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SHORT COMMUNICATION

Incorporation of a Lysergic Acid Diethylamide Intermediate into Antibody Protein *in Vitro*

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SUMMARY

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Antibody-producing lymphoid cells incubated *in vitro* in the presence of [^{3}H]lysergic acid diethylamide secrete labeled protein. Incorporation of [^{3}H]lysergic acid diethylamide (or a derivative) *de novo* was indicated by precipitation of labeled peptides with trichloracetic acid and by the retention of label after dialysis against denaturing agents and high concentrations of a dissociating ligand. Incorporation of [^{3}H]lysergic acid diethylamide by immune lymphoid cells was inhibited by puromycin and enhanced by homologous antigen.

Previous studies indicated that when D-lysergic acid diethylamide was incubated *in vitro* with rabbit antibody-producing cells, an altered secreted protein pattern was observed (1, 2). Results from these previous studies, summarized below, were not readily interpretable. Whereas antibody-producing cells not exposed to lysergic acid diethylamide secreted 7 S protein which was precipitable by anti-γ-globulin antiserum and reacted with homologous antigen, material secreted by lysergic acid diethylamide-exposed, antibody-producing cells lacked both antigen-binding activity and γ-globulin antigenicity when tested with anti-γ-globulin antiserum. Radioactive amino acid incorporation studies indicated that the drug interfered with tryptophan insertion into protein but not to a significant extent with total protein synthesis (1, 2) or cell viability.

A peptide termination mechanism was proposed for the mode of action of lysergic acid diethylamide (2), but the possibility of a coincident amino acid analogue effect was not dismissed. If the hallucinogen functioned as an incorporable tryptophan analogue, the net result might be either peptide termination after incorporation or uninterrupted synthesis and secretion of complete but modified antibody chains. In either case, incorporation of lysergic acid diethylamide or a metabolic by-product of it would be consistent with noted changes in the antigenicity of the secreted product, and also its lack of reactivity with homologous antigen. Experiments were therefore performed to test the hypothesis that lysergic acid diethylamide (or a metabolic derivative) is incorporated directly into secretable protein from lymphoid cells.

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Rabbit lymphoid cells obtained from hyperimmunized animals were incubated *in vitro* with [^{3}H]lysergic acid diethylamide (1.9 Ci/mmole, New England Nuclear Corporation) in minimal essential medium (Grand Island Biological Corporation) minus tryptophan or leucine (1). Rabbits were immunized with fluorescyl-conjugated proteins (3), and spleen or node cells were obtained as previously described (1, 4). The radiochemical purity of [^{3}H]lysergic acid diethylamide was determined by ascending silica gel chromatography in chloroform–ethanol–acetic acid (18:10:2). [^{14}C]Tryptophan (specific activity, 29 mCi/mmole; Schwarz/Mann), unlabeled L-tryptophan, and unlabeled D-lysergic acid diethylamide (supplied as the tartrate salt by the National Institute of Mental Health) were chromatographed as internal standards. After chromatography, the gels were air-dried and placed in contact with X-ray film (Royal blue, Eastman Kodak) for 1 week. Gels were monitored for location of unlabeled compounds by ultraviolet absorption. Both ^{3}H-labeled and unlabeled lysergic acid diethylamide showed R_F values of 0.66, while [^{14}C]tryptophan and unlabeled tryptophan showed R_F values of 0.29. The radiochemical purity of [^{3}H]lysergic acid diethylamide was greater than 95%, with no detectable tryptophan. To a 0.1-ml volume of packed cells were added 1.0 ml of minimal essential medium minus tryptophan and 10 ml (1.7 μg), 25 μl (4.3 μg), 50 μl (8.6 μg), or 75 μl (12.9 μg) of [^{3}H]lysergic acid diethylamide. Cell aliquots were also incubated with [^{14}C]tryptophan or [^{3}H]leucine as incorporation controls. After 4 hr of incubation at 37° in 7% CO_2, cells were centrifuged at 2500 rpm (refrigerated International PR-2 centrifuge) for 10 min and extracellular supernatants were harvested. After incubation, Trypan blue exclusion viability studies indicated equal cell viability (80–90%) in all cultures (i.e., with or without lysergic acid diethylamide). As previously shown (1, 2, 4), labeled extracellular supernatants from cells not incubated with the hallucinogen contain 80–90% 7 S γ-globulin. The supernatant fractions were dialyzed against 8–12 liters of 0.05 M potassium phosphate, pH 8.0, for 48 hr at 5°. Dialyzed supernatants were analyzed for incorporated

TABLE 1

Incorporation of label in vitro by antibody-producing cells incubated with [^{3}H]lysergic acid diethylamide

Rabbit No.	Cells	Radioactive label	Total radioactivity after dialysis
			cpm/.01 ml cells
22	Spleen	[^{3}H]Lysergic acid diethylamide (8.6 μg/ml)	4.7×10^4
		[^{3}H]Lysergic acid diethylamide (12.9 μg/ml)	6.9×10^4
		[^{14}C]Tryptophan	1.2×10^4
	Node	[^{3}H]Lysergic acid diethylamide (8.6 μg/ml)	3.4×10^4
		[^{3}H]Lysergic acid diethylamide (12.9 μg/ml)	5.0×10^4
25	Spleen	[^{3}H]Lysergic acid diethylamide (4.3 μg/ml)	6.6×10^4
		[^{3}H]Lysergic acid diethylamide (4.3 μg/ml)[a]	10.0×10^4
		[^{3}H]Leucine	30.0×10^4
	Node	[^{3}H]Lysergic acid diethylamide (4.3 μg/ml)	7.5×10^4
		[^{3}H]Lysergic acid diethylamide (4.3 μg/ml)[a]	11.1×10^4
		[^{3}H]Leucine	30.0×10^4
30	Spleen	[^{3}H]Lysergic acid diethylamide (1.7 μg/ml)	4.9×10^4
		[^{3}H]Lysergic acid diethylamide (1.7 μg/ml) + Puromycin (100 μg/ml)	0.6×10^4
Normal[b]	Spleen	[^{3}H]Lysergic acid diethylamide (1.7 μg/ml)	0.01×10^4

[a] Incubations included 25 μg of fluorescyl-substituted porcine γ-globulin (antigen).

[b] Nonimmunized rabbit.

radiolabel by precipitation with 5% trichloroacetic acid, dialysis against both denaturing agents and dissociating ligand, and sedimentation in a sucrose gradient. Table 1 shows the level of incorporation with immune rabbit (anti-fluorescyl) spleen cells in two separate experiments. Cells obtained from rabbit 22 (spleen and node) showed significant incorporation of tritium counts into secreted protein. Greater incorporation was evident at 12.9 μg of [^{3}H]lysergic acid diethylamide than at 8.6 μg, and significant [^{14}C]tryptophan incorporation was measured in the controls. In similar studies with rabbit 25, tritium was incorporated into protein secreted from cells incubated with 4.3 μg of [^{3}H]lysergic acid diethylamide. Spleen and node cell incubation mixtures showed similar levels of incorporation. Increased incorporation of counts was evident when immune cells were incubated in the presence of immunogen (i.e., fluorescyl-substituted porcine γ-globulin). This suggests a booster effect *in vitro* by the fluorescyl group and direct enhancement of tritium incorporation into an antibody or biosynthetic product of this stimulation. Puromycin inhibited [^{3}H]lysergic acid diethylamide incorporation by 88% (Table 1), suggesting an active incorporation process *de novo*. Table 1 shows that incubation of the labeled drug with spleen cells from nonimmunized rabbits yielded very low levels of incorporation. This is consistent with a low rate of nonspecific γ-globulin synthesis relative to the role of antibody synthesis, characteristic of antigen-stimulated cells.

Covalent linkage of the radioactivity measured in the extracellular material was tested by ionic bond denaturation studies (Table 2). Dialysis of trichloracetic acid-precipitable labeled peptides against 1 mM lysergic acid, as a dissociating ligand, resulted in the retention of 78 and 86% of the radiolabel. Dialysis against the denaturing agents 8 M urea, 6 M guanidine HCl, and dioxane–acetic acid (10%:1.0 N) resulted in similar retention percentages. After dialysis against the denaturing agents, the material was 100% precipitable by trichloroacetic acid. Cumulatively, these data indicate that tritium counts were incorporated covalently into the secreted material and were not bound by ionic interactions. Pronase treatment of the labeled material resulted in low molecular weight, tritium-labeled products which were dialyzable and could not be precipitated with trichloroacetic acid. Pronase susceptibility and previous observations (1, 2), revealing competition between lysergic acid diethylamide and tryptophan, suggested that the extracellular material was protein. To assess further the nonspecific binding properties of [^{3}H]lysergic acid diethylamide, the ligand (30 μM, 3.3 × 10^5 cpm/nmole) was dialyzed against purified rabbit serum albumin (0.93 mg/ml) and normal rabbit γ-globulin (1–2 mg/ml) in equilibrium dialysis chambers (5). No binding was detected with either protein.

TABLE 2

Retention of incorporated label after exposure of protein to denaturing agents and dissociating ligand

Rabbit No.	Reagent	Nondialyzable[a]	Normalized to buffer control
		%	%
22	1 mM lysergic acid	62	78
	8 M urea	65	81
25	1 mM lysergic acid	69	86
	6 M guanidine HCl	62	78
	10% dioxane–1.0 N acetic acid	54	68
	0.1 M potassium phosphate, pH 8.0	80	

[a] After 48 hr of dialysis at 5°.

In previous studies (1, 2) it was shown that the extracellular material secreted from antibody-producing cells incubated in the presence of lysergic acid diethylamide sedimented as significantly lighter material in a sucrose gradient than material from cells not exposed to the drug. To determine whether the extracellular labeled material from cells incubated with [^{3}H]lysergic acid diethylamide was also low molecular weight, the material was examined on a 5–35% sucrose gradient (2). Figure 1 shows that the material from cells incubated with [^{3}H]-lysergic acid diethylamide (rabbit 22) was significantly lighter than material from cells

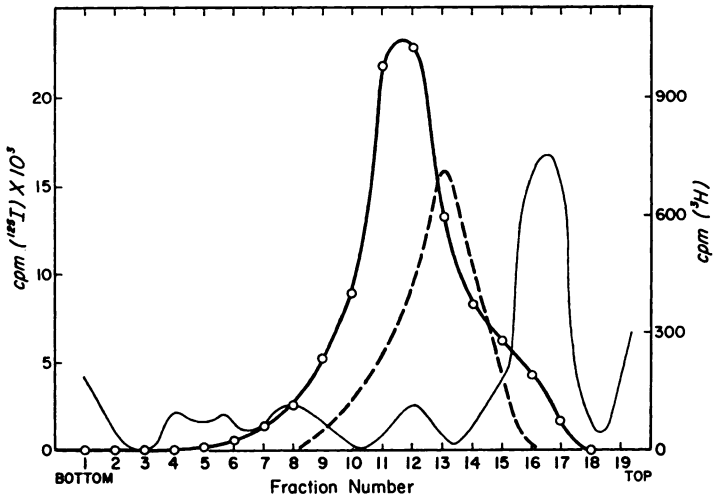

FIG. 1. *Sucrose density gradient profile of purified rabbit* [^{125}I]*anti-2,4-dinitrophenyl-IgG antibody* (O——O), *bovine* [^{125}I]*serum albumin* (- - -), *and* ^3H*-labeled extracellular protein* (——) *derived from anti-fluorescyl antibody-producing cells in the presence of* [^{3}H]*lysergic acid diethylamide.*

The experiment was performed on a 5–35% gradient in 0.05 M potassium phosphate buffer, pH 8.0. Centrifugation was performed in a Beckman Spinco model L centrifuge with a SW-39 rotor at 35,000 rpm for 16 hr.

incubated with [^{14}C]tryptophan. Analysis of extracellular material from rabbit 25 yielded similar results. Relative to the albumin marker the material from the drug-exposed cells had a molecular weight of less than 67,000. The gradient gives insufficient resolution to determine accurately the number of small molecular weight components.

Preliminary studies were conducted to identify the incorporated lysergic acid diethylamide molecules. Isolated extracellular protein labeled in the presence of [^{3}H]-lysergic acid diethylamide was hydrolyzed in 4.25 N NaOH by the method of Hugli and Moore (6). Washed, partially hydrolyzed potato starch was used as an antioxidant to preserve the indole moiety. [^{3}H]lysergic acid diethylamide and [^{14}C]tryptophan were also subjected to alkaline hydrolysis as controls. After hydrolysis at 110° for 16 hr, the hydrolysates were neutralized and analyzed by ascending silica gel chromatography, using the chloroform–ethanol–acetic acid (18:10:2) solvent system. R_F values for [^{3}H]lysergic acid diethylamide and [^{14}C]-tryptophan remained the same as reported above. A single radioactive compound with an R_F value of 0.4–0.5 was obtained from the hydrolyzed extracellular labeled protein. labeled material corresponding to lysergic acid diethylamide and tryptophan was not detected. The labeled derivative has not been isolated in enough quantity to determine its structure.

These results indicate that a modified form of lysergic acid diethylamide is incorporated into protein. Previous results (1, 2) suggested that the modified drug molecule is incorporated competitively with tryptophan molecules. This fact precludes a mechanism of side group modification by lysergic acid diethylamide. Because of the absence of an α-amino or carboxyl group, it is improbable that D-lysergic acid diethylamide can become charged and directly incorporated into protein. Therefore we propose either that the molecule is degraded (catabolic intermediate) or that enzymes are available which by appropriate side group modifications (anabolic intermediate) render the drug molecule incorporable into proteins.

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