Brief Definitive Report

DIFFERENTIAL EFFECTS OF CHLORPROMAZINE ON THE IN VITRO GENERATION AND EFFECTOR FUNCTION OF CYTOTOXIC LYMPHOCYTES*

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Allograft rejection represents a cytotoxic response mediated to a large degree by thymus-derived T lymphocytes (1). The study of such cell-mediated cytotoxic phemonena has been greatly facilitated by the discovery first noted by Häyry and Defendi (2) and Wunderlich and Canty (3), that a natural consequence of allogeneic stimulation in an undirectional mixed lymphocyte culture (MLC) was the appearance of cytotoxic lymphocytes specific for antigens present on the stimulator cell. Subsequent studies have shown that such in vitro generation of cytotoxic lymphocytes was dependent on the proliferative response in an MLC (4), was genetically determined (5), and possibly required the interaction of several subpopulations of T cells (6).

We now report that the surface active agent chlorpromazine: (*a*) inhibits allogeneic stimulation of the proliferative response in an MLC; (*b*) inhibits the MLC generation of cytotoxic lymphocytes, (*c*) has no effect on the recognition, binding, or lysis of target cells by already sensitized lymphocytes; and (*d*) blocks a postproliferative membrane-mediated event, independent of proliferation, and necessary for the MLC generation of cytotoxic lymphocytes.

Materials and Methods

Animals. 3- to 6-mo-old female BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Cell Preparations. Spleens were removed with aseptic technique and minced through a no. 40 stainless steel mesh, washed twice with cold minimal essential medium. Red blood cells were removed by a 3 min incubation at 25°C in 0.01 M Tris-0.83% NH_4Cl.

In Vitro Sensitization. 4×10^6 BALB/c responder cells were added with 0.5×10^6 C57BL/6 stimulator cells (previously irradiated with 2,000 rads) (C57X) in 2 ml to 16mM plastic tissue culture plates (Linbro Chemical Company, New Haven, Conn.). Spleen cells were cultured in a modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS) (Microbiological Associates, Bethesda, Md.), 1% penicillin and streptomycin, 2 mM glutamine, and 2-mercaptoethanol (5×10^{-5} M).

Cytotoxicity Assay. Direct cell-mediated cytotoxicity was assayed by a modification of the methods described by Hodes et al. (7). Cells from the above cultures were collected and washed twice in the Eagle's medium. They were then incubated with ^{51}Cr-labeled EL4 (*H-2*^{b}) cells (maintained by serial passage in C57BL/6 mice) in ratios of 8:1, 4:1, and 2:1 (lymphocytes:EL4

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cells). These mixtures were incubated for 4 h in modified Eagle's medium at 37°C. The mixture was centrifuged at 2,500 rpm; the supernate was removed and ^{51}Cr activity counted in a Nuclear Chicago Automatic gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Maximum obtainable ^{51}Cr release was determined when distilled water was added to ^{51}Cr-labeled EL4 cells. Spontaneous ^{51}Cr release was determined by incubating ^{51}Cr-labeled EL4 cells alone at 37°C for 4 h.

Percent lysis was calculated as follows:

$$\text{percent Lysis} = \frac{\text{experimental } ^{51}\text{Cr cpm} - \text{spontaneous } ^{51}\text{Cr5 cpm}}{\text{total } ^{51}\text{Cr cpm} - \text{spontaneous } ^{51}\text{Cr cpm}} \times 100.$$

Proliferation Assay. Proliferative assays were performed in 0.2 ml of the above described supplemented media in 6-mm plastic culture plates (Linbro Chemical Co.). 8×10^5 responder BALB/c cells were mixed with 1×10^5 stimulator C57BL/6 (previously irradiated with 2,000 rads). 18 h before harvesting, each culture was pulsed with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) sp act 6.7 Ci/mmol (new England Nuclear, Boston, Mass.).

Results and Discussion

Effect of Chlorpromazine (CPZ) on the Proliferative Response in the MLC. If CPZ was added to responder cells in culture at the same time as the stimulator cells, subsequent incorporation of increased tritiated thymidine uptake was diminished or totally inhibited in a dose-dependent manner. At a CPZ concentration of 5×10^{-5} M total inhibition of stimulated ^3H uptake was observed, whereas 5×10^{-6} M CPZ produced only 50% inhibition. If CPZ was added after 24, 48, or 72 h of exposure of the responder and stimulator cells, [^{3}H]TdR incorporation proceeded in a pattern similar to the control untreated cultures, despite the presence of the drug for the majority of the culture period. This was particularly striking for 5×10^{-6} M CPZ (Fig. 1).

Effect of CPZ Upon the MLC Generation of Cytotoxic Cells. If CPZ (5×10^{-6} M) was added to responder cells in an MLC at the same time as the stimulator cells, no demonstrable cytotoxic activity was generated, in addition to the above described partial inhibition of proliferative responses. If, however, the same concentration of CPZ (5×10^{-6} M) was added to cultures containing target cells and already sensitized MLC-generated effector cells (efferent phase), no inhibition of killing was observed (Fig. 2).

The Effect of Delayed Addition of CPZ Upon the Generation of Cytotoxic Cells. The effect of adding CPZ (5×10^{-6} M) to MLC's at successive intervals after exposure of stimulator and responder cells in culture on the subsequent development of cytotoxic activity is shown in Fig. 3. If CPZ was added at 0 time, 24 h, or 48 h after culture initiation, no significant cytotoxic activity was generated during the 5-day culture period. If, however, CPZ was added at 72 or 96 h (after culture initiation), full expression of cytotoxic activity was generated. Again, addition of CPZ (5×10^{-6} M) had no effect on the ability of already sensitized effector cells to lyse target cells.

The Relationship between the CPZ Inhibition of Proliferation and Generation of Cytotoxic Lymphocytes. The differential effect of CPZ-induced inhibition of MLC-stimulated [^{3}H]TdR uptake and generation of cytotoxic lymphocytes is shown in Fig. 4. Using parallel experiments, measuring thymidine incorporation and generation of cytotoxic lymphocytes, it can be seen that if CPZ was added to cultures at 48 h considerable incorporation of [^{3}H]TdR was observed but

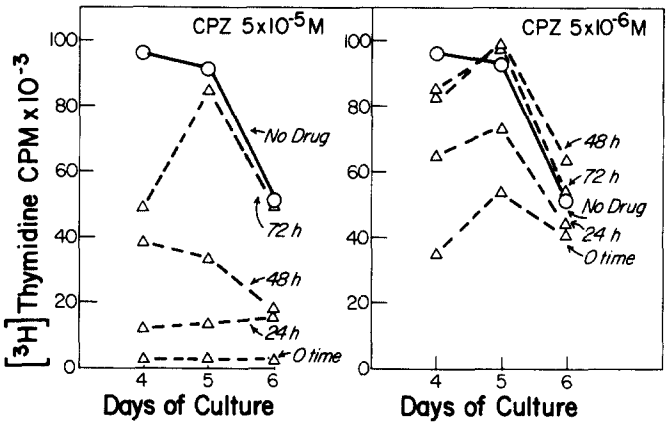

FIG. 1. The effect of the addition of two concentrations of CPZ (5×10^{-5} M and 5×10^{-6} M), at various intervals after MLC initiation, on the subsequent [^{3}H]TdR uptake. MLC's were performed as described. The hours shown represent the hour of culture that CPZ was added. That the drug was not toxic is demonstrated, for a full proliferative response is shown, despite the presence of CPZ from 48 to 96 h before harvest (or 24–48 h after culture). A dose-dependent inhibition is also demonstrated.

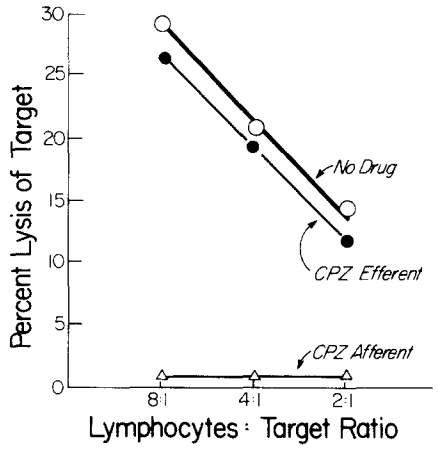

FIG. 2. Effect of CPZ (5×10^{-6} M) on the MLC sensitization of BLBL/c spleen cells and effector function of already sensitized BALB/c cells. Sensitization and cytotoxic assays were performed as described in the Materials and Methods. CPZ was present throughout the entire culture period of the afferent (sensitization culture) and efferent (cytotoxic assay) phases.

virtually no cytotoxic lymphocytes were generated. If, however, CPZ was added after 72 or 96 h of culture a full cytotoxic response in addition to full proliferative activity was observed.

CPZ has been shown to bind to and inhibit a variety of cell surface phenomena (8). It also binds to and inhibits responses of lymphocytes to various mitogens (reference 9 and footnote 1). The data presented here demonstrate differential

[1] Ferguson, R. M., J. R. Schmidtke, and R. L. Simmons. 1976. Inhibition of mitogen-induced lymphocyte transformation by local anesthetics. *J. Immunol.* In press.

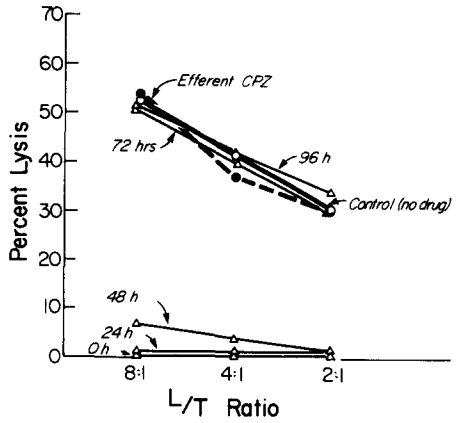

FIG. 3. Effect of adding CPZ (5×10^{-6} M) to the afferent (MLC sensitization) culture at various intervals after culture initiation on the subsequent generation of cytotoxic cells assayed after the standard 5-day culture. (○—○), MLC culture with no CPZ. (●---●), CPZ (5×10^{-6} M) added to the efferent (cytotoxicity) assay and not present in the afferent (MLC) assay. (△—△), CPZ (5×10^{-6} M) added to the afferent (MLC) assay at times indicated after culture initiation.

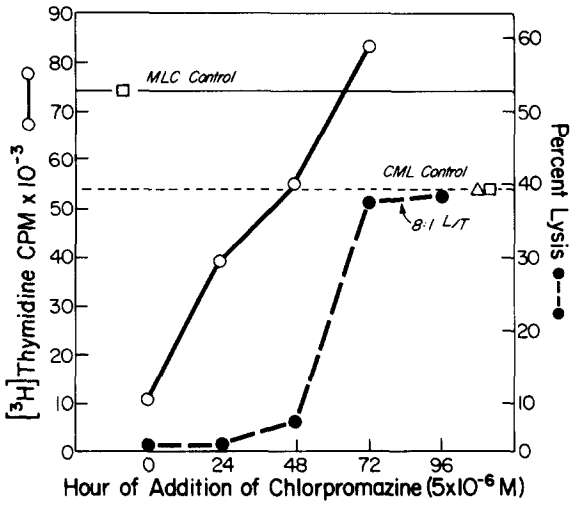

FIG. 4. Time relationship of inhibition by CPZ (5×10^{-6} M) of MLC proliferation and generation of cytotoxic cells. Proliferation and cytotoxicity assayed as described. Counts per minute of MLC are those from day 5. Cells were obtained from day 5 MLC sensitization cultures and assayed for cytotoxic activity.

effects of this surface active drug on two phases of in vitro cell-mediated immune responses. CPZ inhibited the proliferative response of an MLC if it was added within the first 24 h of culture. CPZ, when added at 48 h, also inhibited the generation of cytotoxic lymphocytes by a mechanism independent of proliferation in the MLC. The drug, however, had no effect on the recognition, binding, and subsequent lysis of specific target cells by already sensitized effector cells. The mechanism of the CPZ-induced inhibition of allogeneic sensitization, proliferation, and generation of effector cells is not clear. Also, it is not clear why CPZ

did not interfere with binding and lysis of target cells by sensitized cells. Extensive data from our laboratory have shown that CPZ was not cytotoxic to murine spleen cells and that its effects on mitogen-induced [^{3}H]TdR uptake was completely reversible (reference 9 and footnote 1).

Proliferation in a unidirectional MLC is thought to be less dependent upon serologically defined (SD) surface *H-2K* and *H-2D* genetic desparity than on nonserologically definable loci (LD) which are themselves defined by MLC reactivity (10). Lymphocytes from congenic strains of mice differing only in *SD* loci stimulate a weak mixed lymphocyte reaction (MLR) and will generate very minimal cytotoxic activity in a MLC, whereas, murine lymphocytes from certain *H-2* (and therefore SD), identical strains stimulated a relatively strong proliferative response in an MLC but generated few cytotoxic lymphocytes specific for the stimulating cell population (5). The cytotoxic specificity of MLC-generated effector cells is generally considered to be located within the *H-2* surface antigenic sites (11). However, the nature of the interaction between the *SD* and *LD* loci and proliferation (all involved in the generation of cytotoxic lymphocytes) as well as the role of the LD and SD surface determinants in recognition and killing of effector cells of *H-2* bearing specific target cells is poorly understood at the present time. Cantor and Jandinski (4) recently reported that cell division during a critical early period of sensitization and a subsequent division-independent period of differentiation was necessary for full cytotoxic expression. In addition, it would appear that both LD and SD antigenic disparity must be present to generate a vigorous primary cytotoxic response (5, 9). It has also been demonstrated that synergism can exist between different subpopulations of T lymphocytes as demonstrated in in vitro primary cytotoxic responses showing that two T cells differing by the amounts of theta (Thy-1.2) antigen on their surface have been separated from spleen and found to synergize in cytotoxic responses generated in vitro (11). More recently Cantor et al. (12) and Cantor and Boyse (13) have demonstrated, using the Ly antigen marker on T lymphocytes, that there are three subpopulations of T lymphocytes as demonstrated by differently Ly-marked populations, Ly-1,2,3-; Ly-1-; and Ly-2,3-bearing cells. Ly-1-positive cells have helper function in antihapten antibody responses and in proliferation in MLC's in response to LD differences. Cytotoxic lymphocytes, however, are derived from Ly-2,3-positive cells and have no demonstrable helper function and show little response to LD disparity. In addition, Ly-1-positive cells can synergize with Ly-2,3-positive cells in the in vitro MLR generation of primary cytotoxic responses directed toward the SD antigens.

One possible explanation of CPZ-induced inhibition of these above events could involve an inhibition of cell interactions. In the case of cytotoxic lymphocyte generation it is possible that CPZ prevents a necessary interaction of proliferating the precytotoxic cells. If these are one cell type, and the proliferating cells eventually become cytotoxic cells, CPZ could perhaps inhibit the differentiation of such proliferating cells. If, however, the proliferating cell and the cytotoxic cell come from two different T-cell subpopulations, then perhaps the interaction of the Ly-1 helper cells (LD specific) (13, 14) with the precytoxic Ly-2,3 cells (SD specific) (13, 14) at a critical period between 48 and 72 h of culture in a unidirectional MLC is sensitive to CPZ. A third possibility is that

the secretion of a soluble mediator active in producing differentiation of precytotoxic cells and secreted by proliferating cells could be inhibited. Although the exact mechanism of action is not clear it seems reasonable that some surface-mediated process is involved. The further use of a pharmacologic approach to lymphocyte activation, differentiation, and effector cell killing by drugs such as CPZ could prove useful in attempting to define critical membrane-mediated phenomena involved in such processes.

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