

# Functional Inactivation in the Whole Population of Human V $\gamma$ 9/V $\delta$ 2 T Lymphocytes Induced By a Nonpeptidic Antagonist

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## Summary

Nonpeptidic compounds stimulate human T cells bearing the TCR- $\gamma\delta$  in the absence of major histocompatibility complex restriction. We report that one of these ligands, 2,3-diphosphoglyceric acid (DPG), which induces expansion of V $\gamma$ 9/V $\delta$  T cells *ex vivo*, antagonizes the same cell population after repetitive activation. Stimulation with DPG results in partial early protein tyrosine phosphorylation and a prolonged, but reversible, state of unresponsiveness to agonist ligands in V $\gamma$ 9/V $\delta$ 2, but not in other T cells. These findings show that TCR antagonism is a general phenomenon of T cells. However, in contrast to the clonal specificity of altered peptides antagonizing  $\alpha\beta$  T cells, all the tested V $\gamma$ 9/V $\delta$ 2 polyclonal cell lines and clones become unresponsive, a fact that may be relevant for the regulation of their response *in vivo*.

Human  $\gamma\delta$  cells bearing the V $\gamma$ 9 (TCR $\gamma$ V2S1)/V $\delta$ 2(TCR $\delta$ V102S1) TCR react to a variety of phosphorylated nonpeptidic ligands (1–4), some of which are natural metabolites (5). The TCR participates in the stimulation of the cells by such ligands as evidenced by reconstitution of reactivity when V $\gamma$ 9 and V $\delta$ 2, but not other V $\gamma$  or V $\delta$  genes, are co-transfected into a nonresponder T cell line (6).  $\gamma\delta$  T cell recognition of phosphorylated nonpeptidic ligands has two remarkable characteristics (5): (a) it is highly specific, since minor modifications in the structure of the ligand abolish recognition, and (b) all V $\gamma$ 9/V $\delta$ 2 cells *ex vivo* and clones show the same specificity since they are broadly cross-reactive against different metabolites, and all ligands display the same relative potency on all tested T cell clones and polyclonal lines. Without exception, we observe that *in vitro* cultivated  $\gamma\delta$  T cells progressively lose their capacity to proliferate in response to the weaker ligands first, and to the stronger ones later on, while maintaining their ability to react to mitogens and to express similar levels of TCR (monitored by flow cytometry analysis using antibodies against CD3 $\epsilon$ , C $\delta$ , V $\gamma$ 9, and V $\delta$ 2, data not shown). This consistent loss of proliferative capacity does not necessarily imply a loss of reactivity, but might reflect a change in biological responsiveness. We have investigated the possibility that a ligand which functions as an agonist during the beginning of the immune response, may act as an antagonist on the same cells in later phases.

## Materials and Methods

**Cells and Cell Culture.** Cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamax I, 100  $\mu$ g/ml kanamycin, MEM nonessential amino acids, 1 mM Na pyruvate (all from GIBCO BRL, Basel, Switzerland), human serum 5% (Blutspendezentrum, Bern, Switzerland), and 100 U of recombinant human IL-2 (IL-2), unless mentioned as IL-2-free medium. The V $\gamma$ 9/V $\delta$ 2 cell lines were raised by culturing  $10^6$  freshly isolated human PBMC with a single dose of the indicated ligand (50  $\mu$ g/ml *Mycobacterium tuberculosis* [*M. tub.*]<sup>1</sup> (Difco Laboratories, Detroit, MI), 10  $\mu$ l/ml protein-free extract from *M. tub.* (PFE), 1 mM xylose-1-phosphate (X1P), 1 mM ribose-1-phosphate (R1P), or 100  $\mu$ M isopentenylpyrophosphate (IPP; Sigma, Buchs, Switzerland). After 72 h, 50 U of IL-2 was added to the cultures. T cell clones were established as reported (7) by limiting dilution using PHA (1  $\mu$ g/ml; Wellcome, Dartford, UK), IL-2 (100 U/ml), and irradiated PBMC ( $5 \times 10^5$ /ml). T cell clones were restimulated periodically following the same protocol (7).

**Flow Cytometry.** V $\gamma$ 9/V $\delta$ 2 cells were incubated in the presence of indicated stimuli for 3 h, and then maintained on ice during staining with mAbs against CD3 $\epsilon$  (TR66) or C $\delta$  ( $\delta$ 1), and analyzed using a FACScan<sup>®</sup> flow cytometer. Data analysis was performed using CELLQuest (Becton Dickinson, San Jose, CA).

**Proliferation Assay.** Proliferation assays were performed in IL-2-free medium using  $10^5$  responder cells/well and 30 Gy-irradiated

<sup>1</sup>Abbreviations used in this paper: DPG, 2,3-diphosphoglyceric acid; E/T, effector/target; IPP, isopentenylpyrophosphate; MFI, median fluorescence intensity; *M. tub.*, *Mycobacterium tuberculosis*.

PBMC ( $10^5$ /well) as APC. After 48 h,  $1 \mu\text{Ci}$ /well of [ $^3\text{H}$ ]thymidine (TRK120; Amersham Intl., Little Chalfont, England), was added and the cultures were harvested after an additional 18 h. Results are shown as mean cpm  $\pm$  SD.

**TNF Release Assay.**  $10^4$  responder cells were incubated with the indicated ligand, and culture supernatants were collected after 6 h.  $15 \times 10^3$  WEHI 164.13 cells were incubated for 18 h with appropriately diluted supernatant in the presence of actinomycin D ( $2.5 \mu\text{g}/\text{ml}$ ). After a further 4 h incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT;  $500 \mu\text{g}/\text{ml}$ ), the cells were lysed and the reduced MTT dissolved by adding an equal volume of HCl 0.04 N in isopropanol.  $\text{OD}_{560} - \text{OD}_{650}$  was determined using a THERMOMax Microplate Reader with SOFTmax (Molecular Devices Corp., Menlo Park, CA), and the released TNF calculated by comparison with the OD in the linear range of a standard curve simultaneously acquired with recombinant human TNF. Results are shown as mean  $\text{pg}/\text{ml} \pm$  SD.

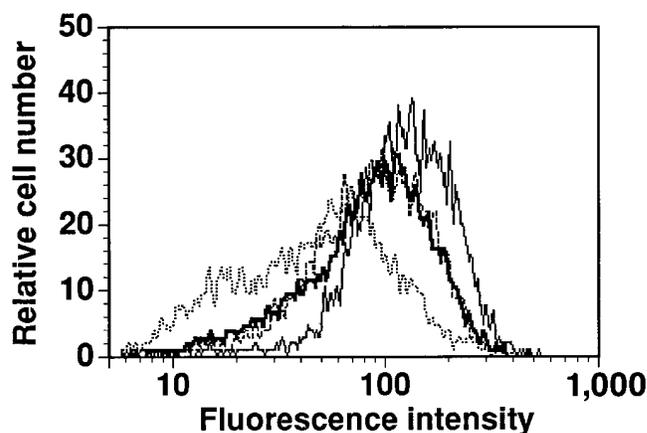
**Cytotoxicity Assays.** In brief, PHA-stimulated T cell blasts were labeled with  $100 \mu\text{Ci} \text{ } ^{51}\text{Cr}$  (Amersham Intl.) and 5,000 cells/well were incubated in round-bottom wells with  $\text{V}\gamma 9/\text{V}\delta 2$  cells at the indicated effector/target (E/T) ratios. After 6 h, the amount of  $^{51}\text{Cr}$  released into the supernatant was measured as cpm and expressed as percent of specific  $^{51}\text{Cr}$  release: (effector induced cpm - spontaneous cpm / maximum cpm - spontaneous cpm)  $\times$  100. Maximum cpm were obtained by target cell lysis with 1 M HCl.

**Analysis of Protein Tyrosine Phosphorylation.** Immunoblot analysis for protein tyrosine phosphorylation was performed with total cell lysates. Briefly, T cells ( $7.5 \times 10^5$  cell equivalents/lane) were lysed with  $100 \mu\text{l}/10^6$  cells of lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, inhibitors of proteases (1 mM PMSF,  $4 \mu\text{g}/\text{ml}$  leupeptin,  $4 \mu\text{g}/\text{ml}$  aprotinin), and inhibitors of phosphates (10 mM EDTA, 1 mM sodium orthovanadate), for 20 min on ice. After removal of nuclear debris by centrifugation, the supernatant was electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C; Amersham Intl.). Protein tyrosine phosphorylation was detected using the 4G10 mAb (Upstate Biotechnology Inc., Lake Placid, NY). The same results were obtained using another phosphotyrosine specific mAb (PTyr1; a gift from V. Horejsi). Blots were developed using horseradish peroxidase-conjugated second antibody (sheep anti-mouse Ig; Amersham Intl.) and enhanced chemiluminescence (ECL system; Amersham Intl.).

## Results and Discussion

A ligand and intermediate potency, 2,3-diphosphoglyceric acid (DPG), was compared with a ligand with high potency, IPP (4, 5). Fresh  $\gamma\delta$  T cells fully respond to both compounds. However,  $\gamma\delta$  T cells in culture gradually lose their proliferative response to DPG without a change in their capacity to proliferate in response to IPP.

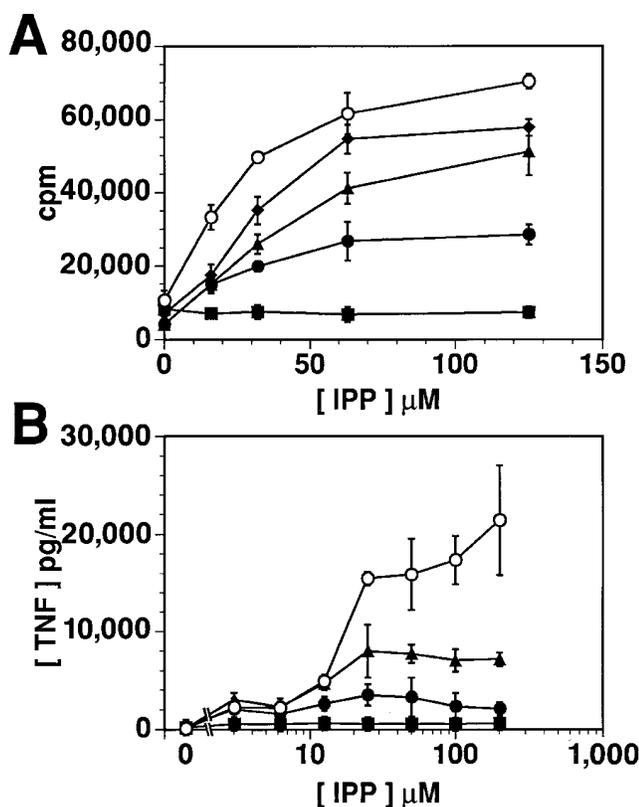
Throughout this study we have used  $\text{V}\gamma 9/\text{V}\delta 2$  T cells which proliferate to IPP, but no longer to DPG. Both DPG and IPP induce downmodulation of the TCR  $\gamma\delta$  on these cells (Fig. 1), confirming that activation by DPG and IPP involves TCR stimulation (8). DPG induces TCR downmodulation without cell proliferation, we investigated whether it might behave as a partial agonist or as an antagonist (9, 10). DPG induces neither release of IFN $\gamma$  and TNF, nor transcription of IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF, IFN- $\gamma$ , and TNF- $\alpha$  genes in  $\text{V}\gamma 9/\text{V}\delta 2$  cells (as-



**Figure 1.** DPG induces downmodulation of the TCR  $\gamma\delta$ . Overlaid histograms of immunostaining of  $\delta 1$  mAb on  $\text{V}\gamma 9/\text{V}\delta 2$  clone Z1P 101 simulated with medium (—, median fluorescence intensity [MFI] 138, 100%), 1 mM DPG (---, MFI 99, 71%), IPP  $100 \mu\text{M}$  (---, MFI 100, 72%), and PHA  $1 \mu\text{g}/\text{ml}$  (- · -, MFI 55, 40%). Similar results were obtained with several different  $\text{V}\gamma 9/\text{V}\delta 2$  cell lines and clones, but not with control clones bearing other types of TCR.

essed by reverse transcriptase-PCR, data not shown), nor killing of target cells by cytotoxic  $\text{V}\gamma 9/\text{V}\delta 2$  clones (data not shown), which are all effects detected after stimulation with IPP. Therefore, it is unlikely that DPG is a partial agonist. In another series of experiments,  $\gamma\delta$  T cells were cultured in the presence of both ligands simultaneously (Fig. 2). Although DPG no longer displays its agonistic properties on cultured  $\gamma\delta$  T cells, it nevertheless has a clear dose-dependent effect whereby it inhibits IPP-induced activation in a noncompetitive manner. DPG lowers the efficacy (maximal response) of IPP, but does not alter the potency of IPP (dose for 50% of maximal effect). These results indicate that DPG does not displace IPP from its binding site, but that it may act as a TCR antagonist.

To investigate whether stimulation by DPG could result in a state of altered responsiveness,  $\text{V}\gamma 9/\text{V}\delta 2$  T cells were preincubated with DPG, and then washed and challenged with IPP. Preincubation with DPG blocks the subsequent ability of IPP to stimulate TNF release and cell proliferation (Fig. 3) indicating that DPG induces a state of unresponsiveness which persists after removal of the ligand. This prolonged unresponsiveness is not due to inadequate wash-out of the antagonist, since control cells are fully reactive in the presence of equal numbers of DPG-preincubated cells (data not shown). Incubation with DPG for only 5 min is sufficient to block IPP-mediated TNF release (Fig. 3 A). The rapidity with which DPG exerts its inhibitory effect is consistent with observations that phosphorylated non-peptidic ligands induce  $\text{Ca}^{2+}$  fluxes in  $\text{V}\gamma 9/\text{V}\delta 2$  T cells within 2 min (11, 12). The IPP-unresponsiveness induced by DPG is not a consequence of cell death since the number of viable cells recovered is the same (70–100%) as controls, and the cells remain responsive to low doses (20 U/ml) of IL-2 (Fig. 3 B). Furthermore, once a state of unresponsiveness is induced, it lasts for 1–5 d (using different

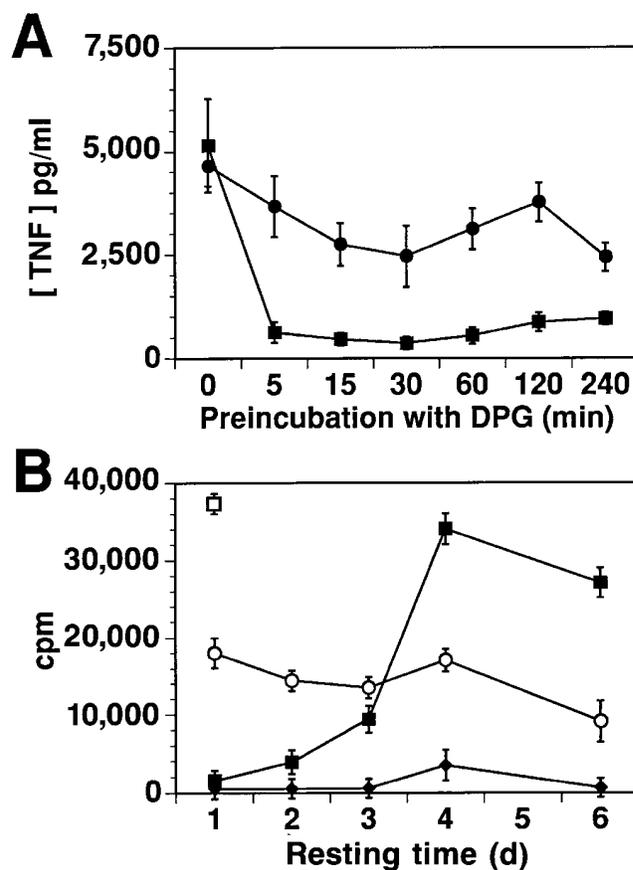


**Figure 2.** DPG reduces the efficacy, but not the potency, of IPP. Proliferation (A) and TNF release (B) of the  $\gamma\delta$  T cell line PG96 coincubated with different concentrations of IPP and DPG (1 mM, ■; 500  $\mu$ M, ●; 250  $\mu$ M, ▲; 125  $\mu$ M, ◆), or medium (○). Comparable dose-response curves were obtained in four independent experiments.

cell lines and clones), after which the cells regain their ability to respond to the agonist (Fig. 3 B). The induction of unresponsiveness by DPG differs from the induction of T cell refractoriness by repetitive stimulation with agonist ligands. Indeed, (a)  $\gamma\delta$  cells stimulated by DPG become unresponsive without induction of effector functions, and (b) the induction of unresponsiveness by DPG has a dominant effect even over simultaneous stimulation by IPP (Fig. 2).

Proliferation and lymphokine release both require nuclear gene activation. To study whether effects induced by earlier signaling events are altered by DPG, we performed cytotoxicity assays. IPP stimulates cytotoxic V $\gamma$ 9/V $\delta$ 2 cells to kill target cells at low E/T ratios; however, IPP does not trigger killing by the same cells preincubated with DPG (Fig. 4). These results suggest that in unresponsive cells, not only late events, but also earlier signaling events leading to cytotoxic activity are affected.

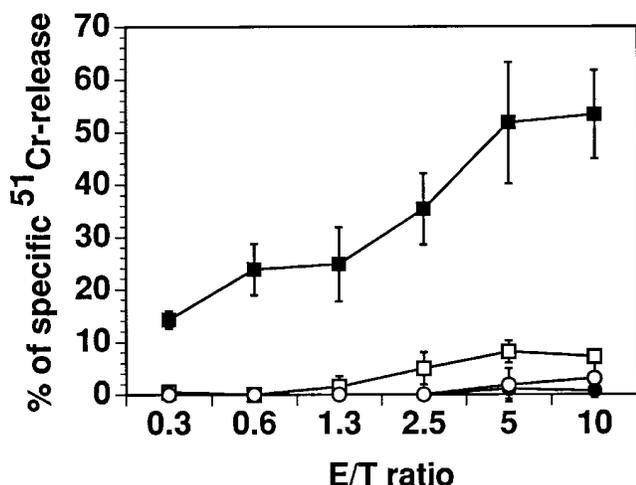
To determine which signal transduction pathways might be affected, we applied various procedures described to prevent anergy induction or restore responsiveness. While cyclosporin A completely prevents anergy with  $\alpha\beta$  T cells (13, 14), it has only a partial effect on  $\gamma\delta$  T cells (data not shown). This suggests that both the late signals blocked by cyclosporin A, as well as other signals, participate in establishing unresponsiveness. IPP-unresponsive cells could be



**Figure 3.** DPG rapidly induces a prolonged, but reversible, state of unresponsiveness to IPP. (A) The V $\gamma$ 9/V $\delta$ 2 cell line BCI 31 was incubated with DPG for the time indicated on the x-axis. After extensive washing, the TNF release stimulated by 50  $\mu$ M IPP (■) or 1  $\mu$ g/ml PHA (●) was measured. (B) V $\gamma$ 9/V $\delta$ 2 clone BCI 49 ( $2 \times 10^6$  cells/ml) was incubated in IL-2-free medium with 1 mM DPG overnight and washed extensively. The cells were then rested in medium for the time indicated on the x-axis. Proliferative responses to medium (◆), 100  $\mu$ M IPP (■), or 20 U/ml IL-2, (○) are shown. □ indicates control proliferation to IPP (100  $\mu$ M) of the same cells not preincubated with DPG. Results obtained in four independent experiments using different V $\gamma$ 9/V $\delta$ 2 clones showed recovery of responsiveness after 1 to 5 d. Preincubation with DPG did not affect the responsiveness of CD4<sup>+</sup> TCR  $\alpha\beta$  cells to their specific peptide (data not shown).

stimulated by PHA or a combination of PMA and Ca<sup>2+</sup> ionophore (Fig. 5). Importantly, reactivity to IPP could be restored by PMA, which was not stimulatory by itself. Thus, IPP can apparently still trigger unresponsive cells with a signal that is mimicked by Ca<sup>2+</sup> ionophore. In addition, the ability of PMA to overcome unresponsiveness indicates that the blockade of signaling in unresponsive cells most likely occurs between the TCR and P21<sup>ras</sup>. Blockade of proximal signaling has been recently shown in two  $\alpha\beta$  T cell anergy models (15, 16).

Alterations of the TCR proximal signals have been previously described for modified peptide ligands which induce unresponsiveness in TCR  $\alpha\beta$  clones (17, 18). To investigate whether early signals are also altered in  $\gamma\delta$  T cells, the IPP- and DPG-induced protein tyrosine phosphorylation patterns were compared. A similar spectrum of phos-

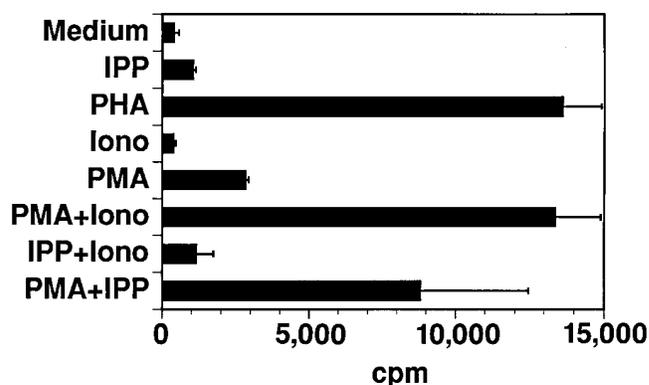


**Figure 4.** Early signaling events, necessary for cytolytic function, are altered in cells made unresponsive by DPG. The figure shows results of killing of PHA-blasts by V $\gamma$ 9/V $\delta$ 2 clone RNM.t.73 preincubated overnight with medium (solid symbols) or DPG 1 mM (open symbols) in the presence (squares) or absence (circles) of 100  $\mu$ M IPP, at the indicated E/T ratios.

phorylated proteins was detected (Fig. 6). However, the overall level of phosphorylation induced by DPG was quantitatively less than that induced by IPP.

The presented data show that TCR  $\gamma\delta$  antagonism has several characteristics in common with TCR  $\alpha\beta$  antagonism induced by altered peptide ligands (9, 19, 20). However, there are also important differences. In V $\gamma$ 9/V $\delta$ 2 T cells, the antagonistic effects of DPG occur only after cell activation and extensive proliferation. Since the effects of DPG are observed without changes in its structure, the change in response must be due to a change occurring in the T cells. Cultured  $\gamma\delta$  T cells, on which DPG has lost its agonistic effects, express normal levels of TCR, which can transduce a full signal after stimulation with IPP. Therefore, it is likely that molecules other than the TCR are involved in the altered responsiveness to DPG. In our case, modified expression of costimulatory molecules seems unlikely, because cells which have lost the capacity to proliferate with DPG still proliferate with IPP, implying adequate costimulation. Yet, triggering the TCR with various stimuli in the absence of costimulation always leads to  $\alpha\beta$  T cell anergy (21, 22). The finding that cytotoxicity, which is not so dependent on costimulation (23), is lost in unresponsive cells also supports this conclusion.

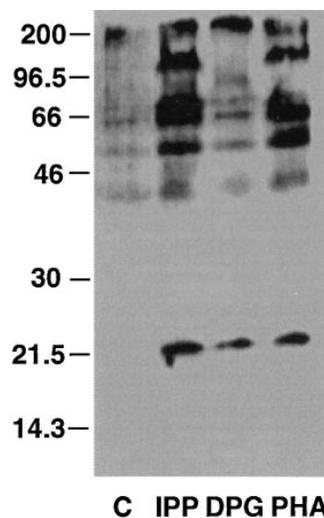
The hypophosphorylation observed after DPG stimulation indicates that the signal transduction cascade is affected, and this might be due to loss of molecules important in the signaling pathway. Interestingly, the potency of DPG as agonist is the same as its potency as antagonist ( $ED_{50}$  250  $\mu$ M), thus implying that DPG interacts with its receptor with the same affinity on both fresh and expanded  $\gamma\delta$  cells and that the nonproliferating  $\gamma\delta$  cells have not modified the receptor for DPG. We hypothesize that responsiveness of V $\gamma$ 9/V $\delta$ 2 cells is modulated by the expression levels of a (unknown) molecule with a coreceptor-like function simi-



**Figure 5.** Unresponsiveness induced by DPG is the consequence of the alteration of TCR proximal signals. V $\gamma$ 9/V $\delta$ 2 clone BCI 49 ( $10^6$  cells/ml) was incubated overnight in IL-2-free medium with 1 mM DPG and washed extensively. Proliferative responses to medium, 100  $\mu$ M IPP, 1  $\mu$ g/ml PHA, 500 ng/ml  $Ca^{2+}$  Ionophore A23187 (Iono) 50 ng/ml PMA, or the indicated combinations were measured.

lar to that described for CD4 and CD8 coreceptors on  $\alpha\beta$  cells (24–29). Based on the presented data, our hypothesis predicts that stimulation of  $\gamma\delta$  T cells with strong ligands (e.g., IPP) is largely independent of coreceptor density, while stimulation with weak ligands (e.g., DPG) would require a high density. If the coreceptor density is reduced, weak ligands switch their properties from being agonists to antagonists. A progressive loss of the coreceptor might allow a given ligand to function as a full agonist in the early phase of the immune response, and subsequently acquire antagonistic properties and regulatory functions.

A second important difference is that while most of the TCR  $\alpha\beta$  antagonists are highly T cell clone specific, DPG is broadly active on human V $\gamma$ 9/V $\delta$ 2 T cells. When many different  $\gamma\delta$  T cell lines and clones from various donors are tested, all the V $\gamma$ 9/V $\delta$ 2 cells become unresponsive, irrespective of the ligand used to expand the original cell lines (Table 1). This is observed with assays detecting either proliferation or TNF release.



**Figure 6.** Upon DPG treatment, tyrosine phosphorylation of TCR-associated proteins is reduced when compared to IPP stimulation. V $\gamma$ 9/V $\delta$ 2 T cells were incubated for 10 min with either medium (C), 100  $\mu$ M IPP, 1 mM DPG, or 1  $\mu$ g/ml PHA. Protein tyrosine phosphorylation is visualized by immunoblotting of total cell lysates. Similar results were obtained in four independent experiments using V $\gamma$ 9/V $\delta$ 2 cell lines and clones. IPP and DPG did not induce any protein tyrosine phosphorylation in TCR  $\alpha\beta$  cells (data not shown).

**Table 1.** Unresponsiveness of V $\gamma$ 9/V $\delta$ 2 Polyclonal Lines and Clones After Overnight Incubation with DPG

| Response to:            |                | Medium        |       | IPP    |       | IL-2   |        | PHA    |        |
|-------------------------|----------------|---------------|-------|--------|-------|--------|--------|--------|--------|
| Preincubation with DPG: |                | No            | Yes   | No     | Yes   | No     | Yes    | No     | Yes    |
| Donor                   | Raised against | Proliferation |       |        |       |        |        |        |        |
|                         |                | <i>cpm</i>    |       |        |       |        |        |        |        |
| OP                      | <i>M. tub.</i> | 2,039         | 1,280 | 15,175 | 1,160 | 11,785 | 10,584 | 13,961 | 15,927 |
| VJ                      | <i>M. tub.</i> | 6,353         | 3,366 | 12,061 | 3,087 | 12,827 | 13,003 | 10,488 | 14,474 |
| GDL                     | <i>M. tub.</i> | 1,320         | 5,075 | 27,385 | 6,899 | 31,537 | 36,617 | 16,890 | 23,029 |
| GDL                     | R1P            | 628           | 1,360 | 19,198 | 1,870 | 12,086 | 16,345 | 15,123 | 17,415 |
| GDL                     | X1P            | 7,324         | 5,757 | 12,104 | 6,171 | 17,094 | 16,486 | 23,042 | 22,941 |
| GDL                     | PFE            | 10,607        | 8,178 | 70,393 | 7,326 | ND     | ND     | ND     | ND     |
| BCI 49*                 | IPP            | 381           | 706   | 37,324 | 1,767 | 44,938 | 50,429 | 19,102 | 19,514 |
| BCI 5*                  | IPP            | 411           | 323   | 24,857 | 1,080 | ND     | ND     | 6,350  | 8,388  |
|                         |                | TNF release   |       |        |       |        |        |        |        |
|                         |                | <i>pg/ml</i>  |       |        |       |        |        |        |        |
| GDL                     | <i>M. tub.</i> | ND            | 744   | 14,746 | 2,295 | ND     | ND     | ND     | ND     |
| GDL                     | IPP            | ND            | 101   | 5,147  | 956   | ND     | ND     | 4,658  | 3,102  |
| BCI 13*                 | IPP            | ND            | 475   | 7,585  | 1,736 | ND     | ND     | ND     | ND     |
| G2C25*                  | PHA            | <15           | <15   | 2,369  | 155   | ND     | ND     | ND     | 1,649  |

\*V $\gamma$ 9/V $\delta$ 2 T cell clone. PFE, protein-free extract from *M. tub.*; R1P, ribose-1-phosphate; X1P, xylose-1-phosphate. Each line or clone was tested two to four times.

The induction of a state of unresponsiveness in a whole lymphocyte subpopulation may have important consequences for the immune response of  $\gamma\delta$  T cells. It has been proposed that  $\gamma\delta$  T cells provide an efficient first line of defense against infectious agents (30, 31). This property may derive from their ability to broadly cross-react with many ligands (5) and with diverse microorganisms (7, 32–36). However, this promiscuous ligand recognition might lead to an uncontrolled expansion of V $\gamma$ 9/V $\delta$ 2 T cells with harmful effects for the organism. The presently described regulatory mechanism could control the whole V $\gamma$ 9/V $\delta$ 2 T cell population in a late phase of the immune response. A paradigmatic example might be malaria infection where either plasmodium ligands or DPG, which is present inside red blood cells at a physiological concentration of  $\sim$ 5 mM, are released after massive erythrocyte rupture. In patients with acute malaria,

a synchronized erythrocyte rupture is followed by hyperactivation of the V $\gamma$ 9/V $\delta$ 2 population (37, 38) and by a massive release of TNF resulting in a severe clinical picture (39). The recurrent release of nonpeptidic ligands from ruptured red blood cells and the successive activation and expansion of  $\gamma\delta$  T cells could change  $\gamma\delta$  T cell reactivity, and thereby result in a beneficial unresponsiveness. Indeed, patients with the chronic form of malaria experience neither V $\gamma$ 9/V $\delta$ 2 cell expansion nor raised TNF serum levels and have an asymptomatic clinical course (38).

In summary, the presented data provide a rationale for manipulating the reactivity of the whole V $\gamma$ 9/V $\delta$ 2 T cell population and offer a new model with which to assess whether TCR antagonism is an important principle of T cell regulation.

We thank A. Lanzavecchia, R. Nisini, and T. J. Resink for reading the manuscript.

This work was supported by the Swiss National Fund grants No. 31-36450-92 and No. 31-045518.95 to G. De Libero and the Marie-Heim Vöglin grant No. 32-38848-93 to L. Mori.

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Received for publication 9 July 1996 and in revised form 17 October 1996.

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