PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 215-218 (1977)

Inhibition of Lymphotoxin- and Lymphocyte-Mediated Cytolysis by High Molecular-Weight Dextran (39640)[1]

CONSTANTINE D. TSOUKAS[2] AND WERNER ROSENAU

Department of Pathology, University of California, School of Medicine, San Francisco, California 94143

Lymphotoxin (LT) is a cytotoxin released by antigen- or mitogen-stimulated lymphocytes *in vitro* (1). The inhibition of lymphocyte-mediated cytolysis by anti-LT serum (2), as well as other lines of evidence (1), suggest that this lymphokine has a role in lymphocyte-mediated cytotoxicity in tissue culture. Recently, Henney (3) and Ferluga and Allison (4) reported the protective effect of dextrans on lymphocyte-mediated target-cell destruction.

In this study, we examined the effect of high molecular-weight dextran (83,000) on lymphotoxin-induced cytolysis in tissue culture and compared our findings with those observed with dextran in cell-mediated cytolysis *in vitro*.

Materials and assays. target cells. Monolayers of mouse L fibroblasts, clone 929, grown for 24 hr in round-bottomed tubes, were used as targets. The tissue culture medium was RPMI 1640 with 5% fetal calf serum (FCS).

Lymphotoxin. Human lymphotoxin was purified from supernatants of phytohemagglutinin (PHA)-stimulated human adenoidal lymphocytes as previously described (5). The activity of LT is expressed in cytotoxic units (CU) as formerly defined (5). Lymphotoxin was dissolved in a buffer consisting of 0.1 *M* glycine–0.15 *M* NaCl, pH 9.5.

Spleen cells. Sensitized spleen cells were obtained from BALB/c mice which had received intraperitoneal and, 3–4 weeks afterward, intrasplenic injections of living L cells, derived from a C3H mouse as previously described (6). Spleens were harvested 3–6 days later; RBCs were lysed in hypotonic solution, and the spleen cells were resuspended in tissue culture medium. Control spleen cells were taken from noninjected BALB/c mice.

Dextran solutions. Dextran, average mol wt 83,000 (Sigma Chemical Co., St. Louis, Mo.), was dissolved in RPMI 1640 and dialyzed against this medium. After dialysis, the concentration of dextran was adjusted to the desired molarity by adding RPMI 1640. Then, the material was filtered through Millipore membranes (pore size, 0.22 μm), and FCS was added to yield a final concentration of 5% serum. Since nondialyzed dextran was found to be slightly cytotoxic, the dialysis was necessary.

Calculation of results. The effect of LT was assayed by the release of ^{51}Cr into the supernatant from L cells [prelabeled as described (7)], and radioactivity was measured in a gamma counter. For each determination, cultures were used in triplicate, and radioactivity was counted to a 1% error. The same technique, employing ^{51}Cr release, was used for assaying the effect of spleen cells on prelabeled target L cells.

The percentage of specific release (SR) of ^{51}Cr from prelabeled target cells was expressed as follows. $SR = (E - C)/T \times 100$, where E is radioactivity in supernatants of cultures receiving LT or sensitized spleen cells, C is radioactivity in supernatants of control cultures, and T is total releasable radioactivity). The percentage of inhibition of specific ^{51}Cr release was calculated relative to cultures without dextran.

Employing an electronic particle counter as described (5, 8), we also determined the numbers of lymphotoxin-treated target cells. Each determination was performed on five replicate cultures.

Results. Release of ^{51}Cr in LT-treated and control cultures as a function of time. The release of ^{51}Cr from LT-treated and control target cells was assessed as a function of time, using LT (700 CU) or control buffer.

[1] Supported by a grant from the National Institutes of Health, U. S. Public Health Service: No. CA-07191-13.

[2] Dr. Tsoukas' present address is: the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115.

ISSN 0037-9727

In this initial experiment, a markedly greater release of ^{51}Cr from LT-treated cells was observed as compared to that in control preparations (Fig. 1). From these data, we calculated the percentage of specific release, which indicated the percentage of release effected by lymphotoxin (Fig. 2).

Effect of dextran on LT-induced cytolysis as measured by ^{51}Cr release. Monolayers of ^{51}Cr-labeled target L cells (60,000 cells/ml) were incubated with 700 CU of LT. We conducted the experiments by two different methods: (i) by incubating the cultures with LT for 2 hr, removing the LT-containing medium, rinsing the preparations with Hanks' balanced salt solution (HBSS), and adding various concentrations of dextran in medium; or (ii) by simultaneously adding lymphotoxin and dextran to the medium. The final concentration of dextran was 0, 0.5, 1, or 1.5 m*M*, respectively. In both types of experiments, after the addition of lymphotoxin at 37°, the incubation period was 17 hr. Control cultures received equivalent amounts of buffer and dextran.

These experiments revealed a dose-related, dextran-induced inhibition of ^{51}Cr release (Fig. 3, Table I). Furthermore, we observed no difference, regardless of whether the cells were exposed first to LT and then to dextran, or both substances were added simultaneously. In one additional experiment (results not shown), inhibition of LT-induced target-cell lysis by dextran was confirmed using cell counting with a Coulter particle counter instead of ^{51}Cr release as a measure of cytolysis.

Effect of high molecular-weight dextran on target-cell lysis induced by sensitized spleen cells. Monolayers of ^{51}Cr-labeled target cells (60,000 cells/ml) were incubated with sensitized mouse spleen cells in a ratio of 1:20 for 2 hr at 37°. The supernatants containing nonattached spleen cells were removed, the monolayers were gently rinsed with HBSS, and various concentrations of dextran in medium were added. The final concentration of dextran was 0, 0.25, 0.5, 1, or 1.5 m*M*, respectively. Following the addition of the lymphocytes at 37°, the cultures were incubated for 17 hr. Control cultures received spleen cells from nonsensitized mice and equivalent amounts of dextran. Three experiments were performed.

Again, dose-dependent inhibition of ^{51}Cr release by dextran was observed in these

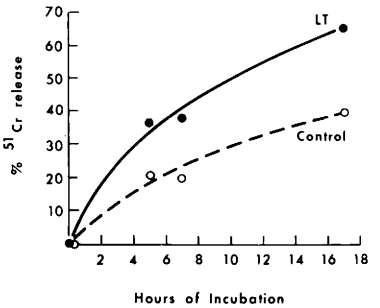

FIG. 1. Release of ^{51}Cr, as a function of time, from prelabeled target cells treated with LT (700 CU) or buffer.

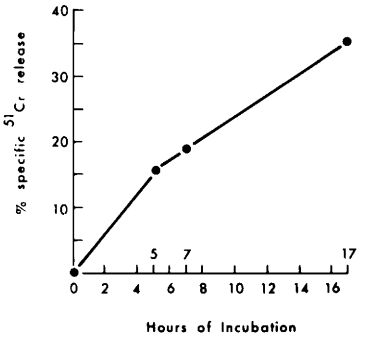

FIG. 2. Specifiic release of ^{51}Cr, as a function of time, from prelabeled target cells reflecting LT-induced injury.

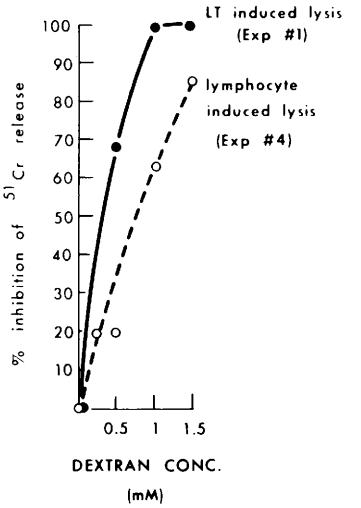

FIG. 3. Dextran-mediated inhibition of ^{51}Cr release induced by LT or sensitized spleen cells.

TABLE I. EFFECT OF DEXTRAN ON CYTOLYSIS INDUCED BY LT AND SENSITIZED SPLEEN CELLS.

	Percentage of inhibition of ^{51}Cr release[a]							
	Inhibition of LT-mediated lysis (%)				Inhibition of cell-mediated lysis (%)			
	Dextran concentration (m*M*)				Dextran concentration (m*M*)			
	0.5	1	1.5		0.25	0.5	1	1.5
Expt 1	68	100	100	Expt 4	20	20	64	85
Expt 2	11	15	60	Expt 5	22	46	61	60
Expt 3	25	38	100	Expt 6	–	32	25	56

[a] In these experiments, the percentage of specific release of ^{51}Cr in the absence of dextran was 35–40.

three replicate experiments (Fig. 3, Table I).

Discussion. In preliminary experiments, we established that ^{51}Cr release can be used as a valid indicator of LT-mediated cell lysis under the experimental conditions employed (Figs. 1 and 2). We had already observed previously that there was a close correlation between the percentage of lysis as determined by cell count and the specific release of ^{52}Cr from cytotoxically affected target cells. The experiments were conducted *in vitro* because only in such a system is it possible to precisely define the macromolecular environment of the target cells and to quantitatively establish the interfering effect of macromolecules on LT-induced cytolysis.

The observations reported here clearly indicate that LT-induced cytolysis can be inhibited by high molecular-weight dextran (83,000) and that the degree of inhibition is dose-dependent. The reason for this inhibition is unknown, but four possible mechanisms are suggested. (i) The polymer may interfere with the binding of LT. (ii) Dextran may affect the cell membrane. (iii) Inhibition may be attributable to the osmotic effect of the polymer. (iv) Inhibition may be due to the diffusion-limiting effects of dextran.

Previously, we noted the binding of LT to sensitive target cells, with 50% of the maximal binding occurring within 2 min and progressing to full maximal binding in 1–2 hr (9). Although the rate of cytolysis is dose-dependent, it becomes marked only several hours after addition of the lymphokine under the given experimental conditions. Therefore, we pretreated the target cells with lymphotoxin in Expts 1 and 2 for 2 hr to allow for uninterrupted maximal binding, removed the unbound LT, and then added the dextran to the target-cell cultures. Inhibition of lysis was observed under these circumstances just as in the case of the simultaneous addition of lymphotoxin and the polymer (Expt 3), intimating that dextran does not act by blocking the binding sites of LT and, thus, speaking strongly against the first mechanism considered.

It is also conceivable that the polymer in some way alters the function of the cell membrane. This hypothesis is difficult to test in view of the current incomplete knowledge of membrane structure and function as well as the various possible sites of action of the polymer on the cellular membrane. At this time, there is still conflicting opinion concerning the mechanism of intracellular fluid regulation. A primary role for the membrane, with pumps for different molecules, is a widely accepted premise (10), but regulation of intracellular fluid according to the state of hydration of macromolecules within the cell has also been postulated (11).

Another explanation which might serve is that high molecular-weight dextran dissolved within the tissue culture medium protects cells with LT-induced membrane injury by its osmotic effect. This theory is based on Henney's studies (3) of target-cell lysis by sensitized allogeneic spleen cells in the presence of dextrans of different molecular weights. He noted that small molecules were released at an early stage of cell damage, with subsequent release of macromolecules or markers bound to them. Moreover, he suggested that the enlargement of initial target-cell lesions by water influx, with the resultant release of cellular macromole-

cules, could be prevented by maintaining the injured cells in a medium containing high molecular-weight molecules. Such molecules, incapable of entering the cells, would prevent the influx of water by their osmotic effect.

Seeman, using erythrocytes, has observed that release of hemoglobin from red cells in a hypotonic environment can be reduced by the presence of macromolecules (12). Such an effect does not depend on the molecular weight of the macromolecule, but rather on its concentration by weight. He has suggested that, since macromolecules are highly hydrated, they exclude the solvent volume accessible to a second macromolecule (e.g., hemoglobin), thereby reducing the solubility and diffusability of the second molecule (13).

In the present study, dextran also inhibited target-cell lysis mediated by sensitized lymphocytes, confirming previous observations by other investigators (3, 4). Likewise, dextran interference with lymphocyte attachment is improbable, because the polymer was added after target-cell exposure to lymphocytes. Inhibition of lymphocyte- and LT-induced cytolysis by high molecular-weight dextran is consistent with the contention that both phenomena are mediated by the same final pathway.

1. Rosenau, W., and Tsoukas, C. D., Amer. J. Pathol. **84,** 580 (1976).
2. Walker, S. M., and Lucas, Z. J., Transplant. Proc. **5,** 137 (1973).
3. Henney, C. S., Nature (London) **249,** 456 (1974).
4. Ferluga, J., and Allison, A. C., Nature (London) **250,** 673 (1974).
5. Russell, S. W., Rosenau, W., Goldberg, M. L., and Kunitomi, G., J. Immunol. **109,** 784 (1972).
6. Rosenau, W., and Moon, H. D., J. Nat. Cancer Inst. **27,** 471 (1961).
7. Peter, J. B., and Dawkins, R. L., Nature New Biol. **232,** 79 (1971).
8. Rosenau, W., Goldberg, M. L., and Burke, G. C., J. Immunol. **111,** 1128 (1973).
9. Tsoukas, C. D., Rosenau, W., and Baxter, J. D., J. Immunol. **116,** 184 (1976).
10. Tosteson, D. C., *in* "The Cellular Functions of Membrane Transport" (J. F. Hoffman, ed.), p. 3. Prentice-Hall, Englewood Cliffs, N. J. (1963).
11. Ling, G. N., and Walton, C. L., Science **191,** 293 (1976).
12. Seeman, P., Fed. Proc. **33,** 2116 (1974).
13. Seeman, P., Canad. J. Physiol. Pharmacol. **51,** 226 (1973).

Received September 1, 1976. P.S.E.B.M. 1977, Vol. 154.