Pancreatic Growth and Cell Turnover in the Rat Fed Raw Soya Flour

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Growth and differentiation of the pancreatic acinar cell was studied in rats fed raw soya flour (RSF) for up to a year. A second group of rats were fed a control diet. After 1 week of RSF feeding there was a 200% increase in tissue RNA and weight, indicating initial hypertrophy, which was maintained for the 1-year study period. By the second week and over the remainder of the period studied there was also a marked increase in total DNA, suggesting hyperplasia. Cell turnover, as measured by the rate of incorporation of ^{3}H-thymidine into pancreatic DNA, was significantly higher in RSF-fed animals only from the second to fourth weeks; it then returned to control values. Autoradiography showed an 18-fold increase in duct cell labeling at the end of the first week and an 11-fold increase by the end of the second week. Acinar cell labeling doubled from the second to the twelfth week. These studies confirm previous reports that RSF produces pancreatic hypertrophy and hyperplasia. They furthermore show that there is initially marked stimulation of DNA synthesis in the duct cell compartment. The results suggest that cells with the morphologic characteristics of duct cells may be the precursors of acinar cells in hyperplastic pancreatic tissue. (Am J Pathol 1982, 108:217-224)

GROWTH AND DIFFERENTIATION of pancreatic acinar cells has been studied in the rat after partial pancreactectomy[1,2] and in a number of models in which pancreatic degeneration has been caused by a variety of chemical substances.[3-6] These studies have shown that the acinar cell, previously thought to be nondividing,[7] is capable of quite marked proliferation when stimulated.

Pancreatic acinar cell hypertrophy and hyperplasia occurs in chickens[8] and rats[9-12] after being fed a diet of raw soya flour (RSF). It has been shown that prolonged RSF feeding leads to pancreatic neoplasia.[13] A possible explanation for this finding is that cell turnover is increased in rats fed raw soya. Since dividing cells are more susceptible to carcinogens,[14,15] and since neoplastic progression requires many cycles of cell division,[16,17] a sustained increase in pancreatic cell turnover in rats fed RSF should lead to an increased incidence of neoplasms. In view of the hyperplasia, increased cell division in RSF-fed rats seems likely; but the extent of any increase in cell turnover, and whether any change persists while the animal remains on RSF, is not known. The present study was therefore set up to measure the growth and differentiation of pancreatic acinar cells by autoradiography and measurement of DNA synthesis in rats fed RSF for varying times up to a year. The results indicate that cell turnover is increased up to 12 weeks on RSF but then returns to control values, and that ductal cells are probably the precursor cells for the more specialized acinar cells.

Material and Methods

Animals

Male Wistar rats, locally inbred for 15 years, were used. The animals were about 3 months old and averaged 225 g in weight at the start of the study.

Diets

A group of 36 rats were fed RSF obtained from Soy Products of Australia Pty. Ltd., Bayswater, Vic-

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toria. The RSF was supplemented with vitamins and minerals as recommended by Folsch and Wormsley.[18] A second group of 10 rats were fed cubes supplied by Milne Food Co. Pty. Ltd., Welshpool, Western Australia, and served as a control group. The rat cubes contained no measurable trypsin inhibitor activity as estimated by the method of Kakade, Simons, and Leiner.[19] The RSF diet contains 20% fat, 40% protein, and 25% carbohydrate, while the cubes contain 5% fat, 22% protein, and 52% carbohydrate.

Biochemical Studies

Groups of 4 rats each were killed at 1, 2, 3, 4, 12, 26, and 52 weeks. A further 8 rats were killed at 8 weeks. A total of 10 rats fed cubes were studied over the same period and served as the control group. Four control rats were killed at 4 and 12 weeks, and a further 2 were killed at 52 weeks. Measured biochemical indices showed little change over the study period. The 10 values were therefore combined to give a mean control value.

The rats were allowed free access to food and water up to the time of killing. After anesthetization with ether, a midline incision was made and the heart cut. The pancreas and liver were quickly removed, trimmed free of fat and lymph tissue, and weighed. A 100–200-mg sample of the liver and pancreas was taken for biochemical assay. Liver indices were studied only up to 6 months.

RNA and DNA were separated by the method of Dembinski and Johnson.[20] We estimated RNA content by measuring the absorption of the RNA extract at 260 nm. We estimated DNA content by the Burton procedure,[21] using calf thymus DNA as the standard. Protein was determined on the solubilized protein-DNA after incubation with 0.3 M KOH by the method of Schacterle and Pollack.[22]

Autoradiography

So that we could achieve optimal fixation for histologic study, RSF-fed rats from a separate group were perfusion-fixed for autoradiography with Karnovsky fixative.[23] Four RSF-fed and 2 cube-fed rats were killed at each of the above intervals. These animals were housed under the same conditions and fed the same batches of food as rats used in the biochemical studies. Food was available up to the time of injection. Tritiated thymidine, 24 Ci/mmol (The radioactive Center, Amersham, England, batches 84–87) was injected intraperitoneally at a dose of 0.5 μCi/g total body weight between 10.00 AM and 12.00 noon. Two hours later the animals were anesthetized with ether, and a midline incision was made. The left ventricle was incised, and a blunt 18-gauge needle was passed into the aorta. The ventricle was clamped about the perfusion needle. The right atrium was cut to permit exit of blood. Under a pressure of 30 cm of water, 100 ml of heparinized saline followed by 100 ml of Karnovsky's fixative was perfused. A 100–200-mg sample of the splenic region of the pancreas was removed and cut into 1-mm cubes while immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The remaining pancreas and a lobe of the liver were stored in 10% buffered formol saline. Tissue for wax embedding was routinely processed by dehydration with increasing concentrations of alcohol and embedded in paraffin. Six-micron sections were cut and floated onto gelatin-subbed slides, then coated with Kodak A.R. 10 stripping film (Kodak, London, U.K.). Exposure time was 1–2 months. The developed and fixed autoradiograph was stained with hematoxylin (Harris's) and eosin (H&E).

Labeling Index

We performed all counting on random fields, using the method described by Fitzgerald and co-workers.[24] Within a given field acinar cells and ductal cells (centroacinar and intralobular duct cells only) were counted. Approximately 2000 nuclei were counted for each animal. The labeling index was expressed as the percentage of labeled nuclei in the total number of nuclei counted.

Definition of Cell Types

Acinar Cells

Acinar cells were identified as pyramidal shaped cells with an eosinophilic cytoplasm in the upper half of the cell due to the presence of protein. The nucleus

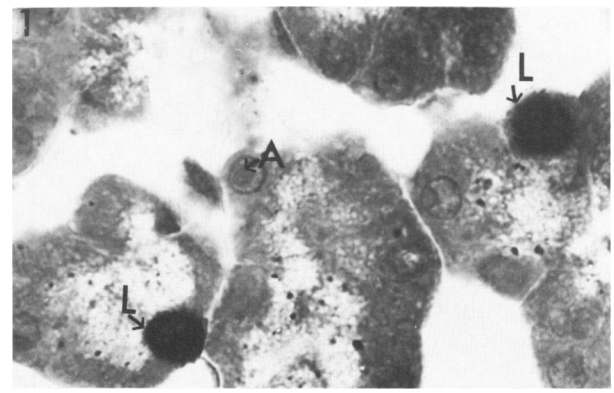

Figure 1 – Autoradiogram of pancreas taken from an animal fed RSF for 2 weeks. Two acinar cells with heavily labeled nuclei (*L*) can be seen. Several unlabeled acinar cells with circular nuclei can also be seen (*A*). Karnovsky's fixative/10% formalin. (H&E, × 2500) (With a photographic reduction of 8%)

was round and moderately basophilic. The surrounding basal cytoplasm was also basophilic because of the presence of rough endoplasmic reticulum (Figure 1).

Centroacinar Cells

These cells were located within the acinus and had a pale-staining cytoplasm. The nucleus was usually elongated and stained more densely than in acinar cells.

Intralobular Duct Cells

These cells had the same morphologic and staining characteristics as centroacinar cells but were distal to the acinus and were seen as lines of cells that connected with several centroacinar cells within the single lobule (Figure 2).

In Vivo *Labeling of DNA*

The incorporation of ^{3}H-thymidine into DNA was determined on either fresh or fixed pancreas and liver with the use of the DNA extraction technique described above. One milliliter of DNA-containing supernatant was counted in a Searle liquid scintillation counter (Searle Analytic Inc., Des Plaines, Ill). DNA synthesis was measured as disintegrations per minute per microgram of DNA. Preliminary studies showed that measured specific activity of isolated DNA was the same in fresh and fixed tissue.

Statistics

Data was analyzed by the Student t test. Significance was considered to be $P = 0.05\%$.

Results

Biochemical changes

During the first week of RSF feeding pancreatic weight and RNA had both significantly increased, but protein and DNA were unchanged (Table 1, Figure 3). However, from the second week of RSF feeding onward, DNA and protein also increased significantly; and all variables remained significantly higher than control values for the rest of the study. Liver parameters varied slightly during the first 12 weeks on RSF, but at no time was there a significant change from control values (Table 2).

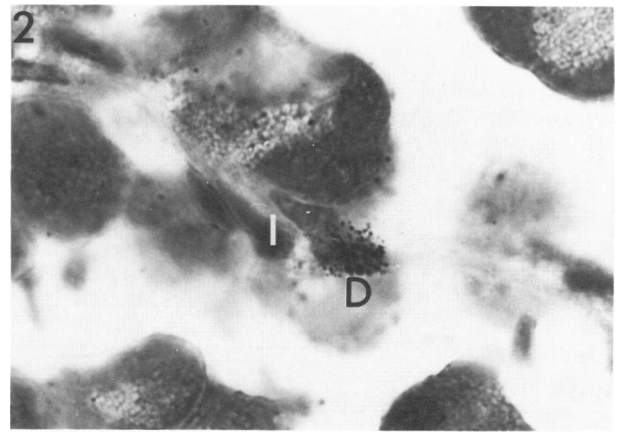

Figure 2—Autoradiogram of pancreas of a rat fed RSF for 1 week. An intralobular duct can be seen running from the upper left to the lower right of the micrograph. One labeled intralobular duct cell can be seen (*I*) next to three unlabeled duct cells (*D*). Karnovsky's fixative/10% formalin. (H&E, ×2500)

DNA Radioactivity

The incorporation of ^{3}H-thymidine into pancreatic DNA in RSF-fed rats was significantly higher than in cube-fed control rats during the first 4 weeks of RSF feeding but then returned to the control value. The incorporation of radioactivity of the liver DNA in RSF-fed rats remained unchanged with RSF feeding and was not significantly different from values in cube-fed controls (Table 2). In Figure 4 the results have been combined to give an average DNA-specific activity for the liver at each interval.

Table 1—Changes in Pancreatic Parameters During Raw Soya Flour Feeding for 52 Weeks

Time (weeks)	Number of rats	Body weight (g)	Pancreatic parameters per 100 g body weight: Pancreatic weight	DNA	RNA	Protein	Specific activity* (dpm/μg DNA)
1	4	215 ± 5.4	0.595 ± 0.040	0.88 ± 0.10	22.0 ± 2.4	73.0 ± 3.3	50.3 ± 6.1
2	4	230 ± 1.5	0.683 ± 0.032	2.05 ± 0.07	24.5 ± 0.1	94.6 ± 4.8	55.4 ± 6.7
3	4	235 ± 1.0	0.645 ± 0.036	1.66 ± 0.11	25.4 ± 1.0	83.0 ± 6.7	44.8 ± 7.3
4	8	263 ± 24.3	0.588 ± 0.035	1.65 ± 0.10	20.0 ± 2.4	110.9 ± 24.1	40.3 ± 7.0
8	4	237 ± 7.9	0.593 ± 0.036	1.56 ± 0.03	18.2 ± 2.1	86.0 ± 3.1	27.4 ± 6.6
12	4	254 ± 3.2	0.588 ± 0.022	1.46 ± 0.10	23.3 ± 1.9	106.0 ± 10.8	28.9 ± 10.3
26	4	349 ± 34.5	0.540 ± 0.025	1.46 ± 0.09	19.3 ± 1.4	106.0 ± 6.7	22.3 ± 7.6
52	4	338 ± 17.8	0.605 ± 0.022	1.70 ± 0.15	24.7 ± 1.8	93.5 ± 7.4	17.6 ± 2.6
Continuous cubes	10	307 ± 16.9	0.299 ± 0.010	0.97 ± 0.06	10.2 ± 0.42	53.2 ± 2.8	20.0 ± 2.0

Pancreatic weight, DNA, RNA, and protein are expressed as mean ± SE.

* Separate groups of 4 RSF-fed and 2 cube-fed rats were used to measure the specific activity of DNA at each interval. Mean ± SE.

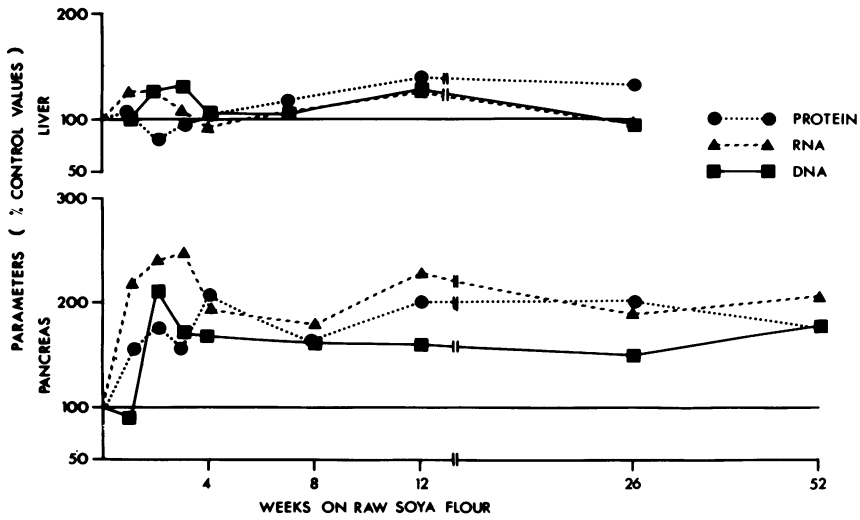

Figure 3—Changes in pancreatic protein, RNA, and DNA during raw soya flour feeding for 52 weeks and liver protein, RNA, and DNA during 26 weeks of RSF feeding. Values are expressed as a percentage of control values from cube-fed rats.

Autoradiography

After the feeding of RSF for 1 week the labeling index of the duct cells had increased 18-fold, while acinar cell labeling had not changed (Figure 5). After 2 weeks duct labeling was 11 times and acinar cell labeling 2 times higher than in control tissue. By 3 weeks on RSF, duct cell labeling had dropped markedly but was still significantly greater than in cube-fed animals. However, duct cell labeling had returned to normal by 4 weeks on the diet. Acinar cell labeling was significantly higher in RSF-fed rats than in control rats from the second to the twelfth week of RSF feeding but returned to control values by 26 weeks (Figure 6).

Macrophages, lymphocytes, fibroblasts, and mast cells were readily recognized and were frequently labeled after the first week of RSF feeding, but labeled cells of this type were rarely seen after the second week. Interstitial cell labeling increased approximately up to 0.5% in RSF-fed rats by 1 week. Labeling of these cells had returned to control values by 4 weeks.

There was a high correlation between specific activity and the total labeling (duct and acinar cells) for each rat. Of the 32 RSF-fed and 16 cube-fed rats studied at the 8 intervals the correlation between the incorporation of ^{3}H-thymidine into DNA and autoradiography for each rat was highly significant ($r = 0.8334$, 48 df). The equation of the regression line was $y = 62.1x + 10.2$, where y is the specific activity in dpm/μg DNA and x the labeling index (Figure 7).

Discussion

After the rats had eaten RSF for 1 week there was a marked increase in pancreatic RNA and weight, indicating hypertrophy of the gland. After the second week hyperplasia was also seen, since from this time onward pancreatic DNA increased to 180% of control values (Figure 3). If cellular DNA remained constant, this represents an 80% increase in the cell number. However, the assumption that cellular DNA remained constant may not be justified, since binucleate cells were common, and there was frequent variation in nuclear size, indicating the possibility of polyploidy. Therefore, it is possible that not all DNA

Table 2—Changes in Liver Parameters During Raw Soya Flour Feeding for 26 Weeks

Time (weeks)	Number of rats	Liver parameters per 100 g body weight				Specific activity* (dpm/μg DNA)
		Liver weight	DNA	RNA	Protein	
1	4	3.74 ± 0.14	5.9 ± 0.5	53.0 ± 3.8	654.7 ± 47.9	19.0 ± 1.5
2	4	3.66 ± 0.04	7.6 ± 0.2	52.0 ± 3.6	520 ± 63.8	18.0 ± 3.0
3	4	3.70 ± 0.15	7.9 ± 0.4	45.9 ± 1.5	594 ± 15.4	20.0 ± 2.1
4	8	2.79 ± 0.35	6.2 ± 1.5	38.2 ± 6.3	636.9 ± 41.3	20.5 ± 1.5
8	4	3.58 ± 0.07	6.2 ± 0.4	45.1 ± 1.4	724.5 ± 25.4	18.2 ± 3.5
12	4	3.64 ± 0.13	7.6 ± 0.5	51.9 ± 1.9	881.5 ± 43.0	23.3 ± 4.4
26	4	3.29 ± 0.20	5.4 ± 0.9	39.2 ± 1.8	828.0 ± 68.3	17.5 ± 5.7
52	0					19.0 ± 3.1
Continuous cubes	10	3.72 ± 0.08	5.9 ± 0.3	42.3 ± 1.9	620.0 ± 36.8	20.3 ± 1.6

Liver weight, DNA, RNA, and protein are expressed as mean ± SE.

* Separate groups of 4 RSF-fed and 2 cube-fed rats were used to measure the specific activity of DNA at each interval. Mean ± SE.

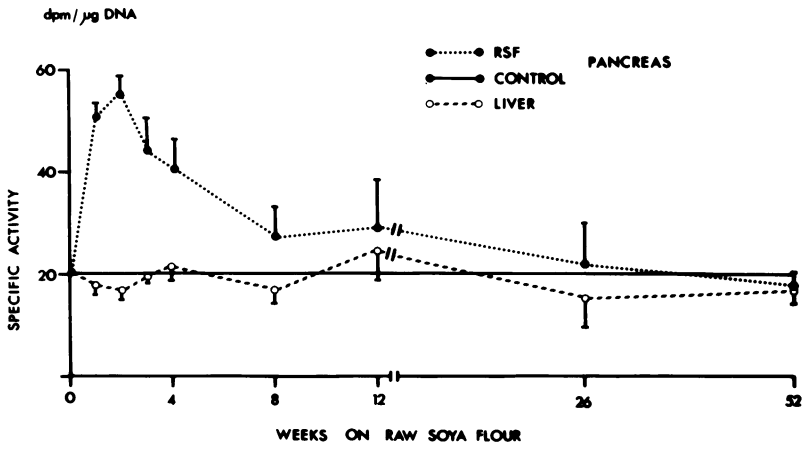

Figure 4—Specific activity of DNA from pancreas and liver of rats fed RSF and cubes for 52 weeks 2 hours after an intraperitoneal injection of 0.5 µCi of ^{3}H-thymidine per gram body weight. Pancreatic values for the two diets are shown separately, but values for liver-specific activity are combined for both diets (see text). Values are expressed as a percentage of the control values ± SE.

synthesized in the growing pancreas was involved in the production of new cells. Nevertheless, a substantial increase in cell turnover seems likely.

It is possible that some of the pancreatic growth seen with RSF feeding was due to the higher protein and fat content present in RSF, compared with cubes, since both of these components are thought to stimulate cholecystokinin (CCK) release, and CCK is a trophic hormone for the pancreas.[25-29] However, in a recent study[25] the effect on pancreatic growth of feeding heated soya flour for up to 9 months was compared with that seen with cubes and RSF. Heated soya flour has the same composition as RSF but differs in that the trypsin inhibitor activity is destroyed by heat treatment. There was little difference in the response to HSF or cubes, but at all times greater than 1 week RSF caused significant growth, compared with the other two diets. It appears, therefore, that the pancreatic growth seen with feeding of RSF, compared with that seen with cubes, is largely due to the presence of active trypsin inhibitor in the diet, rather than differences in composition.

The rate of incorporation of ^{3}H-thymidine into DNA was very high in the first 4 weeks of RSF feeding when the pancreas was growing and then returned to control values after the organ had reached maximum size, despite a maintained hypertrophy and hyperplasia in the organ. This growth is thought to be controlled by increased circulating levels of cholecystokinin.[26] CCK levels are thought to be regulated by a negative feedback mechanism involving intestinal trypsin. In this model, proposed by Green and Lyman,[30,31] CCK release is suppressed by trypsin in the upper small intestine. However, removal of trypsin by its binding to a trypsin inhibitor such as is found in RSF causes an increased release of CCK from the small intestine.[32] Release of CCK is thought to continue until the amount of trypsin secreted by the pancreas is sufficient to exert its inhibitory effect on the further release of CCK. An increased pancre-

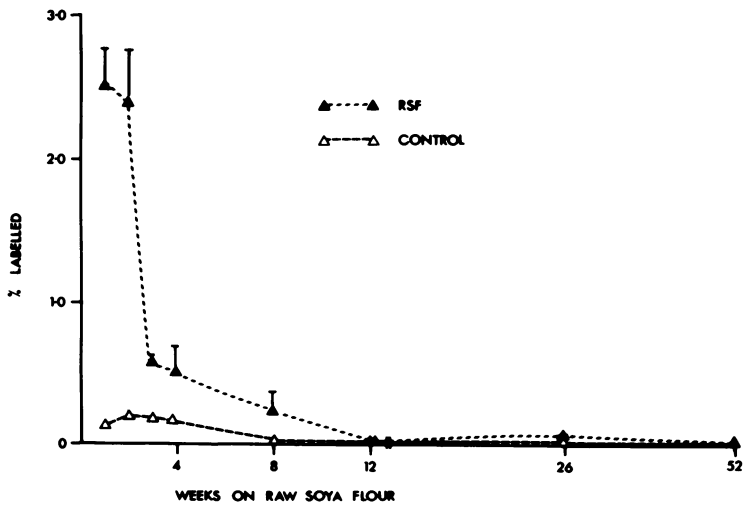

Figure 5—Pancreatic duct cell labeling in RSF-fed and cube-fed rats 2 hours after an intraperitoneal injection of 0.5 µCi of ^{3}H-thymidine per gram body weight. Labeling index is the percentage of labeled duct cells in the total number of duct cells counted. Mean ± SE.

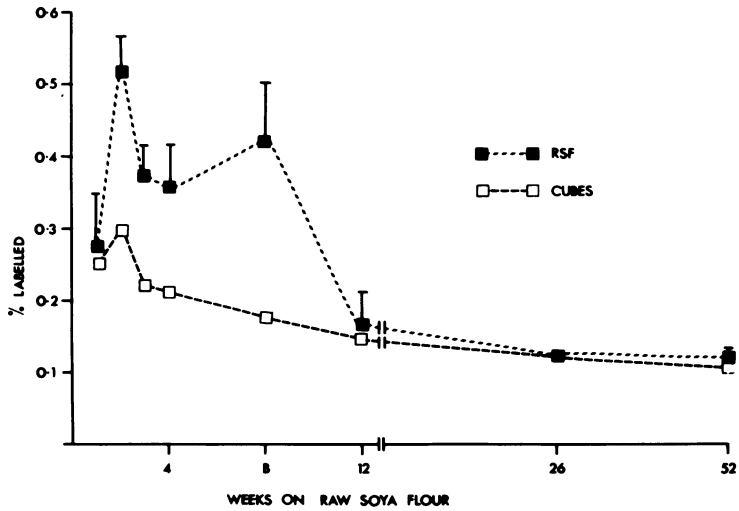

Figure 6 — Pancreatic acinar cell labeling in RSF-fed and cube-fed animals 2 hours after an intraperitoneal injection of 0.5 μCi of ^{3}H-thymidine per gram body weight. Mean ± SE.

atic size and enzyme content and an increased secretion of enzyme in response to exogenous CCK has been shown in rats fed RSF for 4 weeks.[18,33] This growth of the pancreas with RSF feeding was confirmed in the present study.

From the measurement of specific activity of total pancreatic DNA it was not possible for us to determine the type of cell synthesizing DNA. Indeed it was possible that significant amounts of radioactivity could be present in inflammatory cells or in some other "intruder cell" as a result of damage to the pancreas by RSF. The autoradiographic studies allowed the identification of the cell types involved. They showed that after the rats were on RSF for 1 week, duct cell labeling had increased 18-fold and labeling in this cell compartment remained significantly higher than control values for the next 2 weeks. In contrast, acinar cell labeling did not increase until 2 weeks and then remained significantly higher than control values from the second to the twelfth week. The combined labeling index of duct and acinar cells correlated well with the DNA radioactivity at each interval (Figure 7), suggesting that it was largely label in these compartments which was being determined in the specific activity measurements. The rapid proliferation in duct cell labeling during the early stages of the growth phase and the later increased labeling in acinar cells suggests that the dividing cells may originate largely from cells with duct cell morphologic characteristics, which divide and acquire the characteristics of acinar cells. These new acinar cells presumably retain the capacity to divide for some time. The labeling index of interstitial cells increased slightly in the first week but never exceeded approximately

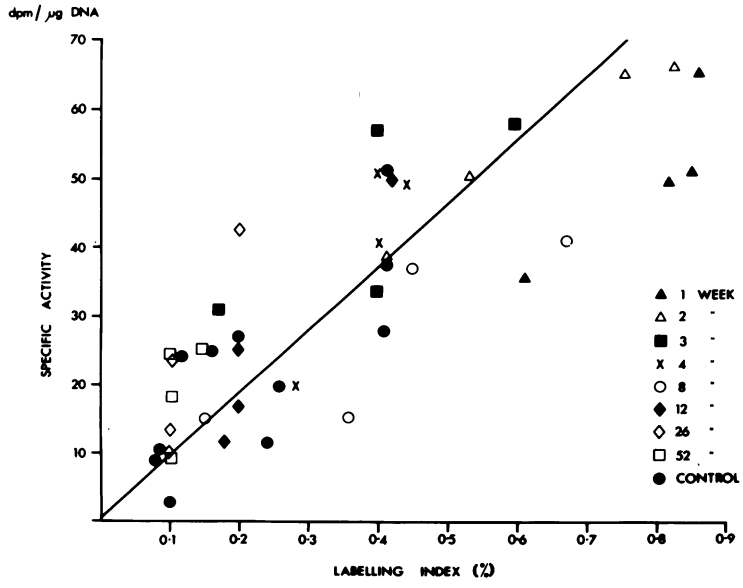

Figure 7 — Correlation of specific activity of DNA with the combined labeling index of acinar and duct cells at 1, 2, 3, 4, 8, 12, 26, and 52 weeks on either RSF or cubes.

0.5%. The possibility of interstitial cells being the precursors of the acinar cells must be considered; but it appears unlikely, since there was only a small increase in labeling of these cells during the first few weeks of RSF feeding, and labeling in this compartment never exceeded that in acinar cells. It is more likely that these cells are involved in producing the connective tissue framework for newly synthesized acinar cells.

The hypothesis that duct cells develop into acinar cells has been advanced previously. Adler and Kern[34] showed that centroacinar cells differentiated into acinar cells after severe damage to the pancreas caused by supramaximal doses of caerulein. In their study centroacinar cells were seen to divide and then undergo a transition in morphologic characteristics and differentiation as evidenced by the formation of rough endoplasmic reticulum and the accumulation of secretory granules.

Under some conditions the acinar cells themselves also appear to divide, and the relative contribution of duct cells and acinar cells to the final acinar cell hyperplasia is not clear. In an early study Fitzgerald et al[3] found that after severe damage to the acinar cell by ethionine feeding, duct cell labeling increased 4-fold, and preceded a more pronounced 20-fold increase in acinar cell labeling 48 hours later. These results are in general agreement with those reported here, though we found that during the peak of the growth phase labeling of duct cells was considerably greater than that of acinar cells. In view of the much higher labeling of acinar cells in the study by Fitzgerald and co-workers, it seems likely that in this experiment acinar cell division was an important source for the increased number of acinar cells. The difference in the magnitude of the contribution of duct cells and acinar cells in the present report and the previous report probably reflects fundamental differences in the two studies. In the present work the pancreas grew from a normal to an enlarged state presumably under the action of the external trophic stimulus CCK, released from the intestine by RSF. In Fitzgerald's study the pancreas regenerated from an atrophic state toward normal size. Under these conditions the initial stimulus to cell division appears to be triggered by loss of tissue mass or cell products,[3] presumably through some local mechanism. In similar studies it has been shown that following pancreatectomy in the rat[35] and after ethionine treatment in the hamster[36] the acinar cell was the cell type responsible for proliferation. In view of these differences, it is conceivable that different stimuli act primarily on different target cells.

Earlier studies showed that prolonged RSF feeding produced neoplastic changes progressing to invasive carcinoma in the rat pancreas[13] and that RSF feeding potentiated the action of the pancreatic carcinogen azaserine.[37,38] It seemed likely that these effects were due to changes in cell turnover in RSF-fed rats. Thus, if acinar cell division was increased even for a short period, the cells should be more susceptible to carcinogens, since DNA in dividing cells is more readily damaged. In addition, if cell turnover was increased for a prolonged period, the progression of a transformed cell through the necessary stages until it became overtly neoplastic should also be speeded up. The findings reported here indicate that acinar cell turnover is increased for up to 12 weeks after starting RSF but then returns to the normal low level. It might be expected, therefore, that by 12 weeks all the effects of RSF in potentiating azaserine would have been seen, and that feeding RSF beyond this time would not further affect the incidence of neoplasm. Such is not the case. In a recent study by McGuinness et al[38] it was shown that only a brief exposure to azaserine was required to produce pancreatic neoplasm (as indeed had been shown earlier by Longnecker and co-workers[5]) but that potentiation of this effect by RSF feeding was only seen if RSF was continued for very long periods.[13,37,38] It seems that even after cell turnover has returned to normal, the RSF diet is still required to maintain the transformed cells. A possible explanation is that these cells require high levels of CCK for survival, and that they are lost if plasma CCK levels fall as a result of stopping the intake of trypsin inhibitor.

In these experiments the earliest time period studied was 1 week after starting RSF. Times earlier than this were not used, because the DNA content of the pancreas does not increase until between the first and second week of RSF feeding. Nevertheless, it is possible that increased cell division does occur at time intervals shorter than 1 week after starting RSF, presumably balanced by increased cell destruction, since total tissue DNA does not increase.

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