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Terminal deoxynucleotidyl transferase and ribonuclease activities in purified hepatitis-B antigen

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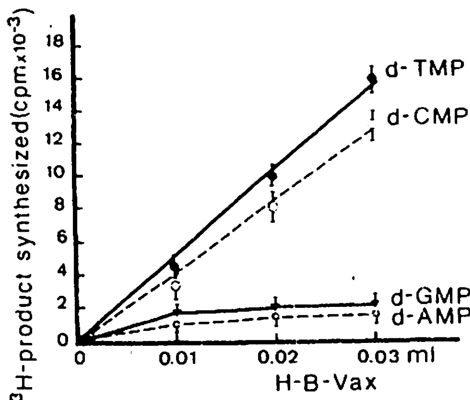
Terminal deoxyribonucleotidyl transferase (TdT) (EC 2.7.7.31) was defined [1] as a template independent DNA polymerase that binds deoxyribonucleoside-5'-monophosphates (dNMP) originating from deoxyribonucleoside-5'-triphosphates (dNTP) in sequential addition to the 3'OH end of the DNA initiator. TdT may exhibit its activity in the presence of oligodeoxyribopolymers. This enzyme has been found in embryonic calf thymus gland [2], cortical thymocytes, brain [3], primitive bone marrow cells, peripheral blood lymphocytes in certain forms of acute leukemia [1, 4, 5], neuroblastoma [6] and retroviruses [7, 8]. A model was proposed according to which TdT may generate somatic mutations in the variable (v) region of immunoglobulin genes [9]. Recently, the mutagenic potential of TdT has been evaluated [10]: TdT inserts noncomplementary bases during repair synthesis by DNA polymerase.

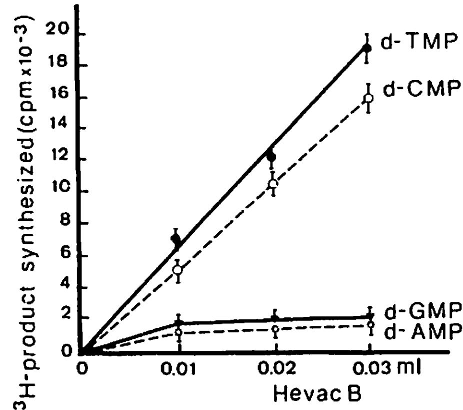
The present work shows that purified and commercially available hepatitis-B antigen (vaccine) contains a very active TdT and ribonuclease (RNase).

Materials and methods: Deoxyribonucleoside-5'-triphosphates were obtained from Miles Laboratory, USA. Oligo dT_{12-18}, [³H]-thymidine-5'-triphosphate (dTTP, sp.act. 17.5 Ci/mmol), [³H]-deoxycytidine-5'-triphosphate (dCTP, sp.act. 37 Ci/mmol), [³H]-deoxyguanosine-5'-triphosphate (dGTP, sp.act. 12.4 Ci/mmol), [³H]-deoxyadenosine-5'-triphosphate (dATP, sp.act. 13 Ci/mmol), [³H]-poly(A) (sp.act. 15 Ci/mmol) and [³H]-poly(C) (sp.act. 15 Ci/mmol) were obtained from Amersham, England. Pancreatic DNase and RNase A came from Worthington Inc., USA, purified hepatitis-B surface antigens Hevac-B from the Institut Pasteur Production, France, H-B-Vax from Merck Co., Germany and Engerix®-B from Smith Kline-RTT, Belgium. Glass millipore filters GF/C were from Whatman, England. [³H]-ribosomal RNA (rRNA) and [³H]-transfer RNA (4S RNA) were isolated and purified from *Escherichia coli* grown in the presence of [³H]-guanine and [³H]-cytosine [11].

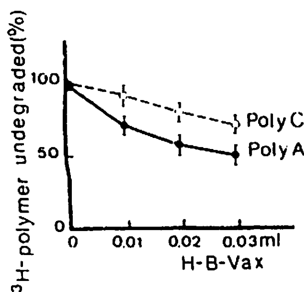
For the assay of TdT activity, the incubation mixture (0.10 ml final concentration) contained Tris-HCl buffer, pH 7.70, 25 μmol; $MgCl_2$, 2 μmol; one [³H]-dNTP, 100 000 counts per minute (cpm) and oligo dT_{12-18} or oligo dA_{12-18}, 0.5 μg, when indicated; antigen-B preparation was as indicated in the legend to the figures. After incubation of the mixture at 36°C for 10 min, trichloroacetic acid (TCA) precipitable labelled material was washed with 5% TCA solution on a glass millipore filter GF/C (three times with 10 ml) then with 95% alcohol. It was then dried and the radioactivity was measured in a Packard (Prias) scintillation spectrometer. The results are expressed as cpm/assay rendered acid-insoluble.

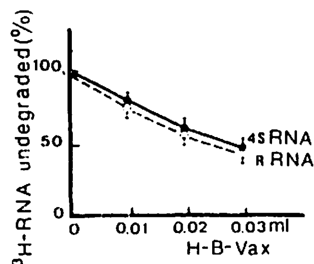
For the assay of RNase activity, the incubation mixture contained per 0.1 ml: Tris-HCl buffer pH 7.5, 25 μmol; [³H]-Poly C 10 μg (15 000 cpm); [³H]-Poly A 10 μg (15 000 cpm); [³H]-4 S RNA, 100 μg (20 000 cpm); [³H]-RNA, 100 μg

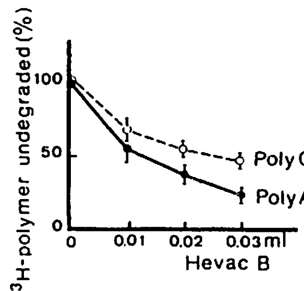


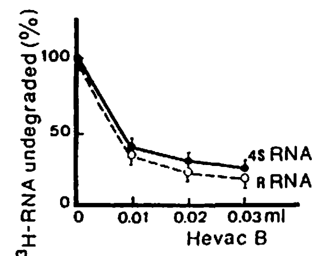


Figures 1 and 2: TdT activity in HB-Vax and Hevac-B antigens respectively (means ± SD of four different samples of each antigen). HB-Vax sample contained 20 μg of protein/ml. Both antigens are used without dilution.









Figures 3 and 4: RNase activity in HB-Vax and Hevac-B antigens respectively (means ± SD; n = 4). Incubation conditions (see text).

(20 000 cpm). Antigen-B preparation was as indicated in the legend to the figures. Incubation was for 10 min at 36°C. TCA precipitate was filtered and washed with TCA on a glass millipore, then with ethanol and dried. Precipitated radioactivity was measured.

Results and discussion: Antigens-H-B-Vax (Figure 1) and Hevac-B (Figure 2) contain TdT which *in vitro* actively polymerizes dTMP or dCMP originating from dTTP and dCTP used respectively as substrates. The amount of product increased with antigen concentrations. Exogenous oligodeoxyribonucleotides (dT_{12-18} or dA_{12-18}) are not required and dGMP or dAMP were poorly polymerized by either antigen-B. [^{3}H]-dTMP incorporation into TCA precipitable material was not inhibited by dCTP added in excess, neither was [^{3}H]-dCMP incorporation prevented by an excess of dTTP. When [^{3}H]-dTTP and [^{3}H]-dCTP were together incubated with antigen B, the radioactivity found in the acid precipitable material was additive (Table 1). The absence of competition between two pyrimidine deoxyribonucleotides indicates the presence in the antigen B of two accceptors, one for dTMP and the other for dCMP. Addition of three other dNTP besides [^{3}H]-dTTP or [^{3}H]-dCTP into the incubation mixture did not influence the incorporation of pyrimidine nucleotides, thus demonstrating the absence of need for a DNA template (Table 1). Both antigens seem to contain some initiators as we estimated by the amount of UV absorbing material (= 5%) (280/260 nm, ratio = 0.82) [12]. In fact preincubation of antigen-B with RNase free DNase results in a strong decrease in the polymerizing capacity (Table 1) while DNase free RNase had no effect. Since exogenous oligodeoxy-initiators are not required, antigen-B provides by itself the necessary initiator for pyrimidine deoxyribonucleotides polymerisation only. Engerix®-B, recombinant hepatitis-B vaccine exhibited higher TdT activity (Table 2) than that observed with H-B-Vax and/or Hevac-B. Antigen H-B-Vax and antigen Hevac-B also contain an RNAse which is capable of degrading naturally occurring RNAs such as rRNA or tRNA and synthetic Poly(A) and Poly(C) polymers as shown respectively in Figure 3 and Figure 4. Antigen Hevac-B contained more RNase activity than antigen H-B-Vax. Similar results were obtained with Engerix®-B antigen (data not shown here). RNase, probably belonging to viral particles, may though generate undesirable RNA or poly(A) fragments, perturbing the translation process and gene expression in those cells that will receive antigen-B.

The importance of TdT in modifying gene expression has been stressed in different laboratories: (i) TdT can provide to retroviral DNA the terminal nucleotides required for its integration into the cell genome [13]; (ii) the mutagenic potential of TdT has been demonstrated in DNA *in vitro* synthesis [10]. In this system, TdT can perform sequential addition of random bases, the number of which varies from 5 to 31; (iii) a model for generating somatic mutations by TdT in the variable (v) region of immunoglobulin genes was proposed [9]. The expected consequence will be a modification of the primary structure of immunoglobulin. Moreover, when in a cell DNA chains are destabilized (unpaired areas appearing under the influence of many different molecules) [14], nucleases may cleave single DNA chains into which TdT inserts noncomplementary bases before DNA chain breaks are repaired. Thus noncomplementary strands remain unpaired and destabilize cell DNA. This may initiate transformation of a healthy cell into a cancer cell [14]. We have also detected the presence of TdT activity in cytomegalovirus monoclonal antibodies produced by hybridome (results not presented here). With purified antigen-B used as a vaccine, the risk of introducing TdT and RNase into cells must be seriously evaluated. Are TdT and RNase integral parts of the chemical entity that constitutes antigen-B?

Table 1: Effect of dNTP, DNase and RNase on the activity of TdT present in H-B-Vax antigen (means ± SD; n = 3)

Substrates	Incorporation of [^{3}H]-dTMP and/or dCMP in 10 min at 36°C: cpm	Inhibition (%)
[^{3}H]-dTTP	5166 ± 170.5	–
[^{3}H]-dTTP + dCTP	5249 ± 306.8	0
[^{3}H]-dTTP + dCTP, dGTP, dATP	5268 ± 199.7	0
[^{3}H]-dTTP + DNase[a]	1221 ± 106.9***	77
[^{3}H]-dTTP + RNase[b]	5268 ± 261.2	0
[^{3}H]-dCTP	4182 ± 243.1	–
[^{3}H]-dCTP + dTTP	4139 ± 157.8	0
[^{3}H]-dTTP + [^{3}H]-dCTP	9149 ± 453.7**	additivity

*For incubation conditions, see text. [a]0.01 ml of H-B-Vax antigen was preincubated with 100 μg of DNase and 2 μg of $MgCl_2$; [b]0.01 ml of H-B-Vax preincubated with 50 μg of RNase. Incubation at 24°C for 10 min. H-B-Vax preparation contains 20 μg of proteins/ml. Experiments were performed in triplicate. In comparison with the control values: p < : **0.01; ***0.001 (Student's t test).*

Table 2: Presence of terminal deoxynucleotidyl transferase in the recombinant hepatitis-B vaccine (Engerix®-B): incorporation of [^{3}H]-dTMP or [^{3}H]-dCMP in 10 min at 36°C (cpm; means ± SD; three independent experiments)

Diluted Engerix®-B	[^{3}H]-dTMP	[^{3}H]-dCMP
0.01 ml	7577 ± 207.6	2047 ± 166.6
0.02 ml	15700 ± 496.1**	3237 ± 295.6*
0.03 ml	21067 ± 857.6**	5767 ± 441.3**

*Engerix®-B vaccine (20 μg/ml) was diluted with Tris-HCl buffer 0.01 M pH 7.3 to 2 μg/ml. For incubation conditions, see text. In comparison with the control values, p < : *0.02; **0.01 (Student's t test).*

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