Propanil Exposure Induces Delayed but Sustained Abrogation of Cell-Mediated Immunity through Direct Interference with Cytotoxic T-Lymphocyte Effectors

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The postemergent herbicide propanil (PRN; also known as 3,4-dichloropropionanilide) is used on rice and wheat crops and has well-known immunotoxic effects on various compartments of the immune system, including T-helper lymphocytes, B lymphocytes, and macrophages. It is unclear, however, whether PRN also adversely affects cytotoxic T lymphocytes (CTLs), the primary (1st) effectors of cell-mediated immunity. In this study we examined both the direct and indirect effects of PRN exposure on CTL activation and effector cell function to gauge its likely impact on cell-mediated immunity. Initial experiments addressed whether PRN alters the class I major histocompatibility complex (MHC) pathway for antigen processing and presentation by antigen-presenting cells (APCs), thereby indirectly affecting effector function. These experiments demonstrated that PRN does not impair the activation of CTLs by PRN-treated APCs. Subsequent experiments addressed whether PRN treatment of CTLs directly inhibits their activation and revealed that 1st allogeneic CTLs exposed to PRN are unimpaired in their proliferative response and only marginally inhibited in their lytic activity. Surprisingly, secondary stimulation of these allogeneic CTL effectors, however, even in the absence of further PRN exposure, resulted in complete abrogation of CTL lytic function and a delayed but significant long-term effect on CTL responsiveness. These findings may have important implications for the diagnosis and clinical management of anomalies of cell-mediated immunity resulting from environmental exposure to various herbicides and other pesticides.

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Numerous studies concerning the health-related effects of environmental toxicants demonstrate that the immune system, in addition to other organ systems including the reproductive, nervous, pulmonary, and circulatory systems, is often compromised (Carp et al. 2000; Costa 1997). Our understanding of such adverse immunologic effects, however, is largely limited to the immediate and early consequences after exposure to such agents. The principal contribution of this present article to the field of immunotoxicology research is its demonstration that the potential long-term impact of propanil (PRN) exposure on cell-mediated immunity is far more severe than its short-term consequences. This delayed appearance of irreversible PRN-induced immunotoxic effects may be important for diagnostic and therapeutic measures in assessing exposure to environmental toxicants in general.

PRN is a postemergent herbicide used extensively around the world in the cultivation of rice and wheat crops. Its particular effectiveness is due to the high level of acylamidase expression in a rice plant that allows it to detoxify PRN, whereas common grass-type weeds lack this enzyme and are killed by this herbicide (Matsunaka 1968). PRN is routinely applied several times during a growing season without detrimental effects to the plant (Casida and Lykken 1969; Smith 1961), with 3–6 lb/acre applied annually in the United States (Costa 1997; Matsunaka 1968). Thus, a high environmental exposure of humans to PRN normally occurs as an occupational risk.

Earlier reports by Barnett and co-workers (Barnett et al. 1992; Barnett and Gandy 1989; Frost et al. 2001; Theus et al. 1993; Xie et al. 1997; Zhao et al. 1995, 1998) indicated that PRN exposure results in adverse effects on most compartments of the immune system, including macrophages, B lymphocytes, and T-helper lymphocytes. Curiously, however, there appeared to be little, if any, effect on cellular immunity mediated by cytotoxic T-lymphocyte (CTL) effectors (Barnett et al. 1992; Barnett and Gandy 1989).

Given that the responsiveness of the other immune compartments examined is inhibited by PRN exposure, we hypothesized that acute PRN exposure might yet impair CTL function, albeit in a manner that is initially difficult to detect under the in vitro conditions used. To test this hypothesis we considered that the adverse immunotoxic effects of PRN exposure on cell-mediated immunity might be observed in one or more of three parameters: a) presentation of peptide antigen to CTLs by antigen-presenting cells (APCs), b) proliferation and differentiation of CTLs, and/or c) functional lytic response of activated CTL effectors.

The immune activation and functional responsiveness of CTLs can be examined and assessed independently in vitro. CTL activation is based on the capacity of APCs to efficiently process and present peptide antigens to CTLs and thus indirectly affects CTL responsiveness. Conversely, the functional lytic response of CTLs emerges as a result of the differentiation of naive CD8+ T cells into effector CTLs capable of responding through lysis of the target cell, thereby serving as a direct measure of CTL activation.

In this present article, we demonstrate three important consequences of PRN exposure on the in vitro parameters of CTL activation and their functional activity as effectors of cell-mediated immunity: a) antigen presentation to CTLs is not impaired, b) the functional lytic activity of primary (1st) CTLs is only marginally impaired, and c) upon restimulation of 1st CTLs in the absence of PRN, the secondary (2nd) CTL response is completely abrogated. On the basis of these observations, we conclude that the immunotoxic effects of PRN exposure on CTLs are delayed in their appearance and directly impair the functional activity of these effectors of cell-mediated immunity. These results may have serious and important direct implications for both diagnosis and clinical management of the acute and chronic effects of PRN exposure. Furthermore, these findings warrant examining similar acute versus delayed exposure effects with respect to the immunotoxic potential of other environmental toxicants.

Materials and Methods

Animals. In this study we used C57BL/6 (B6; H-2b) and BALB/c (H-2b) female mice 10–12 weeks of age from Charles River Breeding Laboratories, Inc. (Wilmington, MA) or from our own breeding colony at the West Virginia University Health Sciences Vivarium. All animals used in this study received humane care in compliance with the “Guidelines for the Care and Use of Experimental Animals” of the Office of Research and Graduate Studies. Address correspondence to J.M. Sheil, Department of Microbiology, Immunology, and Cell Biology, P.O. Box 9177, 2095 Health Sciences North, West Virginia University School of Medicine, Morgantown, WV 26506-9177. Telephone: (304) 293-7416. Fax: (304) 293-7823. E-mail: jsheil@hsc.wvu.edu

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were treated humanely and with regard for alleviation of suffering.

Cell lines. Two tumor cell lines, designated P815 (H-2d) and EL4 (H-2b), were used as targets for alloreactive cytotoxic T cells. The EL4 cell line expresses class I H-2b molecules and is derived from a B6 lymphoma originally induced in a C57BL/6N mouse by 9,10-dimethyl-1,2-benzanthracene (Gorer 1950). P815 is a cell line derived from a mastocytoma in DBA/2 (H-2b) mice, and it expresses class I H-2b molecules (Plaut et al. 1973; Ralph and Nakoinz 1974). Both the EL4 and P815 cell lines have been used extensively by us and others as suitable targets for lysis in cytotoxic T-cell assays. N1 is derived from EL4 cells transfected with the vesicular stomatitis virus nucleoprotein (VSB-N) gene (Puddington et al. 1986).

Monoclonal antibodies and fluorescence activated cell sorting analysis. We used the following H-2K-specific monoclonal antibodies (mAb): 5F1 (Sherman and Randolph 1981), Y-3 (Jones and Janeway 1981), EH144 (Bluestone et al. 1985; Geier et al. 1986), Y-25 (Jones and Janeway 1981), and 28-1-3-3 (Ozato and Sachs 1981). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (heavy- and light-chain-specific) was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Citic acid treatment of APCs. The acid treatment protocol used in these studies to strip the APC cell surfaces of class I peptide/major histocompatibility complex (pMHC) complexes is essentially the same as that described by Sugawara et al. (1987), as modified by Storkus et al. (1993). Briefly, APCs are a) collected and pelleted by centrifugation; b) resuspended in 0.5 mL citrate-phosphate buffer, pH 3.0 (citrate-phosphate buffer consists of 1:1 mixture of 0.263 M citric acid, pH 1.8, and 0.123 M Na₂HPO₄); c) incubated in citrate-phosphate buffer for 1 min at room temperature; d) resuspended in 10 mL RPMI-1640 media. After incubation for 18–24 hr at 37°C in a 7% CO₂ humidified incubator, the amount of prolif-

Alloreactive CTLs were induced by 1° stimulation of B6 spleen cells with irradiated (2,000 rads) spleen cells from BALB/c mice. Briefly, spleens were removed and processed into single-cell suspension preparations; BALB/c spleen cell suspensions were irradiated in a Gammacell 1000 cesium-137 irradiator (Atomic Energy of Canada Ltd., Kanata, Ontario, Canada) to deliver 2,000 rads. For 1° alloreactive stimulation, 25 × 10⁴ B6 spleen cells per flask were added to upright T-25 flasks with 25 × 10⁴ BALB/c irradiated spleen cells in 10 mL RP-10 media. Alloreactive cul-
tures were placed in a 37°C humidified incubator at 7% CO₂ for 7 days.

Secondary alloreactive cultures were prepared similarly in RP-10 media except that 2.5 × 10⁴ 1° effectors per flask were added together with 25 × 10⁴ irradiated BALB/c spleen cells to upright T-25 flasks. Cultures were incubated for 7 days in the same manner as the 1° alloreactive cultures. Subsequent cul-
tures beyond the 2° alloreactive effectors were maintained in 24-well dishes (Corning-Costar; Corning Life Sciences, Corning NY), by the addition of 1 × 10⁵ effectors plus 1 × 10⁴ irradiated BALB/c spleen cells per well in 2 mL RP-10 media supplemented with 5% rat concanavalin A supernatant as a source of interleukin-2.

Mixed lymphocyte reaction assay. To measure the extent of alloreactive T-cell stimulation in mixed lymphocyte cultures (MLCs) and the effect of adding PRN on the induc-
tion of alloreactive CTL effectors, we used the mixed lymphocyte reaction (MLR) assay, as previously described (Sheil et al. 1987). T-cell proliferation was determined in a one-way MLR assay on day 4 of culture by the incor-
poration of tritiated thymidine (³H-TdR) by proliferating T cells. Briefly, after 72 hr of cul-
ture, 5 × 10⁴ viable 1° MLC cells in 100 µL plus 1 µCi ³H-TdR in 100 µL RP-10 were added per well to four wells per sample in a 96-well plate (Corning-Costar; Corning Life Sciences). After incubation for 18–24 hr at 37°C in a 7% CO₂ humidified incubator, the cells were harvested, and the amount of prolif-
eration was determined by measuring ³H-TdR uptake, as reflected by the total radioactive counts per sample in liquid scintillation fluid.

³Cr-release assay. We determined the lytic activity of peptide-specific and alloreactive effector CTLs using a standard 4-hr in vitro ³Cr-release assay, as previously described (Sheil et al. 1987). Briefly, tumor cells to be used as targets were labeled with radioactive sodium chromate (Na³⁵Cr) and mixed with titrated doses of peptide-specific or alloreactive CTLs in 200 µL RP-10/well in 96-well round-bottom microtiter plates (Costar-Costar; Corning Life Sciences). The plates were incubated at 37°C in 7% CO₂ for 4 hr and centrifuged, and 100 µL supernatant was collected from each well. The amount of specific lysis was determined according to the following formula: % specific lysis = (experimental release – spontaneous release) + (maximum release – spontaneous release) × 100.

PRN exposure. PRN (3,4-dichloropropio-

PRN-exposed APCs are recognized effi-
ciently by VSV-N peptide-specific CTLs. We examined the functional capacity of PRN-
exposed APCs to determine whether PRN
exposure of APCs in vitro adversely affects their ability to process and/or present antigen in the class I MHC pathway. The VSV-N transfectant model system (Puddington et al. 1986) was used, as described previously (Sheil et al. 1987), to determine whether exposure of VSV-infected cells to PRN interferes with their ability to effectively present viral peptide antigens to CTLs. In these experiments, CTL clone 33, specific for VSV-N p52-59 (Sheil et al. 1987), was tested against the target VSV-N transfected EL4 (H-2b) tumor cell line, designated N1. Initially, N1 cells were exposed to PRN for 18 hr before their use as CTL targets; however, because of undesirable levels of toxicity to the N1 cells (i.e., up to 30%), the period of incubation with PRN was decreased to 2 hr. After incubation in the presence of PRN for either 2 or 18 hr, N1 cells were tested as targets for lysis by clone 33. The results depicted in Figure 1 demonstrate that incubation of N1 cells with PRN does not adversely affect their capacity to serve as targets for lysis by VSV-N peptide–specific CTL effectors.

Another possible effect of PRN is its interference with the ability of N1 cells to serve as stimulators for the induction of VSV-N peptide–specific CTL effectors, even though they are undiminished in their capacity to serve as targets for CTL lysis. To address this possibility, we used N1 cells as APC stimulators for the VSV-N p52-59 peptide–specific, H-2Kb-restricted CTL clone 33. After a 2-hr exposure to 200 μM PRN, N1 cells were added to culture flasks as stimulators for clone 33 CTLs. The 4-hr 51Cr-release assay results depicted in Figure 2 demonstrate that PRN-treated N1 cells are effective stimulators, in that they are undiminished, compared with control EtOH-treated N1 cells, in their capacity to stimulate lytic activity in clone 33 CTLs. This conclusion is reinforced by the observation that cell viability (as determined by trypan blue dye exclusion) and proliferative capacity (as determined by 3H-TdR uptake in the MLR assay) of clone 33 CTLs are not significantly different after culture with either control EtOH-treated or PRN-exposed N1 cells (data not shown).

Primary alloreactive CTLs show limited inhibition after PRN exposure. Given that we observed no overt adverse effects of PRN exposure on APC function, we directed our attention to whether PRN exposure interferes directly with CTL function itself. To address this point, an alloreactive C57BL/6 (B6) anti-BALB/c mouse model system, as described in “Materials and Methods,” was used to obtain CTL effectors. The effect of PRN exposure on alloreactive CTL activation was examined in two ways. First, we measured the proliferative capacity of BALB/c-stimulated B6 CTLs in a standard 3H-TdR uptake assay. Second, to detect any functional changes in CTL effector activity, we measured allospecific CTL lysis of P815 targets in an in vitro 4-hr 51Cr-release assay (Sheil et al. 1987).

The in vitro PRN-exposed 1° B6 anti-BALB/c CTLs are unchanged in their proliferative capacity compared with EtOH-treated control CTLs (Figure 4A), thereby indicating that there is no effect on their ability to proliferate in response to antigen stimulation after exposure to a range (16, 33, or 66 μM) of PRN concentrations. Furthermore, their functional lytic reactivity is only marginally inhibited after PRN exposure, and only at the highest (66 μM) concentration used in these experiments, as shown by the CTL lytic response in a 4 hr in vitro 51Cr-release assay (Figure 4B).

Proliferation and reactivity of secondary CTLs are markedly impaired after PRN exposure. We next addressed whether the subsequent in vitro exposure of these alloreactive CTLs to PRN, during their 2° stimulation, might reveal an increased adverse effect on CTL proliferation and/or CTL lytic activity. To
examine this possibility, 1° allogeneic CTLs were harvested on day 7 of culture, washed, and restimulated with the addition of fresh PRN as described in “Materials and Methods.”

In the same manner as with allogeneic CTLs from 1° MLCs, we tested 2° allogeneic CTLs for their proliferative capacity (Figure 5A) and their lytic responsiveness (Figure 5B). Secondary B6 anti-BALB/c MLCs were exposed to 66 or 165 µM PRN during *in vitro* 2° stimulation. Because we saw no effects at the lower PRN concentrations of 16 and 33 µM in 1° MLCs, they were excluded from further analysis with 2° CTLs. Instead, 2° MLCs were set up using the effective 66 µM PRN concentration, as well as a higher concentration of 165 µM.

We tested secondary CTLs on day 4 for proliferation (Figure 5A) and on day 5 for lytic activity against syngeneic P815 targets (Figure 5B). We included an important control group in which PRN-exposed 1° MLC effectors were washed and restimulated in 2° MLCs without additional exposure to PRN. As shown in Figure 5A, the proliferative capacity of the 1° EtOH-treated/2° 66 µM PRN group (second bar) is fully intact, whereas the 1° EtOH-treated/165-µM PRN group (third bar) shows no proliferative capacity above background (i.e., media control). Interestingly, for the lytic response of the 1° EtOH-treated/2° 66-µM PRN group (Figure 5B, left), the level of inhibition also is approximately double that seen with CTLs exposed to 66 µM PRN in 1° MLC (Figure 4B). On the basis of this observation, we are presently examining whether activated CTLs in 2° MLC might be more susceptible to PRN-mediated inhibition than are naive CD8+ T cells.

It is unlikely that the unresponsiveness observed in these groups is due to a generalized PRN-induced toxicity to the exposed CTLs because the viable cell yield of the 1° EtOH-treated/165-µM PRN group is approximately 70% of the EtOH-treated group, and that of the 1° EtOH-treated/2° 66-µM PRN group is approximately 90% that of the EtOH-treated group. Furthermore, even the marginal decrease in cell viability observed in these groups has been taken into account in determining the total number of viable cells used in both the MLR and 51Cr-release assays. Cell populations used in both assays were equalized based on these total viable cell determinations; thus, the number of cells added is the same for each group. It is possible, however, that these cell viability determinations do not take into account damaged cells whose cell membranes are still intact because these cells would exclude the trypan blue dye until such point as membrane damage has occurred.

Another important, although initially unanticipated, finding concerns the control allogeneic CTLs in the 1° 66-µM PRN/2° EtOH-treated group. These CTLs were initially exposed to 66 µM PRN during 1° stimulation, followed by restimulation in 2° MLC in the absence of PRN. The lytic activity of this group is almost completely ablated (Figure 5B, middle), even though the 1° response is only marginally inhibited compared with EtOH-treated 1° allogeneic CTLs (Figure 4B). Thus, the CTL lytic response of this group is nearly 20-fold lower in than that of 66 µM PRN-treated 1° CTLs.

To determine whether this unanticipated decline in CTL reactivity is irreversible, 3° MLCs were established without the addition of PRN as was done for the 2° MLCs. So, in this case we have allogeneic CTLs that have been activated multiple times, but they were exposed to PRN only during their initial activation. Among the 3° MLC-derived CTL effectors, both proliferation and lytic activity remained severely diminished (data not shown), as seen with the 2° CTLs. Thus, the profound PRN-induced defect incurred during their 1° MLC stimulation appears to render these CTLs irreversibly impaired.

**Discussion**

Most herbicides and other pesticides exert a diverse array of immunotoxic effects on exposed individuals, including compromised humoral and cellular immunity (Banerjee et al. 1996; Rodgers 1995; Vial et al. 1996; Voccia et al. 1999). Earlier studies on the immunotoxic effects of PRN exposure by Barnett and co-workers (Barnett et al. 1992; Barnett and Gandy 1989) indicate that, although other important immune parameters are adversely diminished, the immunotoxic effects of PRN...
do not include impairment of cell-mediated immunity. This apparent anomaly in the immunotoxic impact of PRN exposure on different immune compartments prompted us to consider whether the effects of PRN on cell-mediated immunity might be more subtle or less easily detectable than effects on other immune compartments.

In the present study, we addressed the immunotoxic potential of the herbicide PRN on the effector cells of cell-mediated immunity, CTLs. A rigorous in vitro analysis of CTL activation and function was applied to determine whether and how PRN might induce immunotoxic effects in this regard. We approached this problem with the understanding that impaired cell-mediated immunity can result from the inhibition in antigen presentation to CD8+ T cells and/or, more directly, from a diminished functional CTL response.

Antigen processing and presentation defects have been implicated as the basis for impaired cell-mediated immunity induced by viruses (Früh et al. 1997; Hewitt and Dungan 2004; Mylin et al. 1995) and by antioxidants (Gong and Chen 2003; Pernat-Seauve et al. 2003), as well as in tumor development (Bennink et al. 1993; Cohen et al. 2003; Restifo et al. 1993; Seliger et al. 1998) and aging (Plowden et al. 1993; Cohen et al. 2003; Restifo et al. 1993; Seliger et al. 1998) and by antioxidants (Gong and Chen 2003; Pernat-Seauve et al. 2003), as well as in tumor development (Bennink et al. 1993; Cohen et al. 2003; Restifo et al. 1993; Seliger et al. 1998) and aging (Plowden et al. 1993; Cohen et al. 2003; Restifo et al. 1993; Seliger et al. 1998). The indirect consequences of these agents on antigen presentation can adversely affect the proliferation, differentiation, and effector functions of T lymphocytes—including cell signaling mechanisms, cytokine secretion, developmental maturation, and target cell lysis by CD8+ CTLs.

Thus it was important to examine the indirect immunotoxic effects of PRN exposure on the antigen processing and presentation component of cell-mediated immunity. In addition, the most common direct measures of CTL activation are proliferation and lytic activity. The experiments conducted in this study incorporate both indirect and direct approaches to determining the immunotoxic effects of PRN exposure on antigen presentation and CTL activation.

The most important findings of this study are that a) exposure to PRN during 1st CTL activation results in a dramatic delayed abrogation of CTL lysis that is irreversible, and b) the immunotoxic effects of PRN exposure under these conditions are limited to the functional activity of CTLs and do not affect antigen processing and presentation to CTLs. This study is unique in that it demonstrates such a striking difference between the short-term and delayed appearance of the immunotoxic effects of this herbicide. The issue of potentially delayed immunotoxic effects of pesticides has not been a focus of most studies, although some changes have been reported after in utero exposure that manifested after development (Colosio et al. 1999; Vial et al. 1996). This study, however, relates directly to the impaired activation of mature effectors of cell-mediated immunity. It is also important that these effects impair the proliferation and lytic activity of CTLs without interfering with the presentation of antigen by APCs.

In the initial approach to address whether PRN exposure inhibits cell-mediated immunity, we examined its possible impact on antigen processing and presentation in the class I MHC antigen presentation pathway, and indirectly on CTL induction and responsiveness. This approach used VSV-N gene-transfected N1 cells treated with PRN as targets for CTL-mediated lysis by CTL clone 33 (Sheil et al. 1987), which is H-2Kb restricted and specific for the VSV-N p52-59 peptide. Results depicted in Figures 1 and 2 reveal that exposure of APCs to PRN does not interfere with their ability to target CTL-mediated lysis in an antigen-specific manner. Nevertheless, it is possible that PRN could adversely affect the ability of APCs to effectively stimulate CTLs in culture. The results depicted in Figure 2 demonstrate that the responses between clone 33 CTLs stimulated with EtOH-treated (Figure 2A) or PRN-exposed (Figure 2B) N1 cells are similar in their responsiveness to N1 targets, indicating that PRN exposure also does not interfere with antigen presentation in a stimulatory capacity. Thus, the antigen presentation characteristics of N1 cells both as stimulators and as targets for clone 33 CTLs are unaltered by PRN exposure.

The absence thus far of any adverse effects of PRN exposure on antigen presentation, however, could be misleading because of the large number of potential pMHC complexes on N1 cells that can be engaged by the clone 33 T-cell receptors (TCRs). Previous studies have shown that minimally approximately 50–200 pMHC complexes need to be engaged for a CTL effector to lyse its target (Christinck et al. 1991; Sykulev et al. 1994); it is likely that many more pMHC complexes are formed and available for engagement by the clone 33 TCR on the N1 cells. Thus, if PRN exposure only partially interferes with antigen processing and presentation, its adverse effect may be masked under these in vitro assay conditions. To circumvent this problem, we replaced N1 cells as targets in the CTL lysis assay with untransfected EL4 cells plus titrated amounts of the target peptide VSV-N p52-59 (Figure 3). With the addition of lower peptide concentrations during this titration assay, fewer pMHC complexes will be formed. If there is a defect in antigen presentation, it should become apparent at the lower peptide concentrations.

As shown in Figure 3, we observed significant lysis even when a peptide concentration as low as 7.8 pM is added to EL4 targets, yet there is no significant difference in the level of activity in PRN-exposed groups compared with the EtOH-treated control group. With the higher concentrations of added peptide (up to 32 μM), the apparent difference between EtOH-treated and PRN-exposed EL4 targets is not significant. And even so, the critical point to be made is that at lower concentrations with fewer surface pMHC complexes formed, the sensitivity of the assay is much greater, and at peptide concentrations < 125 pM, the experimental and control groups are virtually indistinguishable (Figure 3). Thus, there is no observable effect of PRN exposure on in vitro antigen presentation, and we concluded that APCs exposed to PRN are unimpaired in their ability to serve both as stimulators and as targets for peptide-specific CTLs.

In the next phase of our study, we addressed whether there is a direct effect of PRN exposure on CTL reactivity alone. One potential complication with the peptide-specific CTL model system is that when peptide is added to in vitro cultures, it can bind to MHC molecules expressed on the surface of CTLs themselves as well as to those on the APCs, thereby complicating the interpretation of experimental results. To circumvent this problem we used an alloreactive B6 anti-BALB/c MLC model system to examine the effect of PRN on CTL proliferation and function.

In this model the alloreactive B6 CTLs respond directly to the allologenic class I MHC molecules expressed on the BALB/c stimulator cells without the need for added peptide. As shown in Figure 4B, alloreactive CTLs exposed to PRN during 1st MLC are largely unaffected in their lytic reactivity, with only a limited decrease in reactivity observed at the highest (66 μM) concentration tested. Although there is a 2.5-fold difference between the response of the 66 μM PRN-exposed group and control CTLs, given the similarities in magnitude of their overall response, this apparent difference is probably minimal. We also noted the concomitant observation that the proliferative responses, as measured by 3H-Thymidine uptake in the in vitro MLC assay, among all three PRN exposure groups are not significantly different from the EtOH control group (Figure 4A). These findings are similar to those reported initially by Barnett and co-workers (Barnett et al. 1992; Barnett and Gandy 1989) and seem to support their suggestion that PRN might have very little, if any, effect on cell-mediated immunity.

We next examined the impact of prolonged PRN exposure on these alloreactive CTL effectors by adding fresh PRN during their restimulation in 2nd in vitro MLCs. The overall effect of this longer-term exposure to PRN is the appearance of a significantly increased adverse effect on both CTL proliferation and lytic activity. Although PRN exposure during 1st MLC activation has only a limited
effect on these parameters, the subsequent exposure to PRN during 2° MLC activation has a much greater adverse effect on CTL effectors. Thus, the exposure of 2° MLC-derived CTLs to 66 μM PRN induced an effectors. Thus, the exposure of 2° MLC-derived CTLs to 66 μM PRN induced 

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