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Effect of β-3-Thienylalanine on Antibody Synthesis III. Inhibition of RNA and Protein Synthesis in Immunized Rat Spleen Cells* (34004)

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Previous work from our laboratory (1–3) has indicated that β-3-thienylalanine (β-3-TA), an analog of the essential amino acid phenylalanine, when administered to rats by stomach tube induces marked depression of the production of specific antibody to sheep erythrocytes. This depression is seen when the analog is administered during the induction period and is not noted if β-3-TA is given beginning on the third day after antigen injection. The results of our previous experiments were interpreted as meaning that β-3-TA, like other compounds (4), has an effect on some events occurring during the induction period and will not act on antibody synthesis *per se* when given during the production phase. The purpose of the work reported here was to investigate further the mechanism by which B-3-TA depresses antibody production and to identify the step or steps which are affected by this analog.

This investigation has pointed out that one of the inhibitory effects is at the level of the synthesis of ribonucleic acid.

Materials and Methods. Sprague-Dawley, young adult, male, albino rats were used in all experiments. They were fed the diet described by Hruban and Wissler (5), prepared without addition of water, and with phenylalanin omitted when B-3-TA was administered. β-3-TA (Nutritional Biochemicals) was suspended in normal saline and heated to the boiling point until dissolved. The solubility of this compound in saline is poor, but by using this method, up to 70 mg/ml can be dissolved, being careful to restore the solution to the original volume after boiling. The preparation was always made up fresh as β-3-TA is rapidly decomposed in solution. Rats were immunized with 1 ml of 2% sheep erythrocytes intravenously on Day 0 and serial bleedings were done from the tail vein.

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Sera from one experiment were titriated together to avoid variations in technique. They were stored at —20° until titration was performed. Methods for collection of sera and antibody titration have been previously described (6).

The incorporation of ^{3}H uridine of ^{14}C amino acid mixture into spleen cells was performed as follows: Rat spleens were removed and minced onto a piece of 80-mesh stainless-steel screen, cells were pushed through gently with a syringe plunger and washed three times in Hanks' balanced salt solution (BSS) prior to incubation. The first wash contained 0.05% EDTA. Two ml of cells (2×10^7/ml) were incubated for 45 min in the amino acid solution from Eagle's medium at 1/10 concentration supplemented with 5 mg/ml glucose. At this point 100 μCi of ^{3}H uridine (Schwarz Bioresearch) (20.0 Ci/mmole) or 20 μCi ^{14}C cholorella hydrolysate (Schwarz Bioresesearch) (1.5 mCi/mmole) were added and incubation continued for 15 min. Incubation was terminated by addition of excess cold saline. RNA was prepared by the hot phenol method modified from Mach and Vassalli (7) as follows: Cell homogenates were extracted with phenol saturated with 0.1 Tris buffer pH 5.0 containing 0.5% sodium dodecyl sulfate (SDS) and 1 mg/ml hydroxyquinoline. The homogenate was heated to 63° for 6 min, chilled, and centrifuged. The aqueous layer was removed and saved and another extraction was performed on the remaining material with 0.1 *M* Tris buffer pH 5.0 at 20°. The aqueous phase was again recovered by centrifugation, combined with the previous one, and extracted twice with phenol at room temperature. RNA was precipitated with 2.5 vol of chilled ethanol. The RNA was pipetted into counting bottles and counted in a liquid scintillation spectrometer. Incorporation of ^{14}C amino acids into protein was determined by homogenizing the cells and precipitating the homogenate with 5% trichloracetic acid onto Millipore HA filters for counting in a gas flow counter. Serum proteins were analyzed by electrophoresis employing polyacrylamide gel disks using a modification of the procedure of Davis (8) previously described (9). After destaining the gels were scanned with a Leeds and Northrup densitometer at 660 mμ and patterns of the scans were recorded.

TABLE I. Effect of Length of Time of Administration of β-3-TA on Antibody Production.

Days injected[a]	Antibody titers[b]
Controls	3360
—2 to 0	2880
—1 to +1	1640
—1 to +2	360
—1 to +4	60
—2 to +4	30

[a] Animals were injected ip with 50 mg β-3-TA in 1 ml saline on the days indicated. All animals received 1 ml (2%) SRBC iv on Day 0.

[b] Each value represents average titer of 4 rats.

Results. Initial experiments indicated that antibody titer of animals receiving 30 mg β-3-TA per day was not significantly different from that of controls, while 40 and 50 mg gave a considerable reduction of titer. The dosages in excess of 50 mg proved lethal to all animals before the end of the experiments. As shown in Table I, a reduction in titer is quite apparent in all animals receiving the drug up to the second day after antigen injection. Maximum reduction was always present when the drug was given for the duration of the experiment. On the basis of these data, we chose a dose of 50 mg/day given from Day —2 to Day +4 with the antigen injected on Day 0.

To obtain further information on the inhibitory activity of β-3-TA on immunoglobulin synthesis, polyacrylamide gel electrophoresis was performed on sera from nonimmunized, immunized, and immunized β-3-TA–treated rats. The results of a representative experiment are presented in Fig. 1. Thre is clear evidence that while immunized rats show some increase in gamma globulin, as a result of immunization, β-3-TA treatment produces an almost complete disapperaance of this protein from the serum.

The results of ^{3}H uridine incorporation into total cellular RNA at various times after immunization are presented in Fig. 2a. Synthesis of RNA in immunized rats increases

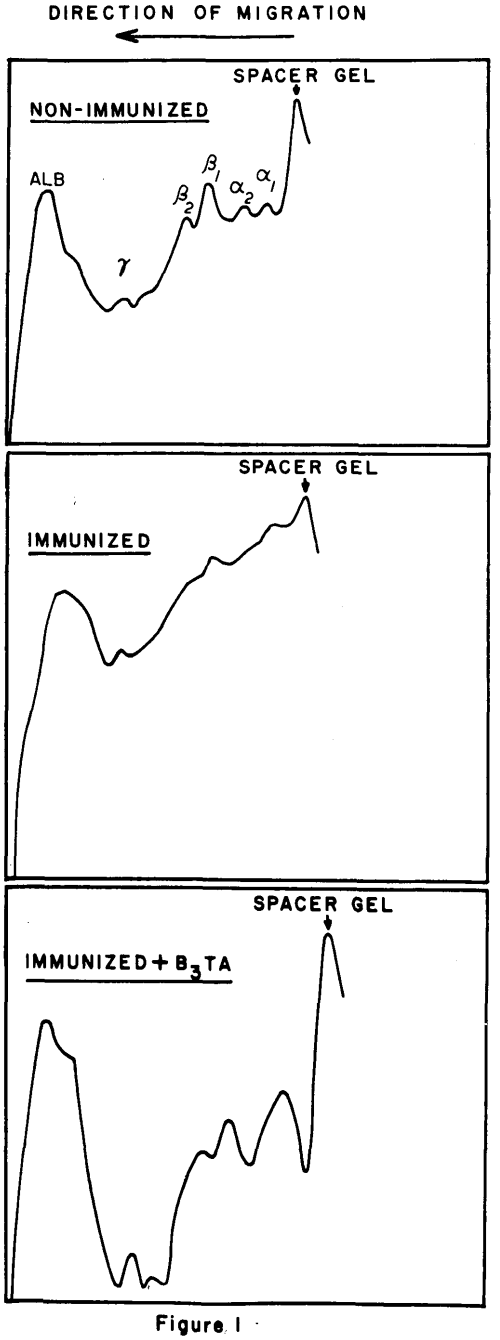

Figure 1

FIG. 1. Electrophoretic patterns of the serum from control rat and immunized rats with or without β-3-TA treatment. There is a considerable increase in the α, β, and γ globulin regions in the immunized rat serum, while the immunized β-3-TA–treated rat shows marked depression in the same regions.

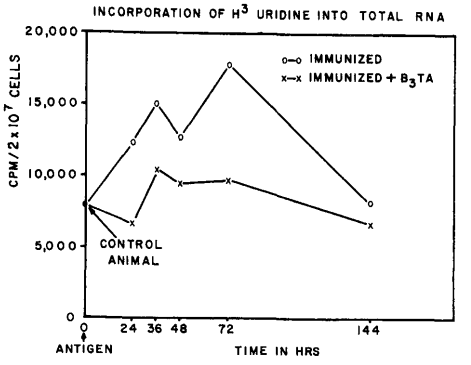

Figure 2a

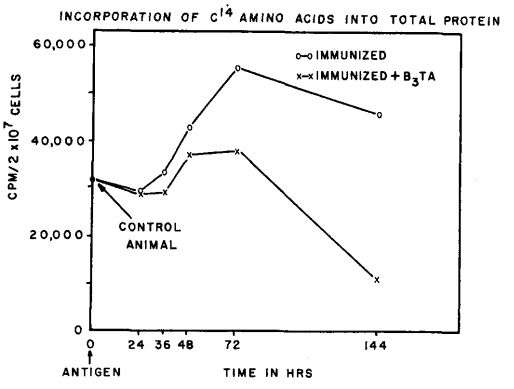

Figure 2b

FIG. 2.A. RNA synthesis in spleen cells from immunized rats treated or not treated with β-3-TA. The synthesis in immune spleen cells rises rapidly and returns to near normal levels by Day 6, while in the β-3-TA–treated cells, it never rises much above the control values seen in nonimmune cells. B. Protein synthesis in immunized, β-3-TA–treated and nontreated rat spleen cells follows the same pattern shown by the RNA synthesis. In addition, there is a profound depression of synthesis after β-3-TA treatment, reflecting the lack of RNA to support protein synthesis.

about 50% over that of nonimmunized controls by 24 hr after immunization and it continues to increase up to Day 3 when it is more than double the control value. By Day 6 it has returned to normal. In the immunized β-3-TA–treated spleen cells, on the contrary, only a slight initial increase in RNA synthesis is observed and a rapid return to normal values follows rapidly. The effect of this inhibition of RNA synthesis is reflected in the concomitant depression of total protein synthesis as measured by incorporation of

TABLE II. Incorporation of ^3H Thymidine into Spleen Cells from Immune β-3-TA–treated and Nontreated Rat after 3 30-min Pulse.

Animal	cpm/2 × 10^7 cells
Immune	565
Immune + β-3-TA	579

^{14}C amino acids into protein. This phenomenon is illustrated in Fig. 2b. In spleen cells from immunized animals protein synthesis starts increasing around 36 hr after immunization and has doubled by 72 hr with a slight decrease observed 72 hr later. In β-3-TA–treated immunized rats, on the other hand, there is an attempt at protein synthesis increase but this soon fails and synthetic activity levels off and drops to a fraction of normal in 6 days.

The results of fractionation of RNA from immunized, β-3-TA–treated and untreated spleen cells are illustrated in Fig. 3. The optical density pattern is typical of an undegraded RNA preparation. The radioactivity incorporated into RNA seems to indicate that β-3-TA has an inhibitory effect on molecular species migrating between approximately 20*S* and 4*S*. This was accompanied by the incorporation of a large amount of label into RNA having an *S* value of approximately 2.5.

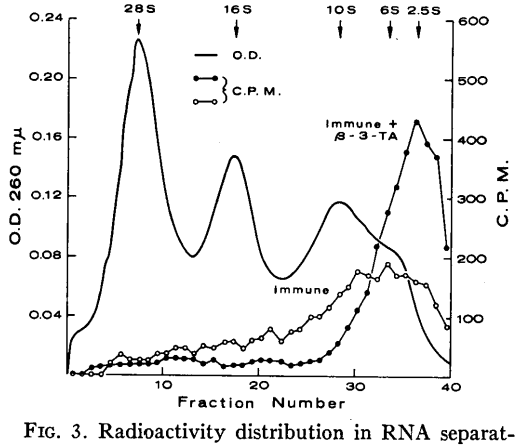

FIG. 3. Radioactivity distribution in RNA separated by sucrose density gradient. The RNA from immunized, β-3-TA–treated spleen cells shows incorporation in the lower molecular weight species as compared to RNA from untreated spleen cells.

The possibility that the defect in RNA synthesis was at the level of DNA was considered and a preliminary experiment was carried out to examine this. Cells were incubated the usual manner except that 10 μCi of ^3H thymidine (Schwarz Bioresearch) (14.0/mmole) were used as the labeling material and the pulse length was 30 min instead of 15. As indicated in Table II, there is no inhibition of DNA synthesis in β-3-TA–immunized rats.

Discussion. The results of these experiments point clearly to a defect in the synthesis of rapidly labeling RNA which follows immunization as one of the important events in the depression of antibody synthesis which is induced by β-3-TA. It appears from our preliminary data that DNA synthesis is not inhibited. It is not clear whether only one type of RNA or more are affected although the fact that the defect seems to be present at the level of rapidly labeling RNA (*i.e.* after 15 min pulse) points to mRNA as the species most likely inhibited. This conclusion is supported by the data on inhibition of protein synthesis. This follows RNA inhibition by about 12 hr, and if one accepts current data indicating that mammalian cell mRNA has a relatively long life span, the lag observed may indicate that mRNA is the affected RNA type.

One drawback of these experiments is the heterogenicity of the cell population used. In a spleen, one finds in addition to cells making antibody, a variety of hematopoietic cells, macrophages, lymphocytes, all of which must have metabolic activities. The fact that the changes observed follow immunization, however, suggest that the phenomenon observed is related to activities needed for antibody production. The results presented here cannot be taken as indicating a damage exclusively to the RNA synthesis necessary for immunoglobulin synthesis. The antibody-forming cells synthesize other proteins as well, and it is at the level of this synthesis that the action of β-3-TA may be exercised. It is clear, however, that ultimately the inhibition of the synthesis of some RNA, which may be mRNA, leads to inhibition of immunoglobulin production as indicated by antibody titer

reduction and by depressed gamma globulin peak in gel electrophoretic patterns.

This system, as modified in the present work by eliminating the cumbersome tube feeding technique, will be very useful in further studies of the early events occurring after immunization and of the mechanism of action of β-3-TA.

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Influence of Uranium-Induced Renal Injury on Flow and Composition of Renal Lymph (34005)

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This laboratory has utilized renal lymph flow and composition as an index of changes occurring in renal interstitial fluid volume and composition (1–3). Renal lymph concentrations of dextran fractions of known molecular weight have also given information on renal peritubular capillary "pore size" and permeability (4). Several years ago we made a preliminary report on the changes occurring in lymph flow and composition after uranium nitrate induced tubular necrosis (5). Recurrence of interest in the mechanism of oliguria or anuria which occurs in acute tubular necrosis (6), as well as the introduction of micropuncture approaches to the problem (7, 8) rekindled our interest in this subject and prompted the present report.

Many investigators have studied the histological and physiological changes which occur in the kidney after uranium intoxication. MacNider, the most prolific of these, reviewed his own work in the Harvey Lectures of 1929 (9). He reported that the epithelium of the convoluted tubules showed the selective action of uranium by developing edema and necrosis which varied in degree with the duration of the injury. The main site of injury was the proximal tubule. The cells of the descending limb of Henle's loop rarely showed evidence of damage. He also found that glomerular changes, unlike those of the tubules, were neither marked nor progressive. The changes in glomeruli are purely degenerative during the acute stages of the process and both degenerative and proliferative phenomena are seen in later stages. Glomerular basement membrane damage is seen in later stages and probably accounts for the observed proteinuria.

The histological changes found in the present study are similar to those reported by others (see "Appendix"). The primary damage at the time of the experiment appeared to be localized in the terminal portion of the proximal tubule and the pars recta. Apparently necrosis and regeneration occurred earlier in the ascending limb and distal tubule. The present results suggest that changes in