An immunoreactive peptide in milk contains bombesin-like bioactivity

L. H. Lazarus[1], G. Gaudino[2], W. E. Wilson and V. Erspamer[3]

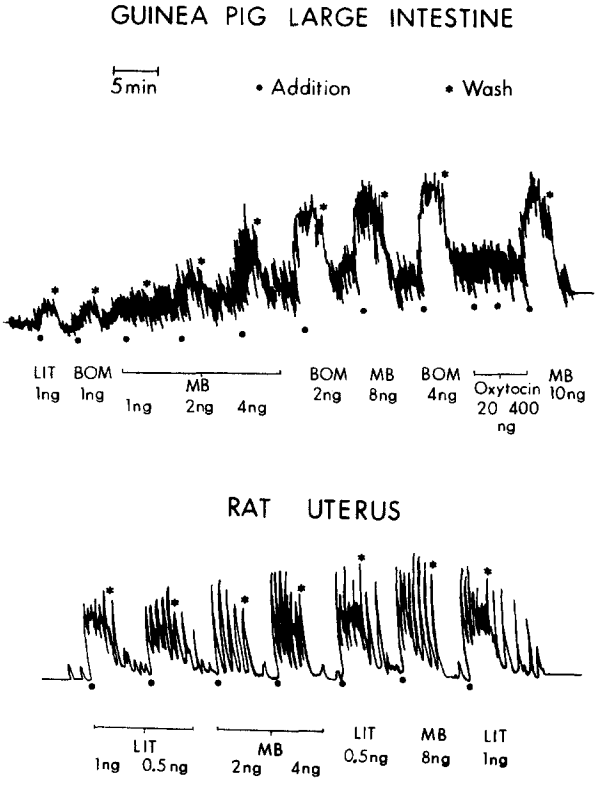
Peptide Neurochemistry Group, Laboratory of Behavioral and Neurological Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park (North Carolina 27709, USA), 10 September 1985

Summary. Parallel in vitro bioassays using rat uterus and guinea pig large intestine tissues specific for the bombesin family of peptides, demonstrated that the bombesin-like peptides present in bovine milk can produce a dose-related response similar to bombesin and litorin. The bioactivity of this type of milk peptide appeared to be approximately 20–50% as active as the amphibian peptides. These data support the proposal that a bombesin immunoreactive peptide in milk contains bombesin bioactivity.
Key words. Bombesin; litorin; bovine milk; smooth muscle.

Recently, evidence has indicated the existence of bombesin-related peptides in bovine[4,5] and human[6,7] milk by the use of selective immunoassays. It is known that serum levels of many gastrointestinal hormones increase after ingestion of milk by the human neonate[8] and that similar effects may be elicited by intravenous infusion of bombesin in adults[9,10]. Furthermore, such physiological phenomena as satiety, blood sugar levels, serum levels of many gastrointestinal hormones, and stomach acidity may be effected by intravenous or intracerebraventricular administration of amphibian bombesin or its mammalian counterpart[9–12]. Bombesin-like peptides have also been suggested to play a role as a growth factor[13], in addition to their involvement in releasing other peptide hormones, neurotransmitters or prostaglandins[14]. Since the absorption of prolactin[15] and thyrotropin-releasing factor (TRF)[16] into plasma occurs with retention of their hormonal, biological properties, it is conceivable that a milk bourne peptide(s) containing bombesin determinants [milk bombesin (MB)] may be absorbed intact and function in a modulator capacity. This possibility could be of particular importance in the case of the developmental processes in the neonate. In order to supplement our previous RIA evidence[4], an effort was undertaken to determine whether the biological activity of MB mimics that of amphibian bombesin. This involved subjecting MB to in vitro bioassays which are especially useful for testing bombesin activities[17,18].

Methods. Milk bombesin was recognized using a rabbit antibody that cross-reacts with the bombesin tetrapeptide sequence -Gly-Asn-Gln-Trp- (residues 5–8) which is partially included in the bioactive domain of amphibian bombesin[11,18,19]. MB was purified from 500 g lots of commercial dry skim bovine milk[5]. Synthetic amphibian bombesin and litorin were obtained from Farmitalia Carlo Erba Res. Labs., Milan, Italy. Bioassays were performed according to methods described by Erspamer et al.[17]. A preparation of guinea pig large intestine or rat uterus was bathed in 10 ml Tyrode's solution, at 32 °C, in a glass chamber and aerated with an oxygen (95%)-carbon dioxide (5%) mixture. Contractile responsiveness, recorded with an isometric transducer coupled to a Unirecord (U. Basile, Milan), was allowed to stabilize prior to introduction of the various peptides. The amplitude and frequency of the contractile response of the preparations were compared against a dose-response curve using the amphibian peptides. Quantitative estimates of MB, based on immuno-cross-reactivity with our antibody to amphibian bombesin (BM-XII-165-4)[4], are in dimensions of bombesin-equivalents.

Results. Contractile responsiveness of guinea pig large intestine to MB was qualitatively indistinguishable from that to bombesin or litorin (fig.); quantitative estimates suggested that MB bioactivity was 25–50% that of the amphibian peptides. Each peptide effected rapid initiation of contractility of the large intestine; removal of the peptides resulted in a return to baseline conditions. The large intestine was unresponsive to 400 ng of oxytocin, nor did oxytocin influence intestinal responsiveness to MB. MB also appeared to have a progressive effect on the rhythmic movement of guinea pig large intestine. With rat uterus, the rate of onset of the contractions was similar to that for litorin and MB; however, the persistence of the contractions after exposure to and washing out of litorin may indicate that MB differs structurally from that peptide.



Bioassay of MB on guinea pig large intestine and rat uterus. The contractility of guinea pig large intestine (top) or rat uterus (bottom), was recorded as given in the text. Time of addition of peptides is indicated by a dot (.); washout is indicated with an asterisk (*). The MB tested was the peak of immunoreactivity that eluted from a Bio-Gel P-10 column[4,5]. The bar represents 5 min. The abbreviations are: LIT, litorin; BOM, bombesin; MB, milk bombesin immunoreactivity.

Discussion. These pharmacological data show that the response characteristics of these two tissues to MB is very similar to that which would be expected from a bombesin-like peptide. These results, coupled with the cross-reactivity of MB to a bombesin-specific antibody[4], support the contention that MB has the potential for being a physiologically important peptide in the nutrition of the neonate. The amount of immunoreactive bombesin ingested during breast feeding varies from 0.1–1 ng equivalents/ml[6,7], a quantity sufficient to affect blood levels of several gastrointestinal hormones[8,9,20–22] as well as gastric acid production[9]. If the neonate consumes approximately 50 ml milk at each nursing, the quantity of MB consumed would be well within the range of the minimum effective dose (circa 5 ng/kg) of amphibian bombesin required to elicit a response during i.v. infusion in adult humans[9]. Formula fed (supplemented with cow's milk) of 'bolus-fed' neonates[21,22] would obtain considerably more MB[4].

The susceptibility of MB to enzymatic digestion is not presently known, but could be an important concern. Several peptides, such as prolactin[15], TRF[16], in addition to milk- and food-derived morphinomimetic peptides[23], may be absorbed from the stomach within minutes, permitting physiologically active forms to reach the blood stream with minimal proteolytic degradation. Other factors in milk may contribute to the stability of peptides as well.

In view of the known pharmacological and physiological effects of amphibian bombesin in mammals[9-12, 17, 18], MB may also function as a 'nutrient hormone'[13] and, by analogy with amphibian bombesin, may be capable of influencing smooth muscle contractility, release of gastrointestinal hormones and prostaglandins[14, 24] and regulation of tissue growth. It may also be capable of acting synergistically to potentiate the effects of other hormones such as the GRP-stimulated release of ACTH by CRF[25].

1 Address for correspondence: Dr L.H. Lazarus, Peptide Neurochemistry Group, Laboratory of Behavioral and Neurological Toxicology, National Institute of Environmental Health Sciences, P.O. Box 1233, Research Triangle Park, NC 27709, USA.
2 Visiting scientist from the Department of Animal Biology, University of Turin, Torino, Italy, supported in part by a NATO Fellowship and by the Fogarty International Center of the National Institutes of Health, Bethesda, MD, USA. Present address: Department of Biomedical Science and Human Oncology, University of Turin, Torino, Italy.
3 Institute of Medical Pharmacology, University of Rome, Rome, Italy, supported by a grant from the Consiglio Nazionale delle Ricerche.

4 Jahnke, G.D., and Lazarus, L.H., Proc. natn. Acad. Sci. USA *81* (1984) 578.
5 Lazarus, L.H., Gaudino, G., Wilson, W.E., and Erspamer, V., Biochemistry *23* (1984) 3377.
6 Gaudino, G., Lazarus, L.H., Wilson, W.E., and Tully, M., Protides of the Biological Fluids, p.235. Ed. H. Peeters. Pergamon Press, Oxford 1984.
7 Ekman, R., Ivarsson, S., and Jansson, L., Regul. Pept. *10* (1985) 99.
8 Aynsley-Green, A., Lucas, A., and Bloom, S.R., Acta chir. scand. *507* (Suppl.) (1980) 269.
9 Varner, A.A., Modulin, I.M., and Walsh, J.H., Regul. Pept. *1* (1981) 289.
10 Erspamer, V., and Melchiorri, P., Neuroendocrine Perspectives, Vol.2, p.37. Eds E.E. Miller and R.M. MacLord. Elsevier, Amsterdam 1983.
11 Erspamer, V., and Melchiorri, P., Pure appl. Chem. *35* (1973) 463.
12 Taché, Y., and Brown, M., Trends Biosci. *5* (1982) 431.
13 Lazarus, L.H., Wilson, W.E., Gaudino, G., Irons, B.J., and Guglietta, A., Regul. Pept. *6* (suppl. 3) (1986) in press.
14 Heinz, J., Sametz, W., Petronijevic, S., and Lembeck, F., Naunyn-Schmiedebergs Arch. Pharmak. *326* (1984) 64.
15 Whitworth, N.S., and Grosvenor, C.E., J. Endocr. *79* (1978) 191.
16 Strbak, V., Alexandrova, M., Macho, L., and Poneč, J., Biol. Neonate *37* (1980) 313.
17 Erspamer, V., Falconieri Erspamer, G., Inselvini, M., and Negri L., Br. J. Pharmac. *45* (1972) 333.
18 Erspamer, V., Gastrointestinal Hormones, p.344. Ed. G.B.J. Glass. Raven Press, New York 1980.
19 Girard, F., Aube, C., St.Pierre, S., and Jolicœur, F.B., Neuropeptides *3* (1983) 443.
20 Lucas, A., Aynsley-Green, A., Blackburn, A.M., Adrian, T.E., and Bloom, S.R., Acta pædiat. scand. *70* (1981) 201.
21 Lucas, A., Bloom, S.R., and Aynsley-Green, A., Arch. Dis. Child. *55* (1980) 678.
22 Aynsley-Green, A., Adrian, T.E., and Bloom, S.R., Acta pædiat. scand. *71* (1982) 379.
23 Ziondrou, C., and Klee, W.A., Nutrition and the Brain, vol.4, p.125. Eds R.J. Wurtman and J.J. Wurtman. Raven Press, New York 1979.
24 Subbiah, M.T.R., Yunker, R.L., Yamamoto, M., Kottke, B.A., and Bale, L.K., Biochem. biophys. Res. Commun. *129* (1985) 972.
25 Hale, A.C., Price, J.F., Ackland, J.F., Doniach, I., Ratter. S., Besser, G.M., and Rees, L.H., J. Endocr. *102* (1984) R1.

0014-4754/86/070822-02$1.50 + 0.20/0

The potent tremorgenic neurotoxins lolitrem B and aflatrem: A comparison of the tremor response in mice

R.T. Gallagher and A.D. Hawkes

Ruakura Animal Research Station, Ministry of Agriculture and Fisheries, Private Bag, Hamilton (New Zealand), 9 September 1985

Summary. Tremor dose-response curves were determined for mice dosed with the ryegrass neurotoxin lolitrem B, and the tremorgenic mycotoxin aflatrem. A family of characteristic curves was revealed for each tremorgen, with lolitrem B eliciting a sustained tremor response persisting for over 24 h.
Key words. Tremorgenic neurotoxins; indoles; lolitrem **B**; aflatrem; mice; tremor; dose-response, ryegrass staggers.

The lolitrems are remarkable tremorgenic neurotoxins which have recently been isolated from perennial ryegrass (*Lolium perenne* L.) and ryegrass seed[1-3]. When injected i.p. into laboratory mice, these lipophilic neurotoxins induce a neurotoxic syndrome characterized by sustained pronounced tremors[1, 4]. The structure of the major lolitrem neurotoxin, lolitrem B, of mol.wt 685 and formula $C_{42}H_{55}NO_7$, has been determined and shown to be a complex substituted indole (**1**)[5]. The lolitrem neurotoxins are the prime suspect causative agents of ryegrass staggers, a dramatic nervous disorder of sheep, cattle, horses and deer grazing ryegrass-dominant pastures[6-10]. Animals affected by this disorder exhibit tremors, severe incoordination and hypersensitivity to external stimuli, yet there is a consistent lack of observable specific lesions in even severely affected animals, and such intoxicated animals usually show eventual complete recovery and return to normality[3, 8-11]. Since ryegrass staggers occurs frequently in New Zealand, Australia and the United Kingdom, there is considerable interest in the causative neurotoxins[6-14]. A mouse bioassay[1, 4], based on the sustained tremorgenic response induced in mice by the neurotoxins, was originally used to screen and estimate the relative neurotoxicity of pasture samples taken from pastures on which livestock had developed ryegrass staggers. The bioassay was also utilized in the initial isolation and purification work on the lolitrems[1]. Very recently a rapid, sensitive and quantitative method based on high performance liquid chromatography (HPLC) with fluorescence detection[3], has been developed for lolitrem B estimation in pasture samples and ryegrass plant components.

The unique neurotoxicology of the lolitrems (viz. sustained tremor, absence of primary lesions, rapid return to normality of severely intoxicated animals) warrants investigation. The purpose of the present investigation is to provide some quantitative data on the potency of pure lolitrem B by establishing a dose-response relationship in mice and comparing its action with a well-known tremorgenic neurotoxin, aflatrem (**2**)[15-18], produced by the ubiquitous fungus *Aspergillus flavus*.

Materials and methods. An appropriate volume of a stock solution of lolitrem B or aflatrem (available from the authors' re-