ENZYME CAPABLE OF MODIFYING d-LYSERGIC ACID DIETHYLAMIDE (LSD) IN RABBIT LYMPHOID CELLS AND MURINE MOPC 315 MYELOMA CELLS

EDWARD W. VOSS, JR. and
NANCY C. COBB

Department of Microbiology
University of Illinois, Urbana, Illinois 61801

Received October 11, 1974

Enzymatic activity resulting in the modification of LSD was found in the lysates of rabbit antibody producing and murine MOPC 315 tumor cells. The LSD by-product was relatively more hydrophilic in a thin layer chromatography system. In vitro incorporation studies with whole cells indicated that the by-product is probably the incorporable analogue of tryptophan.

In vitro incubation of rabbit antibody producing cells with d-lysergic acid diethylamide (LSD) results in the secretion of low molecular weight peptides containing the lysergyl moiety incorporated covalently (1-4). To explain the incorporation of LSD in place of tryptophan, a pathway hypothesizing enzymatic modification of LSD prior to incorporation was proposed (3). Previous studies comparing the reactivity of the ligand LSD and the incorporated form (i.e. LSD_{DER}) with anti-LSD antibodies, indicated the two moieties were similar in structure (5). This indicated that only minor enzymatic modification(s) of LSD took place prior to covalently bonding to de novo synthesized immunoglobulins. These studies were designed to determine if an enzyme, capable of modifying LSD was present in lymphoid cells.

MATERIALS AND METHODS

Preparation of immunogen. Porcine gamma globulin (PGG) was conjugated with gluorescein (Fl-PGG) or 2,4 dinitrophenyl (DNP-PGG) as previously described (3, 6, 7).

Immunization. Adult albino rabbits received a primary immunization of the appropriate immunogen (5.0 mg) emulsified in complete Freund's adjuvant (Difco). Injections were made in each hind foot pad and intrascapularly. Secondary immunizations (5.0 mg) were administered 6 weeks after the primary injections. Rabbits were sacrificed and cells harvested 4-6 days after secondary immunizations.

Antibody producing cells. Spleen and lymph node cells were obtained as previously described (3).

Murine MOPC 315 plasmacytoma cells. MOPC 315 tumors were maintained in BALB/c mice (Cumberland Farms, Clinton, Tenn.) by in vivo cell transfer every 21 days. Tumor cells were processed as described for rabbit hyperimmune spleen or lymph node cells.

In vitro enzyme incubations. Washed rabbit lymphoid or murine MOPC 315 tumor cells were suspended in 2 parts buffer (0.15M KCl and 0.2 M phosphate buffer, pH 7.5) per 1 part packed volume cells. Cells were lysed using a teflon serrated pestle tissue grinder (Thomas Co.) driven by a variable speed motor (Model S63C, Tri-R Instruments, Inc.). Cells were lysed in an ice bucket, centrifuged at 12,000 rpm (Sorvall RC2-B automatic refrigerated centrifuge). Supernates were decanted and 1.0 ml aliquots incubated with LSD-2-^{3}H (N) obtained from New England Nuclear (spec. act. 14.2 Ci/m mol), 2 μ moles nicotinamide adenine dinucleotide (NAD), 100 μ moles nicotinamide and 100 μ moles $MgCl_2$. Reactions were incubated at 37°C (4-5 hours) in a Forma Hydro-Jac CO_2 incubator (5% CO_2, 98% rel hum) and were terminated by cooling to 0°C and adding 1.0 ml 1.0 N NaOH. Reactions were immediately extracted with chloroform (2.0 ml), dried, and dissolved in 1.0 ml chromatography solvent (CEA, chloroform:ethanol:glacial acetic acid; 18:10:2 v/v). Samples (50-100 μl) were spotted on thin layer silica gel plates (Eastman Kodak Co.) and chromatographed in the CEA solvent. Chromatograms were visualized under ultraviolet light (365 nm) and then cut into 0.5 cm strips. Each strip was placed in 4.0 ml scintillation fluid (3.79 l dioxane, 2.78 g naphthalene and 12.9 g PPO) and trituim content measured in a Beckman LS-150 liquid scintillation counter. ^{3}H-LSD control in buffer plus cofactors was run in every experiment.

Rabbit lymphocyte purification. Lymphocytes were purified from rabbit spleen and lymph nodes by the Technicon Lymphocyte Separator System. Cells (1.0 ml packed volume) were teased into minimum essential media (MEM, Grand Island Biological) and 0.4 ml lymphocyte separating reagent (i.e. iron filings). Cells were drawn into a 50 ml syringe (20g needle), gently mixed for 25 min and allowed to settle for 20 min. Cell suspensions were passed through the lymphocyte separator and the effluent collected. Cells were analyzed microscopically and found to be greater than 95% lymphocytes.

Purification of specific antibody producing cells. Hyperimmune rabbit spleen or lymph node cells (two parts) suspended in cold Kreb's-Ringer-Phosphate Buffer (KRPB), pH 8.0 (8) were incubated in the presence of either fluorescyl or dinitrophenyl conjugated cellulose (one part) immunoadsorbent (6, 9) for 1 hour at 5°C. Aliquots (1.0 ml) of cell incubation mixtures

were layered onto a wide bore 10 ml glass pipette, closed at the bottom with rubber tubing and a pinch clamp. Vertical-positioned columns contained 3.3% sucrose dissolved in 9 ml KRPB. This allowed settling of the immunoadsorbent within 15-20 minutes at 5°C. Lymphoid cells were buoyant under these conditions. Unbound cells were visible as a buffer coat at the meniscus, while antigen binding lymphocytes sedimented with the immunoadosrbent. Bound cells were greater than 95% lymphocytes, and stained with the appropriate radioiodinated (^{125}I) hapten conjugated proteins. Incubation of these cells with radiolabeled amino acids resulted in the secretion of intrinsically labeled 7S (IgG) antibody molecules, which were readsorbable to the specific immunoadsorbent.

RESULTS

Metabolism of LSD by lysates of rabbit lymphocytes. When ^{3}H-LSD was incubated in the presence of soluble supernates of lymphoid cell lysates, a conversion to a more hydrophilic product was observed chromatographically. Figure 1 shows a silica gel chromatogram in CEA solvent, comparing ^{3}H-LSD control (incubated in buffer) with the ^{3}H-LSD-by-product after incubation with the lysate. A similar profile was obtained with unfractionated spleen cells, popliteal lymph node cells, nonspecifically purified lymphocytes or

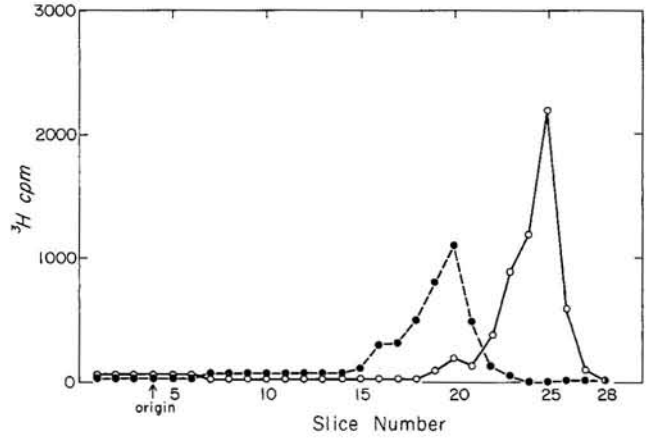

Figure 1. Chromatograms of ^{3}H-LSD after in vitro incubation with lysate from purified rabbit lymphoid spleenic cells on silica gel plate in CEA solvent. After a 5 hour incubation the reaction mixture was extracted with chloroform, dried and dissolved in DEA before spotting. After ascending chromatography, the silica gel strip (14 X 2 cm) was cut into 28 0.5 cm slices and placed into scintillation fluid for counting.
(●——●) ^{3}H-LSD by-product after incubation with cell lysate;
(○——○) ^{3}H-LSD after incubation in co-factor enriched buffer.

immunoadsorbent purified antibody producing cells (i.e. anti-fluorescyl or anti-dinitrophenyl producing cells). These results indicated that an enzyme capable of modifying LSD was present in rabbit lymphoid cells. All cells examined incorporated ^{3}H-LSD into secretable low molecular weight lysergyl peptides. The latter were bound by anti-LSD antibodies using the modified Farr assay (10).

Conversion of LSD by lysates of murine MOPC 315 myeloma cells. Modification of LSD by lysates of purified rabbit lympocytes and antibody producing cells suggested the enzyme was present in lymphoid tissue. To determine if the reaction was unique to rabbit lymphocytes and to further verify cell purity, lysates of MOPC 315 myeloma cells were examined for enzyme activity. Figure 2 shows that lysates of MOPC 315 also converted LSD to a relatively more hydrophilic derivative.

The enzymatic metabolism of LSD was studied. The enzyme reaction required NAD, nicotinamide and Mg. The reaction also proceeded best under aerobic conditions at 37° C and neutral pH (7.2-8.0).

Analysis of the enzymatic by-product. Figures 1 and 2 show that in the CEA solvent system, ^{3}H-LSD showed an R_F of 0.91-0.92. The enzymatically derived metabolite showed an R_F of 0.74-0.75. After purification and elution from silica gel plates, the tritiated derivative was bound by anti-LSD antibodies in the Farr assay. As previously suggested (10) by relating the specificity

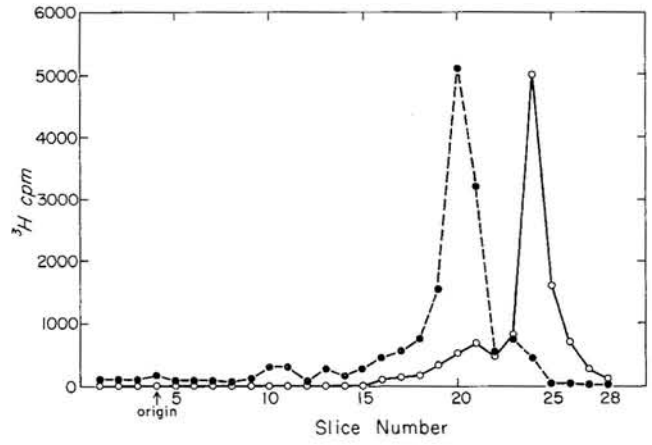

Figure 2. Chromatogram of ^{3}H-LSD after in vitro incubation with lysate from murine MOPC 315 myeloma cells on silica gel plate in CEA solvent. The reaction protocol is as described in the legend to Figure 1. (●——●) ^{3}H-LSD by-product after incubation with cell lysate; (○——○) ^{3}H-LSD after incubation in buffer.

of ligands binding to anti-LSD antibodies, conservation of the 4-ring structure of LSD is indicated. Thus, the enzyme modification observed, is a subtle alteration and is probably confined to side groups. There was evidence of blue fluorescence under the ultraviolet lamp and positive staining with p-dimethylaminobenzaldehyde. Staining with this reagent (Ehrlich's) is indole specific and suggests the lack of a substituent in the 2 position (ring B).

LSD metabolite in the in vitro protein synthesizing system. Radiolabeled metabolite was purified by repeated chromatography on silica gel plates until a chromatographically pure species with an R_F of 0.74 was obtained. The metabolite was incubated with rabbit antibody producing cells to determine if the relatively more hydrophilic metabolite could be incorporated in place of tryptophan during de novo protein biosynthesis. Tritium label was incorporated into low molecular weight secretable peptides which were bound by anti-LSD antibodies in the modified Farr assay. Incorporated label was not dialyzable against 6 M guanidine HCl. Thus, the enzymatically derived metabolite, which accumulates upon in vitro incubation with lymphoid cell (including MOPC 315) lysates is the apparent LSD_{DER} (or precursor to) which functions as a tryptophan analogue (5).

Enzyme isolation studies. Soluble lysates from rabbit lymphoid and murine MOPC 315 myeloma cells were treated in similar manner in all isolation studies. After lysis and clarification (9000x g), enzyme activity was found in the soluble supernate. Molecular sieve chromatography (Sephadex G-200 in 0.05 M phosphate buffer, pH 8.0) resulted in the elution of enzyme activity with the void volume. Precipitation of the soluble supernate or the void volume fraction from G-200 columns with 50% saturated ammonium sulfate resulted in almost quantitative precipitation of enzyme activity.

These results suggested that the enzyme was either of large molecular weight or was associated with the microsomal fraction. Therefore, the supernatant after lysis and clarification was centrifuged at 100,000 xg for 90 minutes. The microsomal pellet (suspended in buffer) and the 100,000 xg supernate were assayed for enzyme activity. Both the supernate and the microsomal fractions contained enzyme activity. Treatment of the 100,000 xg supernate with 50% saturated ammonium sulfate resulted in the precipitation of enzyme activity.

DISCUSSION

These studies show that rabbit lymphocytes contain an enzyme, capable of metabolizing LSD to a relatively more hydrophilic derivative. Although the reaction was measurable with lysates of unresolved spleen cell popula-

tions, similar results were obtained with purified rabbit lymphocytes and antibody producing cells. This indicates that the enzyme is a definite constituent of rabbit lymphocytes. However, similar results with lysates of murine myeloma cells (MOPC 315) indicates that lymphoid tissues of other species also contain the enzyme.

The LSD modifying enzyme required NAD, nicotinamide, oxygen and magnesium for activity. These are similar requirements to similar enzyme(s) described in rat liver (11, 12).

The principal metabolic derivative of LSD was extractable in chloroform and had an R_F of 0.74 in the CEA solvent system. LSD had an R_F of 0.92 in the same solvent system, indicating that LSD is relatively more hydrophobic than the derivative. Chemical analyses of the derivative have not been performed due to inadequate amounts of purified derivative. However, certain conclusions can be made regarding the structure of LSD_{DER} on the basis of nearly identical reactivity of the derivative and LSD with anti-LSD antibodies. It had been previously shown that lysergyl peptides and LSD_{DER} isolated from such peptides (4, 5) also have nearly equal reactivity with anti-LSD. Thus, it is reasonable to assume that enzyme modification does not involve the four ring aromatic structure of LSD.

ACKNOWLEDGEMENTS

This research was supported by National Institute of Drug Abuse (DA 00308) and Illinois Department of Mental Health (533-03).

REFERENCES

1. Voss, E. W. Jr., Babb, J. E., Metzel, P. and Winkelhake, J. L. Biochem. Biophys. Res. Comm. 50, 950 (1973).

2. Voss, E. W. Jr., Metzel, P. and Winkelhake, J. L. Mol. Pharmacol. 9, 421 (1973).

3. Winkelhake, J. L., Voll, E. W. Jr. and Lopatin, D. E. Mol. Pharmacol. 10, 68 (1974).

4. Voss, E. W. Jr. and Winkelhake, J. L. Proc. Natl. Acad. Sci. 71, 1061 (1974).

5. Lopatin, D. E., Winkelhake, J. L. and Voss, E. W. Jr. Mol. Pharmacol. In Press.

6. Lopatin, D. E. and Voss, E. W. Jr. Biochemistry 10, 208 (1971).

7. Eisen, H. N. "Methods in Medical Research", Year Book Medical Publishers, 10: 94 (1964).

8. Umbreit, W. W., Burris, R. H. and Stauffen, J. F. "Manometric Techniques and Related Methods for the Study of Tissue Metabolsim", Burgess Publishing Company, 194 (1945).

9. Robbins, J. B., Haimovich, J. and Sela, M. Immunochemistry 4, 11 (1967).

10. Lopatin, D. E. and Voss, E. W. Jr. Immunochemistry 11, 285 (1974).

11. Niwaguchi, J., Inour, T. and Nakahana, Y. Biochem. Pharmacol. 23, 1073 (1974).

12. Ladu, B. N., Gavdette, L., Trovsof, N. and Brodir, B. B. J. Biol. Chem. 66, 741 (1955).