Mechanism of Lysergic Acid Diethylamide Interference with Rabbit Antibody Biosynthesis

(LSD/amino acid analogue/isoelectric focusing)

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ABSTRACT Lymphoid cells from hyperimmune rabbits producing antibodies to a hapten, incubated in the presence of *d*-lysergic acid diethylamide, continued to synthesize protein at a normal rate. Isoelectric focusing analysis of the low-molecular-weight protein secreted by the cells incubated with lysergic acid diethylamide indicated two components, with pI's of 4.9 and 5.2. Immune cells not exposed to lysergic acid diethylamide secreted only 7S IgG molecules with an average pI of approximately 7.0.

Previous studies (1, 2) indicated that *d*-lysergic acid diethylamide (LSD) significantly affected immunoglobulin synthesizing cells *in vitro*. The LSD "effect" was related to interference with tryptophan incorporation into protein during *de novo* synthesis (3). Antigen-stimulated cells, incubated in the presence of LSD, secreted an inactive protein of lower molecular weight than rabbit IgG antibody molecules (i.e., <150,000). However, sucrose gradient and isoelectric focusing analyses failed to provide evidence that the secreted protein was of immunoglobulin origin. Characterization and correlation of the secreted material to the native multisubunit antibody molecule (IgG), would suggest the drug's mode of action. Whereas, the existence of a few discrete and definable extracellular subunits would indicate that LSD interferes at a few specific points in the assembly of the antibody molecule, a heterogeneous population of low-molecular-weight peptides would indicate random termination at various points of tryptophan incorporation. This report describes experiments designed to determine the nature of the material being secreted by the antibody producing cells, incubated in the presence of LSD. Experimental evidence distinguishing between specific termination and random termination is presented and a mechanism of drug action proposed.

MATERIALS AND METHODS

Adult albino rabbits hyperimmunized (1, 4) with fluorescein conjugated porcine gamma globulin (Fl$_{23}$PGG) were used as the source of immune lymphoid cells. Rabbits were boosted 5–6 days prior to cell harvest with 5 mg of immunogen emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) in the rear foot pads and intrascapularly. Animals were monitored for antifluorescyl antibody activity by serum assays previously described (1). Rabbits were sacrificed by intracardiac injection of 1.0 ml of sodium pento-

barbital (Diamond Laboratories, Des Moines, Iowa). Spleens and popliteal lymph nodes were immediately extracted and placed in minimum essential medium (MEM, Grand Island Biological Co., Grand Island, N.Y.). Lymphoid cells were teased (5) from the organs, passed through a fine wire mesh filter and rinsed twice in Krebs–Ringer Buffer (6). After washing, 0.1 ml (packed volume) of cell aliquots were incubated in a total volume of 1.0 ml as previously described (1). To designate the manner in which the cells were treated *in vitro*, reactions are noted as A, B, and C (see Tables 1 and 2): A indicates cells incubated in MEM minus leucine, B and C indicate cells incubated in MEM minus the amino acids leucine and tryptophan. [³H]Leucine (50 µl, 480 µmol, specific activity 52 Ci/mmol, Schwarz/Mann) was added to all reactions. Fifty microliters of [¹⁴C]tryptophan (96 nmol, specific activity 29 mCi/mmol, Schwarz/Mann) was added to reactions B and C. Thus, A and B serve as controls for net protein synthesis and normal [³H]leucine/[¹⁴C]tryptophan ratios respectively. Reaction C also received various amounts of LSD. Reactions were incubated 18 hr at 37° in a CO$_2$ incubator (7% CO$_2$, Wedco model 2-17B). After incubation, the cells were pelleted (2000 × *g* for 15 min, the supernate (*i.e.*, extracellular material) collected and dialyzed against frequent changes of 4-liter volumes of 0.05 M K-phosphate buffer, pH 8.0 for 72 hr at 5°. Pelleted cells were washed in Krebs–Ringer buffer; suspended in 5.0 ml K-phosphate buffer and lysed in a motor driven Dounce homogenizer at 4°. Cellular debris was sedimented by centrifugation at 10,000 × *g* for 10 min at 0° (Sorvall RC 2-B). The soluble supernate (i.e., intracellular sap) was dialyzed against K-phosphate buffer for 48 hr at 5°. Precipitability in 5% trichloroacetic acid was carried out as described (1).

Cell viability during incubation was determined by the trypan blue method described by Paul (7). Cell samples were obtained at 3.5 and 17 hr.

Buoyant density centrifugation was accomplished on a 5–35% continuous sucrose gradient in 0.05 M K-phosphate buffer, pH 8.0. Protein samples (0.5 ml) were placed on the 5.0-ml gradient and centrifuged at 35,000 rpm (Beckman, Spinco model L, SW-39 rotor) for 20 hr. Three-drop fractions were collected into a scintillation vial containing 8.0 ml of PCS solubilizer (Amersham-Searle). Fractions were monitored in an Isocap/300 liquid scintillation spectrophotometer (Nuclear Chicago).

A modified dodecyl sulfate polyacrylamide disc gel electrophoresis (8) in 7.5% gels was used to analyze material labeled *in vitro*. Samples (20–50 µl) containing 8,000–20,000 cpm were

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Abbreviations: LSD, *d*-lysergic acid diethylamide; MEM, minimum essential medium; BSA, bovine serum albumin; H and L, heavy and light chains, respectively, of immunoglobulin G.

TABLE 1. *Effect of LSD on in vitro incorporation of labeled amino acids into antibody secreted from hyperimmune antifluorescyl producing cells*

Reaction*	μg LSD	Total thousand cpm/0.1 ml of cells	$^3H/^{14}C$†
A	—	202	—
B	—	221	3.9
C-1	10.0	140	10.7
C-2	0.1	171	7.1
C-3	0.01	199	5.3
C-4	10.0 (+ 100 μg of tryptophan)	209	4.5
Control	(puromycin)‡	0.40	—

* Reaction nomenclature described in *Materials and Methods*.
† [^3H]Leucine/[^{14}C]tryptophan ratios.
‡ 200 micrograms of puromycin per 0.1 ml of cells.

electrophoresed in 0.1% sodium dodecyl sulfate in 0.05 M K-phosphate, pH 7.2 buffer with a field strength of 140 V at 4 mA per tube for 3–4 hr. Gels were frozen over dry ice and sliced horizontally into 55–60 1.0-mm fractions, with a sequential gel slicer (Mickle Laboratories, Brinkmann Instrument, Westbury, N.Y.). Each slice was dissolved in 0.5 ml 30% hydrogen peroxide at 50° overnight in a scintillation vial. Eight milliliters of PCS was added and radioactivity measured.

Isoelectric focusing in 4% polyacrylamide gels containing 2% carrier ampholytes (pH 3–10, LKB, Sweden) was performed according to the method described by Righetti and Drysdale (9). Samples containing 10–40,000 cpm were subjected to a starting current of 1 mA per tube. The voltage was slowly increased to 300 V and maintained for 5 hr. Each gel was immediately frozen over dry ice and sliced sequentially in 1-mm slices as described above. Slices were dissolved in 0.3 ml of 30% hydrogen peroxide overnight at 50°. Solubilized slices were assayed in 10% Bio-Solv (Beckman, Fullerton, Calif.)–toluene–4% 2,5-diphenyloxazole (PPO, Packard, Downers Grove, Ill.) scintillation fluid on a Nuclear Chicago scintillation counter. The pH gradient was measured on a control gel from which sets of four slices were combined in 1.0 ml of distilled water in tightly stoppered tubes. After shaking overnight to dissolve the ampholytes, the pH of each tube was measured.

Radioiodination (^{125}I) of purified antihapten IgG (4) and bovine serum albumin (BSA, Miles Laboratories, Kankakee, Ill.) was performed by the chloramine-T oxidation method (10).

Immunoassays for gamma globulin were performed with goat anti-rabbit IgG, goat anti-rabbit heavy (H) chains and goat anti-rabbit light (L) chains antisera. After equivalence point determination in quantitative precipitin tests, the assays were performed in the presence of unlabeled antigen and labeled *in vitro* material in slight antibody excess.

Ammonium sulfate precipitation of labeled fractions was performed by adding 1.0 mg of purified unlabeled antihapten IgG and equal volumes of saturated ammonium sulfate.

RESULTS

Since the radioactively labeled, extracellular material secreted from *in vitro* incubation of immune spleen or lymph node cells is 80–90% immunoglobulin (5), analyses of the labeled extra-

TABLE 2. *Percent precipitation of labeled extracellular material by 50% saturated ammonium sulfate*

Exp. no.	Labeled sample	μg LSD	Percent precipitability
1.	[^{125}I]IgG	—	97
	[^{125}I]BSA	—	0*
	Reaction B (spleen)	—	81
	B (node)	—	87
	C-1	1.0	5
	C-2	0.1	22
	C-3	1.0	
	(+100 μg of tryptophan)		65
2.†	Reaction A		91
	B		76
	C-1	10.0	0
	C-2	0.1	21
	C-3	0.01	49
	C-4	10.0	
	(+100 μg of tryptophan)		80

* All percent precipitability values are corrected for [^{125}I]BSA precipitation control.
† Corresponds to experiment in Table 1.

cellular supernates is a convenient quantitative measure of antibody secretion. Table 1 compares the extracellular material from reactions A, B, and C showing the effects of LSD on antifluorescyl antibody-producing lymphoid cells. Comparison of reaction A with B indicates that, based on the level of [^3H] leucine incorporation into secreted protein, labeled [^{14}C] tryptophan substitutes for unlabeled tryptophan in MEM without altering antibody synthesis. However, comparison of [^3H]leucine and [^{14}C]tryptophan incorporation into the extracellular material from reactions B and C, indicates that LSD affects tryptophan incorporation. Relative to the [^3H]Leu/[^{14}C]Trp ratios in reaction B, a higher ratio is observed in reaction C. Since the total extracellular ^3H incorporation between reactions B and C is equal, the increased ratio has been interpreted as a decrease in tryptophan incorporation. The cells are synthesizing and secreting approximately equal amounts of labeled protein. This fact coupled with equal cell viability over the short incubation period suggests that the ratio change is significant, and not a redistribution of material derived from the heterogeneous cell population. Ratio changes have been observed at 10, 0.1 and 0.01 μg of LSD per 0.1 ml of cells indicating that a significant effect occurs at very low LSD concentrations. Table 1 shows that additions of unlabeled tryptophan reversed the ratio changes.

Sucrose gradient analysis of the extracellular material in reactions B and C is shown in Fig. 1. Labeled protein secreted from reaction C is of lower molecular weight than comparable material from reaction B and the radioiodinated rabbit IgG control. The low-molecular-weight extracellular material secreted from cells incubated with LSD (reaction C) was not precipitable by goat anti-rabbit IgG, anti-H chain, or anti-L chain antisera and was not adsorbable to a fluorescyl-cellulose immunoadsorbent (4). Dialyzed extracellular material was 90–95% precipitable in 5% trichloroacetic acid. Table 2 shows the degree of precipitability at 50% saturation with ammonium sulfate. Since some precipitability was observed at the lower concentrations of LSD in the titration experiments, it was presumed some 7S immunoglobulin was being secreted.
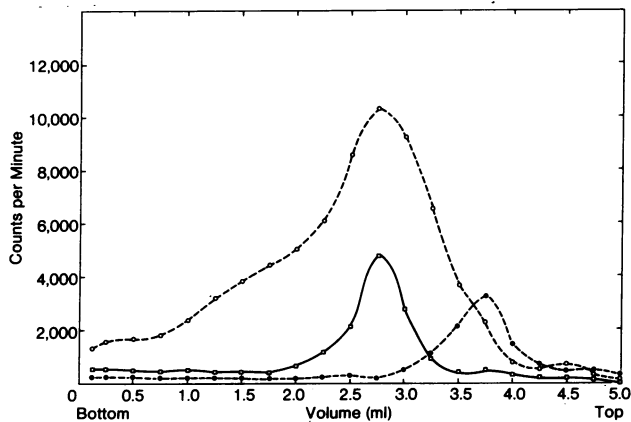
FIG. 1. Comparative sucrose gradient profiles of rabbit [¹²⁵I]IgG and labeled extracellular material secreted from immune cells incubated with and without LSD. (□) secreted material from reaction B without LSD and (●) reaction C with LSD. (O) ¹²⁵I-labeled purified rabbit antifluorescyl IgG antibody. Materials were analyzed on a 5–35% continuous sucrose gradient and centrifuged at 35,000 rpm for 20 hr.
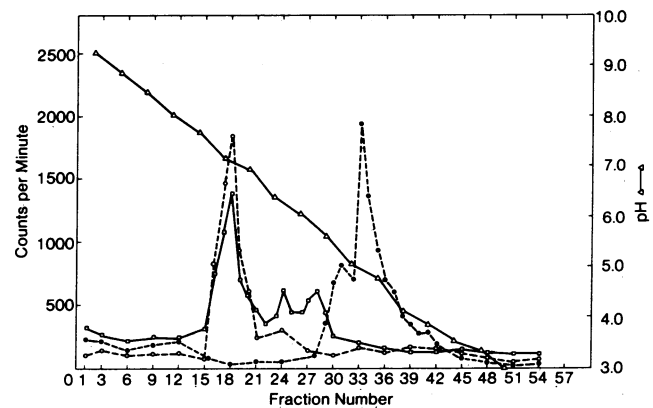

FIG. 2. Analysis of labeled extracellular material by iso-electric focusing polyacrylamide gel electrophoresis. (□) material from reaction B without LSD and (●) reaction C with LSD. (O) ¹²⁵I-labeled purified rabbit antifluorescyl IgG antibody. Focusing was performed in 4% gels containing 2% ampholytes at 300 V for 5 hr.

In the tryptophan-reversal experiments (Table 2) 93% of the extracellular material was precipitable. Experiments have shown that the percent material precipitable by 50% ammonium sulfate corresponds to the amount of 7S identifiable by sucrose gradient analysis.

Labeled extracellular material was further analyzed by iso-electric focusing in polyacrylamide gel electrophoresis. Isoelectric point differentiation was used to determine if the low molecular weight material was (1) a single component, (2) a small number of components, or (3) a complex heterogeneous population of peptides. Fig. 2 indicates that materials from reactions B and C analyzed by isoelectric focusing are dissimilar. Reaction B extracellular material showed the same pI (about 7.0) as the radioiodinated IgG control. Reaction A supernates (not shown) exhibit this same pattern. However, the secreted material from reaction C showed only 2 peaks at pI's 4.9 and 5.2. Little or no material with a pI of 7.0 was evident.

To further define the mode of LSD interference with protein synthesis a comparison was made of the intracellular labeled material from reactions B and C by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Approximately 10% of the intracellular material from reaction B was 7S. Little or no 7S antibody was detectable in the intracellular sap from reaction C. A large 7S peak would have suggested accumulation of IgG molecules within the cell.

DISCUSSION

The secretion of immunoglobulins is generally considered to occur with biosynthetically completed H or L chains (11). However, LSD exposed antibody producing cells appear to secrete low molecular weight proteins (Fig. 1). It is unlikely that the secreted material is a result of extensive cell lysis since microscopic examination and trypan blue viability studies indicated the degree and rate of cell death paralleled normal controls. In addition, control studies with puromycin (Table 1) showed nearly total termination of protein synthesis within the relatively short incubation period. The puromycin control indicates lack of cell lysis in the presence of a protein synthesis inhibitor under the conditions employed. Thus, these data elucidate a dilemma regarding the mode af action of LSD on antibody producing cells *in vitro*.

The "LSD effect" apparently occurs at the translational level by direct involvement with tryptophan incorporation. Table 1 shows that increased $^3H/^{14}C$ ratios are indicative of reduced tryptophan incorporation. Dilution of [¹⁴C]tryptophan by an unlabeled tryptophan-like metabolic analogue of LSD does not satisfactorily explain the ratio change, since a significant decrease in molecular weight of the secreted material may indicate peptide termination. Excess tryptophan specifically inhibits the effects of LSD on protein synthesis, suggesting termination may occur at the point of tryptophan insertion during translation. Kinetics of the drug action are relatively fast, since no labeled IgG (i.e., 7S form) is evident in the analysis of the labeled extracellular material (Fig. 1). A 25- to 30-min lag phase is generally observed *in vitro* before labeled IgG appears in the extracellular supernate (12). Thus, LSD is effective within the time span of the lag phase.

LSD is probably not exerting its action at the DNA·RNA levels. Antibody producing cells do not divide during the incubation period employed, and antibody related messenger RNA is relatively stable, negating the effect of actinomycin D (13). The near normal biosynthesis of total protein observed in these studies supports the actinomycin D analogy.

Isoelectric focusing of extracellular material showed two components with pI's of 4.9 and 6.2. This result is important in assessing the peptide-termination mode of action by LSD on antibody synthesis. Rabbit IgG antibody contains approximately 25 tryptophans (14) potentiating a spectrum of 2–26 peptides depending on the efficiency of LSD interference with tryptophan incorporation, if peptide termination is the mechanism. However, continual [¹⁴C]tryptophan incorporation and the yield of only two components by isoelectric focusing suggests alternatives. Relative to the concentration of LSD employed, interference with tryptophan incorporation is not absolute, indicating an equilibrium situation. Conversely, the secreted material may be complete H and L chains, indicating no peptide-termination. However, the measured acidic isoelectric points for the secreted components would apply only to L chains. Rabbit H chains possess isoelectric properties similar to the 7S IgG molecule (i.e., about 7.0). These alternatives await substantiating since direct experimental proof identifying the secreted components as antibody related is lacking. Precipitation with antigamma

globulin antisera and reactivity with fluorescyl antigen yielded negative results.

In summary, the data do not adequately support a peptide termination mechanism and may be more consistent with an amino acid analogue effect being simulated by LSD.

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