation of cell–virus relationships in lymphoblasts infected with poliovirus revealed some unusual features. Results of these studies are presented here.

Materials and Methods. Tissue cultures. Human lymphoblasts (strain RPMI 7466), derived from peripheral blood of a person without malignant disease (6), were obtained from Dr. George Moore, Roswell Park Memorial Institute. These cells, which consist predominantly of immature lymphoid cells, and a few large multinucleate cells and mature lymphocytes, multiply freely in the fluid medium of agitated or stationary suspension cultures.

Cells were propagated in stationary Blake culture bottles[1] in Moore's 1640 medium (6), supplemented with 1% by volume of bovine fetal serum and the following per liter:

[1] Corning Glass Cat. No. 1285.