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SENSITIVITY OF MURINE B- AND T-LYMPHOCYTES TO OXAZAPHOSPHORINE AND NON-OXAZAPHOSPHORINE NITROGEN MUSTARDS*

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Abstract—The relative sensitivities of murine B- and T-lymphocytes to the oxazaphosphorine nitrogen mustards, cyclophosphamide and ASTA Z 7557, and to the non-oxazaphosphorine nitrogen mustards, melphalan and chlorambucil, *in vivo*, were determined. B- and T-lymphocytes were defined by selective mitogen-induced proliferation. Lipopolysaccharide (LPS)-induced B-lymphocytes were approximately twice as sensitive to the cytotoxic effects of cyclophosphamide and ASTA Z 7557 as were phytohemagglutinin (PHA)- and concanavalin A (Con A)-induced T-lymphocytes. LPS-induced B-lymphocytes and PHA-induced T-lymphocytes were approximately equisensitive to the cytotoxic action of melphalan and chlorambucil, but the former were somewhat more sensitive to these agents than were Con A-induced T-lymphocytes. The relative sensitivities of murine B- and T-lymphocytes to ASTA Z 7557 and the non-oxazaphosphorine metabolite of cyclophosphamide, phosophoramide mustard, *ex vivo*, were also determined. LPS-induced B-lymphocytes were approximately twice as sensitive to the cytotoxic action of ASTA Z 7557 as were PHA- and Con A-induced T-lymphocytes. The three mitogen-induced lymphocyte populations were approximately equisensitive to the cytotoxic action of phosphoramide mustard. These observations suggest that the differential effect of cyclophosphamide on murine B- and T-lymphocytes is uniquely exhibited by oxazaphosphorine nitrogen mustards. Furthermore, the results suggest that 4-hydroxycyclophosphamide is the cyclophosphamide metabolite that mediates the differential immunotoxic effect of the parent compound.

Cyclophosphamide, the prototype oxazaphosphorine nitrogen mustard, has been the subject of extensive investigation and has seen widespread use as an antineoplastic agent. Recently, attention has focused on the immunomodulatory properties of cyclophosphamide, as well as on its clinical utility in transplantation [2–4] and in the treatment of various autoimmune disorders [5–7].

Of particular interest are the numerous observations that cellular elements of the humoral and cell-mediated immune systems are differentially sensitive to the cytotoxic action of cyclophosphamide; investigations employing different criteria to define lymphocyte populations have shown that B-lymphocytes are more sensitive to the cytotoxic action of cyclophosphamide than are T-lymphocytes [8–11].

While differential sensitivity of B- and T-lymphocytes to cyclophosphamide is well established, it is not known if the differential effect is unique to the oxazaphosphorine nitrogen mustards or if it is a general property of all nitrogen mustards. This question was addressed in the present investigation.

MATERIALS AND METHODS

Materials. Male BALB/c mice, 8–12 weeks of age and weighing between 23 and 32 g, were obtained from the Minnesota Mouse Colony, University of Minnesota. They were housed in conventional cages fitted with filtered lids and were given free access to food and water; 12-hr light/dark cycles were maintained.

Melphalan, ASTA Z 7557‡§ and phosphoramide mustard·cyclohexylamine were provided by Dr. G. M. Lyon Jr., Burroughs-Wellcome, Research Triangle Park, NC, Dr. P. Hilgard, ASTA-Werke AG, Bielefeld, F.R.G., and Mr. L. H. Kedda, Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, respectively. Human serum was donated by healthy volunteers and was heat inactivated (56° for 30 min). All other materials were purchased from commercial sources.

PBS was prepared by dissolving $CaCl_2$ (0.9 mM), KCl (2.7 mM), $MgCl_2 \cdot 6H_2O$ (0.6 mM), KH_2PO_4

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‡ Abbreviations: ASTA Z 7557, 2-[bis-(2-cholorethyl)-amino]-4-(2-sulfoethylthio)-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide cyclohexylamine salt; LPS, lipopolysaccharide; PHA, phytohemagglutinin; Con A, concanavalin A; DMEM, Dulbecco's Modified Eagle's Medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PBS, phosphate-buffered saline; ID_{50} and IC_{50}, dose or concentration of drug required to reduce [^{3}H]thymidine incorporation to 50% of control; and cpm, scintillation counts per minute.

§ ASTA Z 7557 is a stable precursor of 4-hydroxycyclophosphamide. It rapidly and spontaneously gives rise to 4-hydroxycyclophosphamide under physiologic conditions.

(0.3 mM), $Na_2HPO_4 \cdot 7H_2O$ (1.7 mM) and NaCl (0.15 M) in triple-distilled water. The pH was adjusted to 7.4 and the solution was sterilized by passage through a 0.22 μm Millipore filter.

Cyclophosphamide and melphalan were dissolved in a 0.85% NaCl solution and were each given i.p. in a volume equal to 2.5 ml/kg. ASTA Z 7557 and chlorambucil were dissolved in a 0.85% NaCl solution and in a 1:10 (v/v) solution of 95% ethanol–Intralipid 10% [12], respectively, and were each injected in a volume equal to 2.5 ml/kg into the ventral tail vein of mice that were lightly anesthetized with ether. Control mice received vehicle by the appropriate route of administration.

ASTA Z 7557 and phosphoramide mustard were prepared for *ex vivo* use by dissolving each in PBS. Sterilization was effected by passage through a 0.22 μm Millipore filter. Drug solutions were kept on ice and used within 30 min of preparation.

Spleen cell preparations. Animals were killed by cervical dislocation, and spleens were removed aseptically, placed into PBS, and passed through a stainless steel wire mesh. The resulting suspension was repeatedly aspirated through a sterile pasteur pipet and then allowed to stand for 10 min to sediment tissue debris. The supernatant fraction, containing spleen cells in suspension, was removed and viable mononuclear cells were quantified by means of a trypan blue exclusion test. Spleen cells prepared in this manner were used when the proliferative capacity of lymphocytes following *in vivo* drug administration was to be determined.

Supernatant spleen cell suspensions from untreated animals were further purified when the desire was to test the antiproliferative effect of drugs *ex vivo*. Portions (5 ml) of the spleen cell suspension were layered onto 2.5 ml of Histopaque-1083. After centrifugation at 650 *g* for 30 min, mononuclear cells were removed from the density interface, washed twice with 10 ml of PBS, and resuspended in PBS. Viable mononuclear cells were again quantified by means of a trypan blue exclusion test.

Ex vivo *drug exposure.* Drug or vehicle, in a volume of 0.1 ml, was added to 2×10^7 viable mononuclear cells suspended in 4.9 ml of PBS, and incubation was allowed to proceed for 30 min in air at 37°. Subsequently, cells were harvested by low speed centrifugation and washed once with 5.0 ml of drug-free PBS.

Assay for proliferative capacity. Following harvest, spleen cells were suspended (4×10^6 cells/ml) in DMEM supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 14 μM folic acid, 140 μM asparagine, 1.1 mM arginine, 1.5 mM glutamine, 50 μM 2-mercaptoethanol, and 2% human serum.

Portions (0.1 ml) of the spleen cell suspensions were placed into individual wells of Costar flat bottom tissue culture clusters containing an equal volume of supplemented DMEM ± LPS, PHA or Con A. Final concentrations of LPS, PHA and Con A were 125 μg/ml, 7.5 μg/ml and 1.5 μg/ml respectively. After incubation for 48 hr at 37° in a fully humidified atmosphere containing 10% CO_2, 1 μCi thymidine[^{3}H-*methyl*] (49 Ci/mmole) in supplemented DMEM (25 μl) was added to each well and the incubation was continued for an additional 12–18 hr. Cells were then collected onto glass fiber filters with the aid of a Brandel M24 cell harvester. After drying, the filter disks were placed into polypropylene vials along with 3.5 ml of Aquasol-2. Thymidine[^{3}H-*methyl*] content was quantified by means of a Beckman LS 8000 liquid scintillation counter. Counting efficiency, consistently between 42 and 45%, was determined by means of an external standard technique using a ^{137}Cs gamma source. Mean control cpm values for background and LPS-, PHA- and Con A-stimulated samples were 3,475, 35,809, 84,892 and 216,797 cpm respectively.

RESULTS

Initial experiments were designed to identify a time point at which maximal inhibition of LPS-induced B-lymphocyte and PHA- and Con A-induced T-lymphocyte proliferation by two oxazaphosphorine nitrogen mustards, cyclophosphamide and ASTA Z 7557, and two non-oxazaphosphorine nitrogen mustards, melphalan and chlorambucil, would occur following the *in vivo* administration of these agents (Fig. 1). Mitogen-induced lymphocyte proliferation was maximally inhibited by 4 hr after nitrogen mustard administration. Accordingly, this time point was chosen as the endpoint for the subsequent dose–response determinations.

The next series of experiments was designed to determine if the differential effect of cyclophosphamide on B- and T-lymphocytes is exhibited only by the oxazaphosphorines or if it is exhibited by both oxazaphosphorine and non-oxazaphosphorine nitrogen mustards (Fig. 2 and Table 1). As expected, LPS-induced B-lymphocytes were found to be more sensitive to the cytotoxic action of cyclophosphamide and ASTA Z 7557 than were either PHA- or Con A-induced T-lymphocytes; this difference was found to be approximately 2-fold. LPS-induced B-lymphocytes and PHA-induced T-lymphocytes were found to be approximately equisensitive to the cytotoxic action of melphalan and chlorambucil. However, LPS-induced B-lymphocytes were somewhat more sensitive to the cytotoxic action of melphalan and chlorambucil than were Con A-induced T-lymphocytes.

Cyclophosphamide is a prodrug whose cytotoxic action is effected by the metabolite phosphoramide mustard. This metabolite does not contain the oxazaphosphorine moiety. It is generated from aldophosphamide which is the ring-opened tautomer of 4-hydroxycyclophosphamide, a hydroxylated metabolite of the parent compound. The oncotoxic specificity of cyclophosphamide is thought to reside with 4-hydroxycyclophosphamide/aldophosphamide, although it is not cytotoxic to tumor cells as such [14–16].

The question arose as to which of the cyclophosphamide metabolites is responsible for mediating the differential cytotoxic effect of the parent compound on B- and T-lymphocytes. The effects of 4-hydroxycyclophosphamide/aldophosphamide (in the form of ASTA Z 7557) and phosphoramide mustard on mitogen-induced proliferation of B- and T-lymphocytes exposed to these agents *ex vivo* are

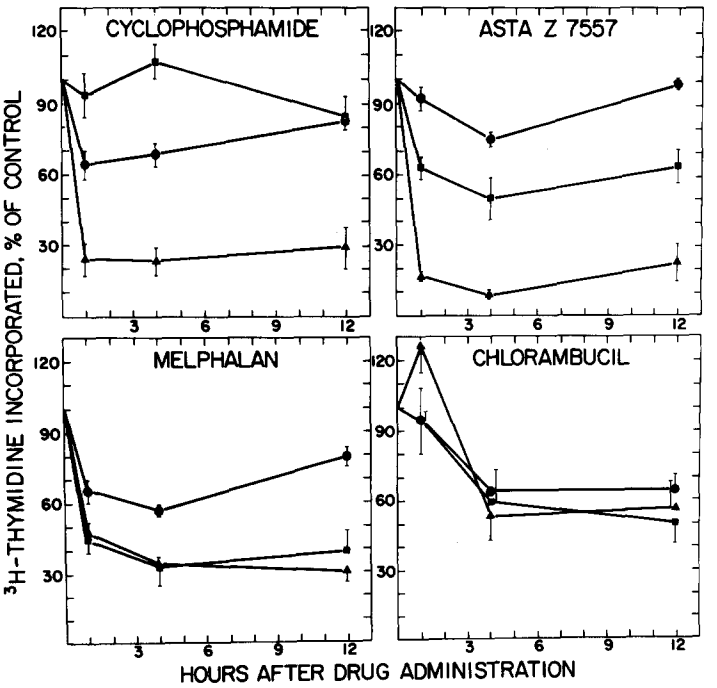

Fig. 1. Inhibition of mitogen-induced murine B- and T-lymphocyte proliferation by cyclophosphamide, ASTA Z 7557, melphalan and chlorambucil: Time courses. Male BALB/c mice were given vehicle (i.p. or i.v.), cyclophosphamide (50 mg/kg, i.p.), ASTA Z 7557 (100 mg/kg, i.v.), melphalan (6 mg/kg, i.p.) or chlorambucil (12 mg/kg, i.v.), and were killed at the times indicated. Spleens were immediately removed and processed, and proliferative responses to LPS (▲), PHA (■) and Con A (●) were determined in triplicate as described in Materials and Methods. Points: mean ± S.E. of three to five animals receiving the drug. Control (one or two vehicle-treated mice) responses were determined at each time point.

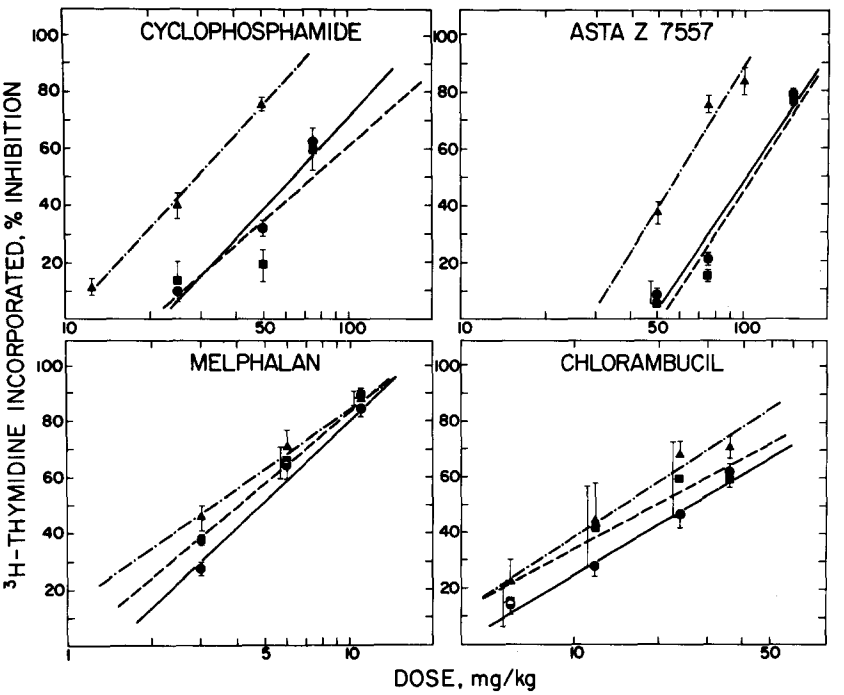

Fig. 2. Inhibition of mitogen-induced murine B- and T-lymphocyte proliferation by cyclophosphamide, ASTA Z 7557, melphalan and chlorambucil: Dose–response relationships. Male BALB/c mice were given vehicle (i.p. or i.v.), cyclophosphamide (i.p.), ASTA Z 7557 (i.v.), melphalan (i.p.) or chlorambucil (i.v.) and were killed 4 hr later. Spleens were then immediately removed and processed, and proliferative responses to LPS (▲), PHA (■) and Con A (●) were determined in triplicate as described in Materials and Methods. Mice receiving vehicle only (N = 3–4) were used to determined control responses. Unweighted log-linear regression analysis was used to obtain straight-line functions. Points: mean ± S.E. of four or five animals receiving the drug.

Table 1. Inhibition of mitogen-induced B- and T-lymphocyte proliferation by cyclophosphamide, ASTA-Z-7557, melphalan and chlorambucil: ID_{50} values*

Agent	ID_{50} (mg/kg)		
	LPS	PHA	Con A
Cyclophosphamide	30 (27–33)	76 (51–112)	63 (56–71)
ASTA Z 7557	58 (53–64)	108 (93–125)	103 (95–113)
Melphalan	3.3 (2.6–4.1)	4.1 (3.6–4.6)	4.9 (4.5–5.4)
Chlorambucil	15 (13–18)	19 (12–30)	26 (22–31)

* ID_{50} Values were obtained from the data presented in Fig. 2. Computer assisted, graded dose–response analysis according to the methods of Tallarida and Murray [13] was used to obtain these values. Parentheses, 95% confidence intervals.

shown in Fig. 3. As was the case *in vivo*, B-lymphocytes were approximately twice as sensitive as T-lymphocytes to the cytotoxic effects of ASTA Z 7557. IC_{50} Values and 95% confidence intervals were 7.6 (7.0–8.1), 16.3 (15.0–17.8) and 17.1 (15.8–18.5) μM when LPS-, PHA- and Con A-induced proliferation, respectively, was examined. LPS-induced B-lymphocytes and PHA- and Con A-induced T-lymphocytes were approximately equisensitive to the cytotoxic action of phosphoramide mustard. IC_{50} Values and 95% confidence intervals were 295 (275–317), 262 (244–280) and 312 (299–317) μM respectively.

DISCUSSION

Several laboratories have reported that B-lymphocytes are more sensitive to cyclophosphamide than are T-lymphocytes [8–11]. In the present investigation we sought to determine whether the differential sensitivity exhibited by B- and T-lymphocytes to cyclophosphamide extended to non-oxazaphosphorine nitrogen mustards. The results of these investigations indicate that B- and T-lymphocytes are differentially sensitive to oxazaphosphorine nitrogen mustards, but are essentially not, or minimally so, differentially sensitive to non-oxaza-

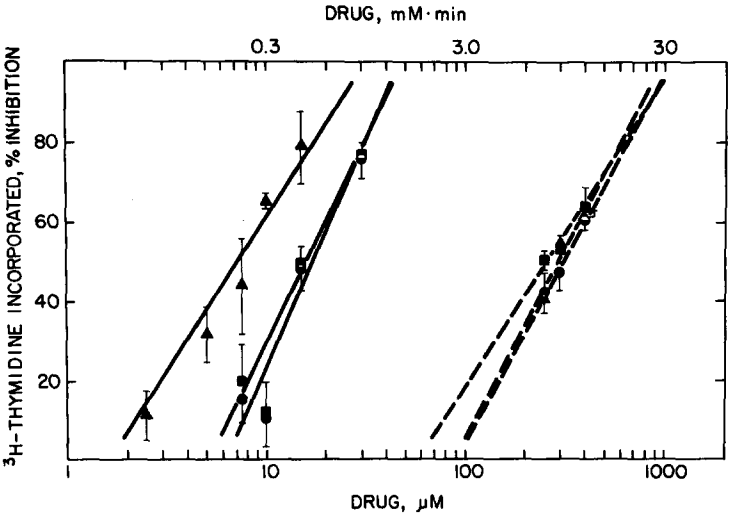

Fig. 3. Inhibition of mitogen-induced B- and T-lymphocyte proliferation by ASTA Z 7557 and phosphoramide mustard: Concentration–response relationships. Splenocytes obtained from ten male BALB/c mice were pooled and incubated with either ASTA Z 7557 (——), phosphoramide mustard (– – –) or vehicle for 30 min at 37°. Following incubation they were harvested and resuspended in drug-free medium. Proliferative responses to LPS (▲), PHA (■) and Con A (●) were then determined as described in Materials and Methods. Unweighted log-linear regression analysis was used to obtain straight-line functions. Points: mean ± S.D. of six replicate samples. Control values were the mean of six replicate samples.

phosphorine nitrogen mustards. Furthermore, they indicate that 4-hydroxycyclophosphamide is the principal metabolite mediating the selective immunotoxic effect of cyclophosphamide on B- and T-lymphocytes.

The phenotypic basis for the difference in sensitivity to oxazaphosphorines exhibited by B- and T-lymphocytes has not been identified. Our observation that a difference in sensitivity is obtained when oxazaphosphorine nitrogen mustards are used, but not when non-oxazaphosphorine nitrogen mustards are used, strongly suggests that the relevant phenotypic determinant relates to the distribution and/or metabolism of the oxazaphosphorines rather than to events common to both oxazaphosphorine and non-oxazaphosphorine nitrogen mustards such as DNA cross-linking or DNA repair. Other cell pairs that exhibit differential sensitivity only to the oxazaphosphorine nitrogen mustards have been identified [17–21]. Bioinactivation of 4-hydroxycyclophosphamide/aldophosphamide catalyzed by aldehyde dehydrogenases in insensitive cells appears to account for the differential sensitivity to the oxazaphosphorines exhibited by these cells [18, 20–24].

Identification of the phenotypic determinants that are responsible for mediating the specific differential sensitivity of B-lymphocytes and T-lymphocyte subpopulations to cyclophosphamide and other oxazaphosphorine nitrogen mustards may not only help to ascertain the basis for the greater cytotoxic selectivity, more favorable therapeutic index, and greater clinical utility of cyclophosphamide as an immunosupressive agent, but could also serve as the basis for the design of additional, more useful, immunosupressive agents.

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