REUTILIZATION OF LYMPHOCYTE DNA BY CELLS OF INTESTINAL CRYPTS AND REGENERATING LIVER

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ABSTRACT

Lymphoid cells from mice injected 54 hours and 30 hours earlier with ^{3}H-thymidine were washed and transfused into isogenic recipients at 29 to 30 hours after partial hepatectomy. The recipients were killed 28 to 30 hours later, and liver, intestine, Peyer's patch, spleen, and the transfused cells were examined in autoradiographs exposed 6 months. Approximately 80 per cent of the labeled transfused cells were classed as lymphocytes. The labeled DNA contained in the transfused cells was partitioned to about 14 times as many recipient liver and intestinal cells, appearing in 72 to 78 per cent of hepatocyte nuclei, in 30 to 35 per cent of liver reticuloendothelial nuclei, and in 90 to 95 per cent of intestinal crypt nuclei. The label was not comparably widespread in the lymphoid organs, but was limited to a few intensely labeled lymphocytes and a somewhat larger number of very weakly labeled cells. When heat-killed cells rather than living cells were transfused, intensely labeled lymphocytes were absent from the lymphoid organs, but the labeling of cells in the recipients was otherwise identical. The results suggest that (*a*) reutilized DNA is derived from dead cells, (*b*) reutilized DNA is mainly degraded to nucleosides and nucleotides, the usual immediate *de novo* DNA precursors, before reincorporation into DNA, and (*c*) DNA reutilization may occur in the lymphoid organs, but on a less active scale than in intestine or regenerating liver.

INTRODUCTION

In vivo reutilization of leukocytic deoxyribonucleic acid (DNA) by mammalian cells has recently been reported from several laboratories (1–7). It was suggested in studies by Bryant (2) and Rieke (7) that DNA thymidine is reutilized by cells locally at the site of release from leukocytes and that the reutilization is associated with endogenous DNA synthesis. The spatial intimacy of the passage of DNA thymidine from leukocyte to receptor cell will suggest to many workers familiar with recent tissue culture data that intact DNA molecules penetrate intracellularly (8) and are incorporated into the genome without significant breakdown (9, 10). The biochemical level of DNA reutilization and its significance for information transfer and metabolite supply in the intact animal have, however, been poorly defined, and may prove to vary with tissue and cell type. In order to gain further insight into the fate of reutilized leukocyte DNA in intact animals, lymphoid cells from mice previously injected with ^{3}H-thymidine, a specific DNA label, were transfused into isogenic recipients post hepatectomy, and the resultant redistribution of the label to host cells was evaluated autoradiographically.

MATERIAL AND METHODS

Male CBA mice, aged 3 to 4 months and maintained *ad libitum* on a standard laboratory diet, were used. A melange of thymus, mesenteric lymph node, and spleen cells was prepared from donor mice which had received, 54 hours and 30 hours previously, intraperitoneal injections of 1 μc ^{3}H-thymidine (2.6 c/mmole, Schwarz) per gm body weight. The cells were transfused into isogenic mice which had been 65 per cent hepatectomized 29 to 30 hours earlier by surgical removal of the median and left lateral liver lobes. The recipient animals, comprising six mice receiving living cells and six mice receiving heat-killed cells, were killed at 28 to 30 hours post transfusion, and liver, intestine, Peyer's patch, and spleen were prepared for autoradiography.

Transfusion Procedures

Cell suspension pooled from six donors and intended for two recipients was prepared in balanced salt solution (Ringer's) using standard methods. The cells were gently washed once in 40 ml Ringer's to remove toxic components, then resuspended in 2.0 ml. This material was brought into a single syringe and 0.75 to 0.80 ml suspension injected into each recipient via the tail vein. The entire procedure required 40 to 45 minutes. Suspension remaining in the syringe served for a cell count and for smears. Heat-killed cells were prepared by incubating living cells at 50°C for 40 minutes before washing. Cell viability was controlled by eosin tests on an equal number of identically prepared suspensions. Care was taken to use equipment which had not had previous contact with radioactive materials.

A restraining cage was used during transfusion; the mice were very active when released and showed no signs of posttransfusion difficulty; their appearance at autopsy was normal.

Microslide Preparations

Thin strips of a 1:2 dilution in serum of a small portion of the transfused cell suspension were painted onto subbed microslides using an artist's sable hair brush, no. 00. Immediately after air drying, the microslides were placed overnight in Carnoy's fixative for fixation and for removal of non-DNA-associated tritium. Cells from recipient spleen were suspended in serum and painted onto the same microslides that held the brushed transfused cells from the previous day; the microslides were again placed overnight in Carnoy's.

The weighed liver and small intestine from each recipient were fixed in acetic-alcohol-formalin and sectioned at 4 μ in paraffin. Intestine (multiple levels) was sectioned to include a Peyer's patch. Sections of the organs were mounted together on microslides and stained by the Feulgen reaction before autoradiography.

Autoradiographs (AR-10 stripping film, Kodak Ltd.) were prepared according to the method of Pelc (11). All autoradiographs examined for this study were processed and exposed in a single group, the exposure lasting 6 months in dry air at 4°C. Giemsa's stain was used through the film to stain cells in the brushed smears.

Counting Procedures

The autoradiographs were examined at × 1000 using an oil immersion lens. Background silver grains were few, approximating 1 to 2 per 1000 μ^2 in film not overlying cells. Nuclei were considered labeled if labeled by 3 or more silver grains; the actual number of grains over individual nuclei was recorded. In smears of transfused cells, lymphocytes were arbitrarily classed into small and large sizes according to whether their nuclear diameter was less or greater than 8 μ (Table I). A minimum of 200 labeled cells was examined in each of the recipients' organs.

TABLE I

*Labeled DNA in Transfused Lymphoid Cells**

Mean cells labeled (% of all cells)	Mean cells labeled by >50 silver grains (% of labeled cells)‡	Cell types labeled (mean % of identified labeled cells)§			
		Small (<8 μ) lymphocytes	Large (>8 μ) lymphocytes	Erythroblasts	Other
18(17–19)	56(53–61)	52(45–55)	26(21–30)	18(17–20)	4(2–5)

* The figures in parentheses indicate observed range. Approximately 200 labeled cells were examined in each of six preparations; three preparations contained heat-killed cells.
‡ Cells labeled by less than 5 silver grains were rare.
§ Approximately 15 per cent of the labeled cells were eclipsed by silver grains and could not be identified.

RESULTS

Transfused Cells

Little variation was found in the DNA labeling of different preparations of transfused cells: approximately 18 per cent of the cells were labeled, and of these, about 52 per cent were small lymphocytes and 26 per cent were large lymphocytes (Table I). Eosin tests indicated that 88 to 93 per cent and 5 to 8 per cent of the cells in the living and the heat-killed preparations, respectively, were viable at the time of transfusion. Cell counts indicated that 240 to 270 $\times$ 10^6 cells, or 43 to 49 $\times$ 10^6 labeled cells, were transfused into each recipient.

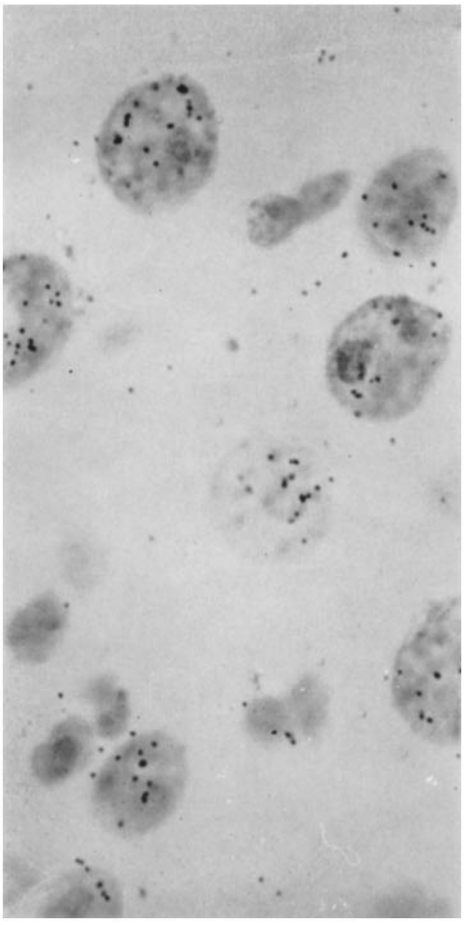

FIGURE 1 Autoradiograph of cells of regenerating liver, showing reutilized labeled DNA. Feulgen-stained section. $\times$ 1250.

Recipient Mice

The sacrificed recipients, which weighed 22 to 25 gm, had liver weights of 0.70 to 0.75 gm and intestinal tract weights of 1.0 to 1.1 gm. The majority of liver cell nuclei contained labeled DNA (Fig. 1), with little variation between recipients: 72 to 78 per cent of hepatocyte nuclei and 30 to 35 per cent of reticuloendothelial nuclei were labeled (Table II). The distribution of intestinal label also showed little variation between individual recipients, occurring in 90 to 95 per cent of crypt nuclei and in nuclei at the bases of the villi (Fig. 2). Counts performed on quadrants of cross-sectioned duodenum from six of the recipients indicated that 28 to 32 per cent of all small intestinal cells were labeled. Mitotic figures in the intestinal crypts and regenerating liver were always labeled. The labeled transfused cells which had presumably localized in these organs at earlier intervals were very rare. Feulgen-stained sections from unlabeled animals, exposed 3 to 6 months as a control for histochemical reduction of the film by liver or intestinal tissue, were negative.

TABLE II

*Labeled DNA in Recipients' Liver Cells**

	Mean cells labeled (% of all cells)	Mean silver grains per labeled cell
Hepatocytes	75 (72–78)	7 (6–8)
Reticuloendothelial cells	33 (30–35)	4 (4–5)

* The figures in parentheses indicate observed range. The mean hepatocyte to reticuloendothelial cell ratio was about 2.5:1. A total of approximately 200 labeled liver cells was examined in each of the twelve recipients.

Only in Peyer's patch and spleen were appreciable differences apparent in the labeling of mice receiving heat-killed and living cells. Intensely labeled cells were absent from the lymphoid organs of the former mice, but were present in mice receiving living cells (Figs. 3 and 4). In brushed spleen smears of these mice, intensely labeled lymphocytes (>5 silver grains) accounted for 0.2 to 0.4 per cent of the cells. In the spleen and Peyer's patch of all recipients, however, a somewhat larger class of very weakly labeled

lymphocytes (1 to 2 silver grains), representing labeling above the general background, was apparent (Fig. 3).

DISCUSSION

Transfused Cells

Much biochemical evidence indicates that synthesis of labeled DNA ceases in most organs, including the lymphopoietic organs, within 1 to 2 hours after radiothymidine injection (12, 13). Intracellular labeled DNA precursor must be exhausted synchronously, as this interval is much briefer than the time required by cells for DNA synthesis. The 30-hour interval between the final ^{3}H-thymidine injection and sacrifice of the donors thus assures the absence of low molecular weight labeled DNA precursor from the transfused cells. Approximately 80 per cent of the labeled transfused cells were lymphocytes (Table I), indicating that the label reincorporated into host cells essentially reflects reutilization of lymphocyte DNA.

Host Cells

In earlier studies (2, 7), reutilization of leukocyte DNA has been interpreted as associated with endogenous DNA synthesis. The label now observed in cells of intestinal crypts and regenerating liver and in mitoses, and, conversely, the absence of label from the intestinal villi, reflect the validity of this interpretation. Moreover, the very infrequent labeling of cells in the lymphoid organs as compared with intestine or regenerating liver indicates the essential absence of labeled DNA precursor (free thymidine) from the blood plasma. Thus, the present data also affirm the validity of the earlier interpretation that leukocyte DNA thymidine is mainly reutilized locally.

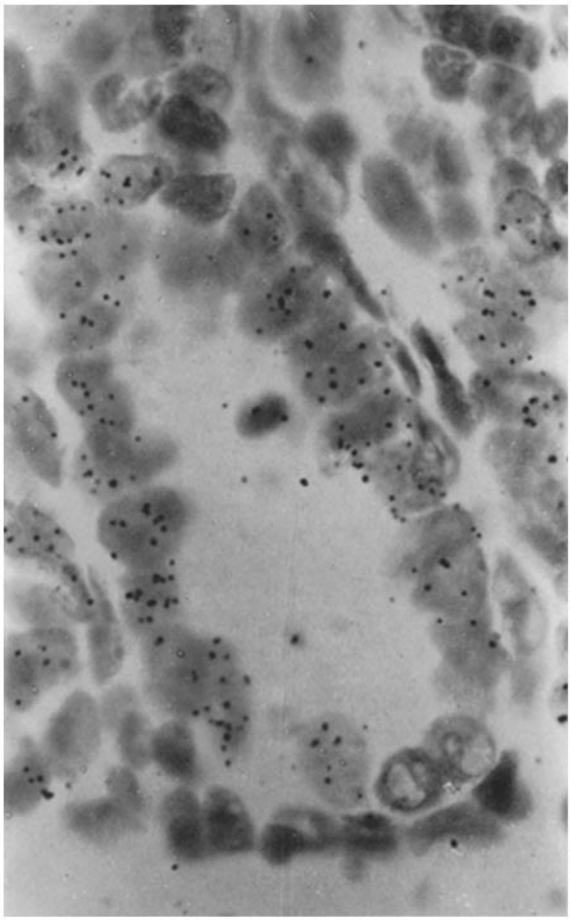

FIGURE 2 Autoradiograph of cells of intestinal crypts, showing reutilized labeled DNA. Feulgen-stained section. × 1250.

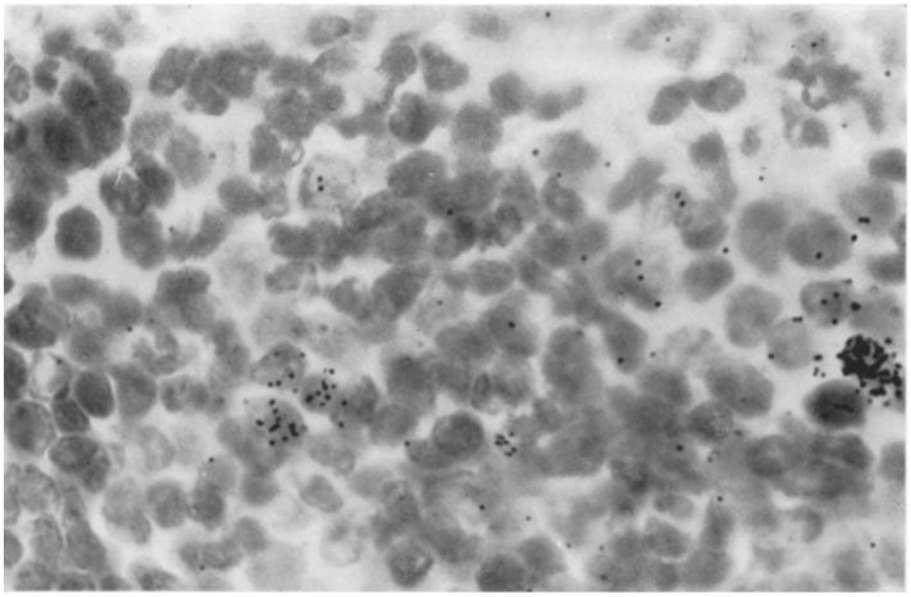

FIGURE 3 Autoradiograph of Peyer's patch, showing distribution of labeling. The intensely labeled cells (>5 silver grains) probably represent viable transfused cells or their progeny. The larger number of very weakly labeled cells (1 to 2 silver grains) are labeled well above background and may contain reutilized labeled DNA. Feulgen-stained section. × 1000.

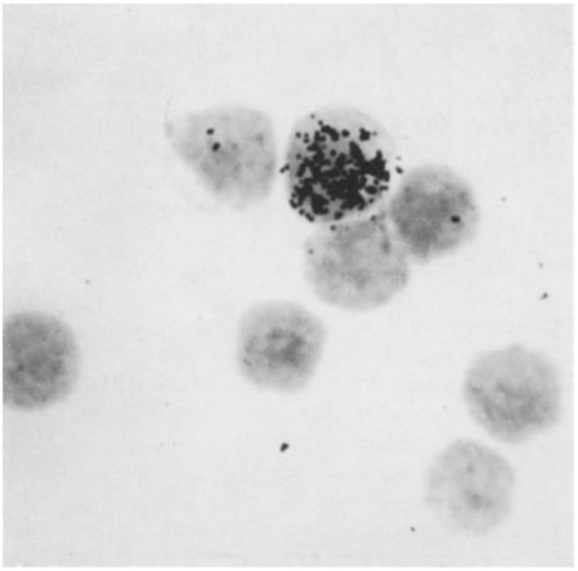

FIGURE 4 Autoradiograph of smeared spleen cells, showing an intensely labeled lymphocyte probably representing a viable transfused cell. Giemsa stain. × 1250.

The scattered intensely labeled lymphocytes in Peyer's patch and spleen of mice receiving living cells must represent viable transfused cells or their progeny (*cf.* also ref. 14), as such label was lacking in mice receiving heat-killed cells. It seems reasonable to conclude that the weakly labeled lymphocytes (1 to 2 silver grains) found in these organs after transfusion of heat-killed cells contain reutilized DNA. In mice receiving living cells, such cells may represent either progeny of the more weakly labeled transfused cells or lymphocytes that have reutilized DNA.

The identical labeling produced in intestine and liver by heat-killed and living cells indicates that release of a DNA precursor fraction from living cells is unlikely; the reutilized DNA is that of dead cells. The identical labeling also indicates that very few of the living lymphocytes reaching these organs survive beyond the 28- to 30-hour posttransfusion interval. The essential absence of viable transfused cells, which were observed in Peyer's patch and spleen, supports this view. Correspondingly, DNA reutilization would appear less active in the lymphoid organs (*cf.* also ref. 15) than in intestine or regenerating liver.

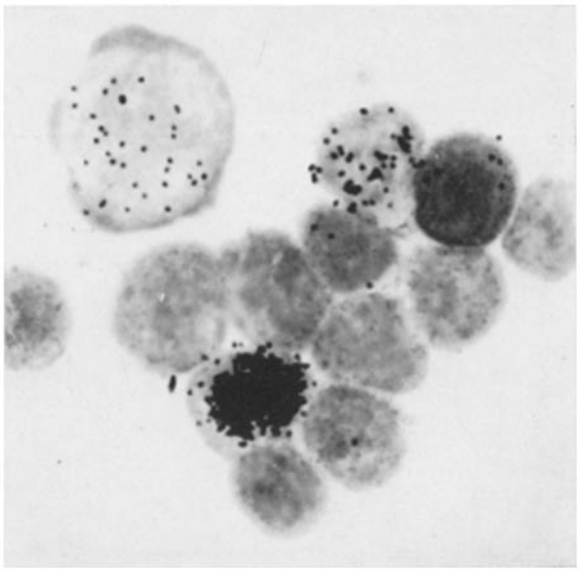

FIGURE 5 Autoradiograph of smeared transfused cells, showing DNA-labeled lymphocytes. In this example, the cells had been prekilled by heat. Giemsa stain. × 1250.

The intestinal crypt labeling emphasizes that lymphocytes not only are shed into the lumen, a previously suggested fate of lymphocytes (16), but that they may also be broken down and reutilized in association with the renewal of intestinal epithelium. Proof that the latter is a normal physiological process, however, must await further experimentation.

Labeled DNA Precursor Availability

Intestinal epithelium is renewed by a cell migration from the crypts to the villus tips. The present distribution of intestinal label (Fig. 2) showed a threefold difference from the pattern expected after ^{3}H-thymidine injection (17, 18): (*a*) label in crypt cells was *more* intense than in the cells which had migrated from the crypts to the villus bases, whereas, after ^{3}H-thymidine administration, one expects the reverse situation; (*b*) the labeled cells at the villus bases represent the initial wave of labeled cells from the crypts and were in transit about 10 to 12 hours, whereas a 28- to 30-hour transit to points midway up the villi is expected with ^{3}H-thymidine labeling; (*c*) labeling in the crypts showed much less variation between individual nuclei than is found with ^{3}H-thymidine labeling, and was essentially general, suggesting continuous availability of labeled DNA precursor during most of a 10- to 12-hour generation period.

The results thus confirm the absence of effective labeled DNA precursor from the transfusion medium and indicate that a cell-derived labeled DNA precursor became available in the crypts during the final 10 to 12 hours of the 28- to 30-hour posttransfusion interval. The initial period of non-availability probably represents the time for release of the transfused cells from the lungs, where many of them first accumulate (19), and the time for elaboration of the labeled DNA precursor.

In regenerating liver of CBA mice, only 5 to 10 per cent of hepatocyte nuclei incorporate ^{3}H-thymidine at 29 to 30 hours post hepatectomy, the transfusion interval (20); maximal incorporation occurs at 36 to 38 hours, when 35 to 40 per cent of the hepatocytes will label. A class of labeled hepatocytes forming 5 to 10 per cent of the total population was, however, not evident among the labeled cells, indicating the essential absence of

contaminant labeled DNA precursor from the transfusion medium. That as many as 75 per cent of the hepatocytes were labeled suggests that the cell-derived labeled DNA precursor may have been available for a period of several hours.

Partitioning of Labeled DNA in Reutilization

Using values of 20×10^6 liver cells and 175×10^6 intestinal cells per 0.1 gm organ (21), the percentages of labeled cells, and the organ weights (*cf.* Results), the total number of labeled cells may be roughly approximated at 100×10^6 liver cells and 550×10^6 intestinal cells per recipient. The combined total of 650×10^6 labeled cells represents a partitioning of the label contained in the 43 to 49 $\times 10^6$ labeled transfused cells to about 14 times as many cells. As these may be pre- or postmitotic cells, the number of premitotics which reutilized labeled DNA for DNA synthesis must fall between 7 and 14 times the number of labeled cells transfused. As a partitioning coefficient, the 7 to 14 figure is minimal, since an unknown number of labeled transfused cells settled elsewhere, some in the lymphoid organs (Figs. 3 and 4). The simplest interpretation of this partitioning of the label is that the transfused cells were extensively broken down in the intercellular spaces, and the label was reincorporated by local host cell pinocytosis.

The Level of DNA Reutilization

The extensive partitioning of transfused DNA to host cells noted in the last paragraph does not indicate in itself the level at which lymphocyte DNA is reutilized. A lead on the DNA "salvage pathway" may be found by noting the general variance between the present results and those of Fichtelius (22), who transfused ^{32}P-labeled lymphoid cells and found them to be more effective as a label for spleen DNA, but no more effective as a label for intestinal DNA, than injected inorganic ^{32}P. These observations are consistent with the present ones only if death of transfused lymphocytes is extensive in the intestine and only if the bulk of the reutilized DNA is first degraded to the nucleoside level. Thus, the internucleoside phosphate linkages would be broken and the liberated P diluted into the large pool of body phosphate. The liberated thymidine, however, may not escape the crypts, as this nucleoside is known to be very efficiently taken up by cells in DNA synthesis (14). Similar reasoning would exclude significant reutilization at the subnucleoside level, as thymine (23) and the labeled catabolites of ^{3}H-thymidine found in the plasma (2, 13, 14) are very poor precursors of DNA.

This interpretation may also be inferred from recent biochemical data: Thymidine and thymidine kinase are known tissue constituents, especially of actively proliferating tissues (24, 25), despite the by-passing of thymidine in the *de novo* DNA synthetic pathway in the methylation of deoxyuridylic acid to thymidylic acid (26). A possible alternative source of tissue thymidine is that arising from dead cells whose DNA has been degraded, at least in part, to nucleosides. The inferred DNA degradation products, nucleosides and their phosphorylated anabolites, are usual *de novo* precursors, and may thus be reincorporated into DNA. Thymidine, uniquely among the nucleosides, however, is not a *de novo* precursor, and its reutilization requires thymidine kinase, by which it is converted to thymidylic acid; incorporation of labeled thymidine into DNA may thus depend on an enzyme of the salvage pathway.

It is well for the reader to keep in mind that some reutilization of DNA polynucleotides remains a distinct possibility, which may, for instance, account for the preservation of immunological memory within the lymphoid cell line.

In vivo reutilization of DNA, as shown by the present and earlier studies, suggests that many of the dead cells supplying DNA precursor to proliferating cells are leukocytes acting as DNA carriers (trephocytes). A large scale reutilization of nucleosides from leukocyte DNA would account for three events observed in regenerating liver: (*a*) the synchronous elevation of thymidine kinase and DNA synthesis levels (25); (*b*) the increased ^{3}H-thymidine incorporation into DNA (20, 27); (*c*) the increased levels of phosphatases (28) and nucleases (29), which may be associated with leukocytic degradation. A necessary corollary is the agreement observed between DNA synthesis as measured by net increase and ^{32}P incorporation (30).

An increase in leukocyte-derived acid-soluble DNA precursors in regenerating liver may be the immediate factor inducing elevated DNA synthetic activity, according to the positive feedback concepts of Potter (31). The best evidence in this

direction is the very recent data of Hiatt and Bojarski, who found greatly stimulated liver DNA synthesis in thymidine-infused rats, apparently limited only by the short supply of other *de novo* precursors (32). Thus, the key to the chain of events controlling liver regeneration may reside in those posthepatectomy factors responsible for the marked increase in lytic enzymes in the liver, as it is presumably these enzymes which bring about leukocytic degradation.

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