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Progress report

Radioimmunoassay of intestinal hormones

Alimentary diseases are common yet often their aetiology is unknown. This ignorance reflects a basic lack of understanding of normal gut function. One area hitherto little studied is intestinal endocrinology although the bowel is known to be an important endocrine organ. Histologists have agreed that there are at least 11 well defined endocrine cell types in the mucosa[1], presumably all producing different hormones. Why is progress in this field so slow? The answer lies in the great technical difficulty in extracting labile peptide hormones from cells scattered sparsely in the gut mucosa and surrounded by highly destructive proteolytic enzymes. The problems of low concentration and rapid degradation are so formidable that nearly 60 years of continuous work were required to purify the first hormone, secretin[2], and even then many thousands of hogs had to be sacrificed to yield a few milligrams of hormone. The more classical endocrine tissues of the body are gathered together as glands and extracts can easily be made to test hormone actions. In the case of gut hormones their actions have to be elucidated first. Only then is it possible to purify a single peptide from the mass of material extractable from the gut, using its assayable biological property as the reference. If a hormone lacks a well defined biological action it cannot be purified. Thus histologists can still point to several endocrine cells in the gut unassociated as yet with any known hormone.

Great progress has been made in the last decade. As well as the success in isolating secretin[2], gastrin[3], and cholecystokinin-pancreozymin (CCK-PZ)[4], three new substances,—motilin[5], gastric inhibitory peptide (GIP)[6], and vasoactive intestinal peptide (VIP)[7]—have been purified. Once a hormone has been isolated rapid advances can be made in its study. One is no longer dependent on bioassays for information. The bioassay is confusing because it measures only effects which may be produced by more than one hormone. It is laborious to perform and often too insensitive to measure plasma levels. A purified hormone allows measurement by radioimmunoassay. This technique is specific for the particular hormone and is so easy to perform that hundreds of specimens can be analysed in a day.

Radioimmunoassay Technique

When the plasma concentration of any hormone is measured by radioimmunoassay in several different laboratories the answers usually differ widely. This reflects the fact that many technical problems still occur with this type of assay. The requirements to set up an assay are basically simple. A quantity of semipure hormone is coupled to a carrier protein mixed with Freund's adjuvant and injected into rabbits. After about three months a few animals will produce the required antibodies. Iodine 125 is attached to a tyrosine

in the hormone to make the radioactive hormone tracer. Unknown samples are then read off against the hormone standards by their ability to block the binding of the radioactive hormone tracer to a small fixed quantity of antibody.

There are three important causes of error in the current radioimmunoassay: methodological errors, factors affecting antibody binding, and factors of antibody specificity. The methodological errors include failure to prevent hormone degradation in blood samples and during assay incubation. Another cause of error is the use of bad radioactive tracer hormone. This can be because the hormone contains an impurity which is iodinated or because the act of iodinating the hormone itself causes significant chemical alteration. Secondly, factors affecting antibody binding may cause error. The answer in a radioimmunosasay is given by the percentage of radioactive hormone bound to antibody; thus anything that affects antibody binding in an unknown sample is read as if it were the hormone. For example, urea depresses antibody binding and so a uraemic patient's plasma may wrongly appear to have a high concentration of hormone. Other substances acting in this way are plasma proteins and haemoglobin, for example, in a haemolysed sample. In addition to these, there are unknown factors present in plasma which also very significantly alter antibody binding. It is such unknown factors which bedevil the measurement of low hormone values. The third main cause of error in radioimmunoassay is antibody specificity. Antibodies may have too broad a specificity. For example, an antigastrin antibody may cross react with, and therefore measure, cholecystokinin-pancreozymin. Antibodies may also have too narrow a specificity and measure biologically inactive fragments more potently than the whole hormone. Antisera are often raised to hormone preparations containing degraded material and then react avidly with degraded hormone.

Thus, while much useful new data can be provided by radioimmunoassay of intestinal hormones, care is needed in its interpretation. There is often an element of error in the measurement and only a single antigenic part of the hormone is being assessed and not necessarily the biological potency.

Individual Hormones

INTESTINAL GASTRIN

The duodenal mucosa of man has been found to contain almost as much radioimmunoassayable gastrin as the gastric antral mucosa[8]. Gastrin has been found to exist in two major forms[9]. The classical form, whose sequence[10] and synthesis[11] was achieved in 1964, has 17 amino acids and a molecular weight of 2200 and is now known as little gastrin or G 17. Synthetic little gastrin is freely available from ICI Ltd, so that many centres have been able to set up radioimmunoassays and several commercial assay kits are available. The second major form of gastrin has 34 amino acids and a molecular weight of about 4000 and is known as big gastrin or G34[12]. Big gastrin contains the entire sequence of little gastrin[13] so that it is usually fully measured by assays for little gastrin[14]. About half of the duodenal gastrin is little gastrin and half big, whereas the gastric antrum contains nearly four-fifths little gastrin[8]. Significant quantities of gastrin are also present in the jejunum where the proportion of big gastrin is very large[8,15]. Recent assay data have demonstrated the presence of three other minor components of gastrin plasma, an even larger gastrin

(big big gastrin)[15], an intermediate big gastrin, and a little little gastrin[16]. The 34 amino acid big gastrin has been found to have approximately equal molar potency with little gastrin for acid production, but has a slower onset and longer duration of action[17]. It has also been found that big gastrin has a longer half life in the circulation[17] and thus forms a large proportion of the fasting plasma immunoreactive gastrin[14]. As most of big gastrin is found in the small intestine it is perhaps not surprising that patients after antrectomy still have significant levels of gastrin in the circulation and that levels are higher when food passes through the duodenum, as in a Billroth I gastrectomy, than when it is bypassed, as in a Billroth II or Polya gastrectomy[18]. The mode of release and the physiological role of the intestinal gastrins, however, are still incompletely understood.

SECRETIN

Secretin is a member of the secretin, glucagon, enteroglucagon, VIP, and GIP family of peptides. These hormones have markedly similar amino acid sequences[19] and it is likely that they have all been evolved from a single ancestral hormone. Their actions overlap and, for example, at various dose levels they inhibit gastric acid[20,21,22,23]: several members stimulate intestinal secretion[22] and several stimulate insulin release[24,25,26,27]. Fortunately an antiserum raised to one shows little tendency to cross react with other members of the group, which may be partly because of their very different charge configurations. The exception to this is, of course, enteroglucagon whose discovery was entirely due to cross reaction with pancreatic glucagon antibodies[28].

Although pure secretin has been available since 1961[2] and synthetic secretin since 1964[29], its radioimmunoassay has been hindered by the lack of tyrosine, an amino acid necessary for coupling to ^{125}I to make the hormone radioactive for use as a tracer. The problem has been overcome either by use of synthetic secretin with a tyrosine put in[30], or by the iodination of the N terminal histidine in natural secretin[31,32], relatively straightforward procedure.

The first published radioimmunoassay reported extremely high plasma secretin concentrations after stimulation, and, in particular, secretin release after oral glucose[33]. Several biological studies, however, have failed to demonstrate any increase in the flow of pancreatic juice after glucose[34,35,36], and subsequent radioimmunoassays have not recorded any plasma secretin rise with glucose[37,38,39]. The fasting plasma levels of secretin reported are variable, eg, 467 ± 150 (SD) pg/ml[31] or well below 100 pg/ml[30], but it has been demonstrated that intraduodenal acid provokes a moderate secretin rise[30,31]. Secretin assay of primate bowel extracts show the greatest total quantity to be in the jejunum but that the duodenal mucosa has the highest secretin concentration[40], which parallels bioassay results[41,42]. Preliminary studies show that intraduodenal infusions of HCl in patients with duodenal ulcer releases only about half as much secretin as in control subjects[43]. This raises the possibility that in duodenal ulceration there may be a primary or secondary failure to release the upper intestinal hormones which inhibit gastric acid production and stimulate alkalinization of the duodenum. Infusion of highly purified porcine secretin, just sufficient to inhibit pentagastrin-stimulated gastric acid (62 ng/kg/10 min), produces a plasma secretin concentration much higher than that resulting from endogenous secretin release induced by intraduodenal infusion of 40 ml 0·1 NHCl, which markedly inhibits gastric acid. Thus if por-

cine and human secretin are equivalent, this would suggest that endogenous secretin may not be an important factor in gastric acid inhibition.

CHOLECYSTOKININ-PANCREOZYMIN

A number of radioimmunoassays have now been reported for cholecystokinin-pancreozymin[40,44,45,46,47,48] (CCK-PZ) but few of these appear to give reasonable values for human plasma concentrations. The lack of success is partly due to difficulty in iodinating CCK-PZ's sulphated tyrosine and partly due to its poor antigenicity. A major problem is the difficulty in obtaining enough purified CCK-PZ for an adequate immunization programme. This is due to the poor yield of CCK-PZ from hog intestine which is only just over half that of secretin because many more steps are required for purification[49]. Great difficulties have also been encountered in the attempted synthesis of the whole hormone, although the C terminal octapeptide, which has the full range of biological activity[50], was synthesized quite early.

There has been a tendency for initial assays of a hormone to indicate higher plasma concentrations than those published subsequently. In retrospect, when considerable correlative data have been reviewed, the higher values have usually been seen to be in error. It is thus helpful to have criteria to assess what plasma concentrations could be expected, and to treat with reserve values reported greatly in excess of these. In the case of CCK-PZ (and indeed of gastrin and secretin) a great deal is known of the biological effect produced by the administration of various amounts of hormone. A 30-minute infusion of either 0·5 or 1·0 Ivy dog units CCK-PZ/kg produces contraction of the human gallbladder similar to that observed radiographically after a large fat meal[51]. If a 10% distribution space and half life in the circulation of five minutes is assumed the steady state plasma increment achieved by the above infusion can be calculated to be approximately 278 and 556 pg/ml respectively (3000 IDU per mg pure CCK-PZ[49]). Under natural conditions hormone levels much lower than this might be fully effective since other factors such as vagal reflexes and the simultaneous release of facilitatory hormones may make the receptor organ more sensitive.

The earliest reported immunoassay found CCK-PZ undetectable in fasting human plasma but a peak increment of 107 to 200 pg/ml occurred after a fatty meal[44] (1280 to 2400 μ U/ml reported and assuming 12 000 CHRU/mg pure CCK-PZ). Subsequently rather higher values have been reported, for example, basal levels well over 1 ng/ml[46], or levels of 8 to 16 ng/ml after stimulation by a milk drink[48]. A preliminary suggestion has been made that levels are higher in patients with pancreatic deficiency[48] and this illustrates the potential role of gut hormone assays in diagnosis, even in diseases in which hormones are not the prime cause of the pathology.

ENTEROGLUCAGON

Enteroglucagon is a convenient name for the substance found in the mucosa of the gastrointestinal tract which, although very different physiologically from pancreatic glucagon, cross reacts with its antibodies. Other less sonorous names that have been used include 'gut glucagon' and 'glucagon-like immunoreactivity of gastrointestinal origin' (GLI). Enteroglucagon has not yet been purified in significant quantities so that it usually has to be assayed with a cross reacting pancreatic glucagon radioimmunoassay. This measures total glucagon and the enteroglucagon content is then calculated by subtraction of

pancreatic glucagon, which is measured with a second, non-cross-reacting, radioimmunoassay. Enteroglucagon is found in greatest quantity in the mucosa of the ileum and thus occurs rather lower in the bowel than the classical gut hormones[40]. The cell of origin is of endocrine type and lies in the middle zone of the intestinal mucosa, closely applied to the basement membrane[52]. Extraction studies of enteroglucagon show that, like gastrin, it exists in a high and low molecular weight form[53]. For this reason it is preferable to express concentrations in molar rather than weight units.

Enteroglucagon has been shown to be most potently released from the bowel by long-chain triglycerides[54] and glucose[26]. Using the 100 gram oral glucose tolerance test it was found that enteroglucagon release was increased in patients after the operation of vagotomy and drainage and very much higher if the symptoms of dumping occurred[55]. Enteroglucagon release also showed a very high degree of correlation with the fall in plasma volume that occurs in these circumstances[56]. In the dumping syndrome there is an initial period of rapid intestinal propulsion followed by a period of hypomotility[57] and this later phase coincides in time with the very high levels of enteroglucagon. A patient with a renal tumour producing enteroglucagon[58] was found to have gross small and large bowel stasis and also a marked hypertrophy of small intestinal villi[59]. After the tumour had been removed plasma enteroglucagon levels returned to normal and the abnormalities disappeared[59]. From such studies as these the possible physiological role of enteroglucagon can be postulated. Enteroglucagon, which is maximally released when unabsorbed food passes into the lower small intestine, may act to produce a slowing of further food transport and also to enhance long-term growth of the absorptive intestinal mucosa.

GASTRIC INHIBITORY POLYPEPTIDE

The presence of a gastric inhibitory component in 10% pure CCK-PZ was first detected by careful comparison of the biological properties of this impure material with a more highly purified CCK-PZ preparation[60]. When a gastric inhibitory polypeptide (GIP) was completely separated from CCK-PZ-like activity it was found to be a potent inhibitor of even histamine-stimulated gastric acid production[23]. The sequence of GIP was published in 1971[61] and seen to have striking similarities with secretin and glucagon. This led to the search for, and discovery of, insulin releasing activity[27]. Radioimmunoassay of human plasma GIP concentrations showed a considerable rise following ordinary meals[62], of which the glucose and fat components appeared to be the main stimulatory agents[63]. The plasma levels achieved were of the same order of magnitude as might have been expected to occur following the exogenous administration of enough GIP to inhibit gastric acid and stimulate insulin release. Clearly further work is required to establish the exact physiological role of GIP, but there is the exciting prospect of the discovery of an important enterogastrone and possible major component of the entero-insular axis.

MOTILIN

When the duodenum of the dog is perfused with an alkaline fluid coordinated gastric contractions result[64]. This effect is seen even in the denervated fundic pouch, and is also mimicked by injection of duodenal extracts[65]. It seems likely, therefore, that a circulating hormone is responsible and the name

'motilin' has been proposed. In 1972 motilin was completely purified and was found to have 22 amino acids[66] and an amino acid sequence quite dissimilar from other gastrointestinal hormones[67]. Preliminary results of radioimmunoassay of plasma motilin concentrations show a distinctly raised level in dogs after intraduodenal instillation of alkali[68]. More sensitive assays will be required, however, before the physiological importance of this new hormonal peptide can be fully assessed.

VASOACTIVE INTESTINAL PEPTIDE

In 1970 a vasodilator activity was discovered in extracts of the upper small intestine of the pig[69] and subsequently purified[7]. The amino acid sequence of this vasoactive intestinal peptide (VIP) had considerable similarities with GIP, secretin, and glucagon[19]. It was also found to have secretin-like actions in stimulating alkaline pancreatic juice production, and glucagon-like actions in raising blood glucose[70]. Further, VIP produced a powerful stimulation of small intestinal secretion and could inhibit histamine-stimulated gastric acid production[22]. Radioimmunoassay of VIP has shown it to be distributed throughout the primate gut, with the total quantity present being greater than that of gastrin, secretin, or CCK-PZ[71]. Human VIP and porcine VIP elute from Sephadex gel columns in an identical position, suggesting that they may differ little in their amino acid sequences[71].

The physiology of VIP is still unknown but some information can be obtained by studying the pancreatic cholera syndrome. In 1958 Verner and Morrison described two patients who died from extremely profuse watery diarrhoea associated with a pancreatic neoplasm[72]. This syndrome, also called WDHA, because of the watery diarrhoea, hypokalaemia, and achlorhydria[73], was found to be associated with high plasma VIP levels[74]. Extracts of the responsible tumours contained large quantities of VIP[71], which behaved as a single substance on gel columns and had the same elution position as VIP from normal human bowel[71]. The finding that the main effect of chronically elevated plasma VIP levels may be to cause watery diarrhoea alters the emphasis on the known actions of this hormonal peptide. Although first purified as a general vasodilatory substance it is probable that the gastrointestinal effects of VIP are at least equally important physiologically. Vasoactive intestinal peptide has a wide distribution in the bowel[71] and it may be that it is not primarily concerned with digestion but, perhaps, acts to protect against injurious substances by increasing intestinal secretions and alimentary blood flow.

Conclusions

The gut appears histologically to be a large and complex endocrine organ. Elucidation of the nature and actions of this endocrine system has proved difficult because the cells are widely scattered and the hormones labile. In recent years the purification and sequencing has been reported on six probable hormones and active investigation is proceeding on several others. When a hormone has been isolated it can be measured by the technique of radioimmunoassay which allows very rapid collection of further data. Unfortunately it is not only quite easy to set up a radioimmunoassay but also very easy to get misleading results. It is important to ensure that all the structures that are being measured by the assay reflect biological activity.

Intestinal hormones probably play a significant role in gut and metabolic diseases. Now that their plasma measurement is feasible, an increase in the current research in this field should add considerably to our understanding of the pathology. Determination of the 'gut hormone profile' will also be an important diagnostic tool in investigating diseases not primarily of endocrine origin. It is predictable that in future most hospitals will need access to a comprehensive and reliable gut hormone radioimmunoassay service.

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