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Source / Izvornik: **Mediators of Inflammation, 2002, 11, 129 - 135**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:184:062463

Mediators of Inflammation, **11**, 129–135 (2002)

Augmentation of NKT and NK cell-mediated cytotoxicity by peptidoglycan monomer linked with zinc

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Background: **Peptidoglycan monomer (PGM), which was originally prepared by biosynthesis from culture fluids of penicillin-treated *Brevibacterium divaricatum*, is an immunostimulator, the activities of which might be improved by addition of zinc (Zn) to the basic molecule.**
Methods: **To test the possible cytotoxic effects of this new analogue, we analyzed the ability of PGM-Zn and PGM to change the phenotypic profile of hepatic and splenic mononuclear lymphatic cells and to affect the growth of malignant T-cell line YAC-1 and syngeneic thymocytes.**
Results: **Pretreatment of C57BL/6 mice primarily with PGM-Zn over 6 days (10/mg/kg intraperitoneally) significantly enhanced the proportions of $NK1.1^{high+}$, $CD4^{-}CD8^{-}$, $CD69^{+}$, and $CD3^{intermediate}/NK1.1^{+}/IL2R\text{-}\beta^{+}$ (NKT) cells in the liver, and major histocompatibility complex class II^{+}, $CD69^{+}$, and $CD8^{+}$ cells in the spleen. Both types of cells were highly cytotoxic against YAC-1 and syngeneic thymocytes, increasing the destruction of YAC-1 by 70% on addition of hepatic cells and by 30% on addition of splenic cells. Destruction of thymocytes increased by 10 and 50%, respectively.**
Conclusion: **The results point to PGM-Zn as a potent cytotoxicity-inducing agent, which also generates autoreactive NKT cells.**

Key words: NK cells, NK1.1, Intermediate T cell receptor expressing cells, Cytotoxicity to YAC-1 and syngeneic thymocytes, Liver and spleen, Self-reacting NKT cells

Introduction

Bacterial cell-wall components, such as lipopolysaccharide derived from Gram-negative bacteria and peptidoglycans (PG) isolated from Gram-positive bacterial cell walls, are well-known immunomodulating agents, which participate in the immune reaction generated on antigen or pathogen stimulation.[1–6] Both substances in the presence of two soluble serum proteins (i.e. CD14 and lipopolysaccharide binding protein) activate lymphoid targets through the CD14 receptor, which might be viewed as the patterns recognition receptor for various bacterial cell-wall components.[3,4] CD14 then recruits peptidoglycan monomer (PGM) to members of Toll-like receptor (TLR)2, which was identified as an essential component of the PG receptor signaling complex that controls innate responses.[5–7]

Stimulation of TLR proteins results in the release mainly of pro-inflammatory mediators (interleukin (IL)-1, IL-6, and tumor necrosis factor-α), which in large quantities might promote infection and induce septic shock.[7] In lower doses, however, bacterial products might have immunostimulating and antimetastatic activities linked with the stimulation of both unspecific and specific immune responses.[8] Adjuvant effects have been tried to be explained by stimulation of co-stimulatory activities in antigen-presenting cells, by mimicking of co-stimulatory signals in T cells, or by enhanced uptake of the antigen by dendritic cells that already express co-stimulatory molecules.[8] Since there is still a paucity of data about the mechanisms leading to inflammatory and immunomodulatory responses induced by Gram-positive bacteria, in the present study we tested the effects of PGM, obtained by biosynthesis from culture fluids of penicillin-treated *Brevibacterium divaricatum* NRRL-2311,[9] and its new analogue PGM-Zn on the innate immune system.

Basically, PGM represents a water-soluble monomeric structural subunit of the polymeric PG, consisting of β1,4-linked *N*-acetylmuramic acid-*N*-acetyl-D-glucosamine disaccharide carrying the pentapeptide. Its immunostimulating and antimetastatic activities have been emphasized in many experimental protocols,[10–12] but the precise mechanism of action of PGM is still unknown. Generally, it is held that it uses similar pathways as other Gram-positive bacteria.[5,6] This is supported also by our own data, showing that PGM *in vitro* has a cytokine-

ISSN 0962-9351 print/ISSN 1466-1861 online/02/020129-07 © 2002 Taylor & Francis Ltd
DOI: 10.1080/0962935022013100

stimulating power on human peripheral blood mononuclear cells, leading to release of IL-1, IL-6, and tumor necrosis factor-α in the presence of human serum or soluble CD14.[13] Previously, however, we also reported that addition of zinc might potentiate the immunostimulatory activity of PGM.[14–17] The new analogue (PGM-Zn) augmented antibody production and isotype switching in mice sensitized with sheep red blood cells,[14] and corrected some immunosuppressive aspects of aging[15] and cholestatic jaundice.[16] We also observed that prolonged treatment of mice with PGM-Zn may result in activation of peritoneal macrophages, which have greater phagocytic activities, as well as the ability to suppress the Con-A-induced blastic transformation of syngeneic splenocytes.[17] Immunomodulatory effects of PGM and PGM-Zn in these experiments correlated with major histocompatibility complex (MHC) class II expression in the liver,[14] in which we also previously noticed an accumulation of zinc during the normal humoral and cellular immune response.[18]

Bearing in mind the crucial role of the hepatic natural killer (NK) cells and NKT cells in the control of immune survielance,[19,20] and evidence about the antitumor effects of PGM alone,[10–12] in this study we attempted to characterize the effects of PGM-Zn in comparison with the effect of PGM alone on processes of cell-mediated cytotoxicity. For this purpose, we determined the phenotype and function of hepatic and splenic mononuclear lymphatic cells (MNLC) activated *in vivo* by PGM-Zn or PGM and analyzed their ability to block the growth of the malignant T-cell line YAC-1 and syngeneic thymocytes. The data showed that pretreatment of C57BL/6 mice, particularly with PGM-Zn, might markedly enhance the cytotoxicity of both types of lymphoid cells against NK-sensitive targets, as well as against syngeneic cells. Simultaneous phenotypic analysis of effector cells also suggested that this cytotoxicity in the liver might be linked with activation of $NK1.1^+$, $CD3^{intermediate}$, IL-$2R\beta^+$ NKT cells, and in the spleen with activation of cytotoxic $CD8^+$ T cells.

Materials and methods

Animals

We used inbred, 2- to 3-month old, male C57BL/6 mice. The animals were housed in standard plastic cages, allowed access to standard mouse food pellets and water *ad libitum*, and exposed to the natural light/dark cycle.

Treatment with PGM-Zn

Immunomodulating agents PGM (GlcNAc-MurNAc-L-Ala-D-iso-Gln-meso-diamminopimelic acid (w-NH2)-D-Ala-D-Ala) and PGM linked with zinc (PGM-Zn), (Pliva, Zagreb, Croatia) were prepared by biosynthesis from the culture fluids of *Brevibacterium divaricatum* NRRL-2311, as an apyrogenic, water-soluble substance devoid of any toxic effects.[9] The samples used in this study contained less than 0.015 ng of endotoxin/mg of PGM, according to the limulus amebocyte lysate test (Pyrostat Kit; Millipore, Bedford, Massachusetts). In the morning every second day, the mice were injected with PGM-Zn or PGM dissolved in phosphate-buffered saline (PBS) (10 mg/kg of body weight, intraperitoneally (i.p.)) for 6 days (total dose, 30 mg/kg). Mice in the control groups were treated with the same volume (0.5 ml) of PBS. Two days after the last injection, the animals were sacrificed to isolate the hepatic and splenic MLNC.

Isolation of intrahepatic lymphocytes, splenocytes and thymocytes

Intrahepatic lymphocytes (IHL) were isolated from intact liver after *in situ* perfusion with PBS, using a modification of the method of Seglen, as we previously described.[21] Resident liver MNLC were isolated by Ficoll–Hypaque density gradient centrifugation (20 min at $800 \times g$). A single suspension of spleen cells was prepared in RPMI 1640 medium (Gipco BRL, Basel, Switzerland), after elimination of erythrocytes by lysing solution. The syngeneic thymocytes were prepared in a similar way.

Cytofluorometric analysis

The surface phenotypes of resident IHL and splenocytes were identified by direct immunofluorescence analysis on FACScan (Becton Dickinson, Immunocytometry Systems, Mountain View, CA, USA), using CELLQuest Software (Quadra 650; Macintosh). As primary monoclonal antibodies (mAbs), fluorescein isothiocyanate-conjugated mAbs (anti-CD8, anti-CD3, anti-class I, anti-class II and anti-T-cell receptor (anti-TCR) αβ) and phycoerythrin-conjugated mAbs (anti-CD4, anti-CD25, anti-CD5, anti-CD44, anti-CD54, anti-CD69, anti-CD30, anti-NK-1.1, and anti-TCR γδ and anti-IL2R-β chain) were used, purchased from Becton Dickinson. All samples had adequate isotypic controls. Propidium iodide (PI) (Sigma, St Louis, MO, USA) (1 mg/ml) stained dead cells were excluded by electronic gating. Relative fluorescence intensities were expressed in the log scale, with 1×10^4 cells.

Flow cytometry cytotoxicity assay

Functional NK cell assays were performed with PKH-26 (orange)-labeled YAC-1 or syngeneic thymocytes as target cells following the manufacturer's instructions (PKH-26 Red Fluorescent Cell Linker Kit; Sigma Biosciences, St Louis, MO, USA). For this purpose, IHL and splenocytes were incubated for 2 h with 1 ×

10^5/ml of labeled YAC-1 cells at different killer-to-target ratios in a final volume of 200 µl at 37°C in a 5% CO_2 atmosphere. After washing in fluorescence-activated cell sorter (FACS) medium, 200 µl of PI (concentration, 10 µg/ml) were added and the percentage of dead cells was measured by flow cytometry. The destroyed cells were counted by detecting cells with both orange (PKH-26) and red (PI) fluorescence.

Statistical analysis

Data were analyzed using the Sigma Plot Scientific Graphing System, Version 1.02. Statistical significance was calculated by Mann–Whitney U test. The differences were considered significant when $p < 0.05$.

Results

Effects of PGM-Zn pretreatment on phenotypic profile of MNLC in the liver and spleen

Six days after i.p. treatment with PGM-Zn (daily dose, 10 mg/kg), the proportion of several subtypes of lymphoid cells in the liver and spleen had changed (Fig. 1). The most prominent changes in the liver were significant increases in the proportion of $CD4^-CD8^-$ (double negative), $NK1.1^{high+}$, and $CD69^+$ cells, which increased from 55.5 ± 1.7 to 65.6 ± 3.1% ($p < 0.05$), from 27.8 ± 3.4 to 37.7 ± 2.5% ($p < 0.01$), and from 35.3 ± 2.6 to 46.5 ± 2.6% ($p < 0.01$), respectively. Double labeling, however, revealed that most of PGM-Zn-induced hepatic MNLC belong to the population of $CD3^{int}$, $NK1.1^{int}$, IL-$2R\beta^+$ cells (i.e. typical NKT cells) (Fig. 2). Their percentage after the PGM-Zn arose more than after treatment with PGM alone (increase from 10.3% to 27.2%, or from 10.3% to 17.2%, respectively). In the spleen, however, significant increases in the proportion of $CD8^+$ (from 14.0 ± 0.3 to 16.3 ± 0.7%, $p < 0.05$), MHC class II^+ (from 53.0 ± 2.6 to 60.3 ± 1.1%, $p < 0.01$), and $CD69^+$ (from 1.5 ± 0.2 to 3 ± 0.3%, $p < 0.05$) cells were observed, together with a decrease in the proportion of $CD3^+$ cells (from 37.1 ± 0.9 to 34 ± 0.6%, $p < 0.01$) (Fig. 1). Double labeling also showed that PGM-Zn-induced changes of NKT cells were in the spleen less expressed than in the liver (Fig. 3). PGM alone in both organs induced similar changes, but they were always significantly lower that these obtained by PGM-Zn.

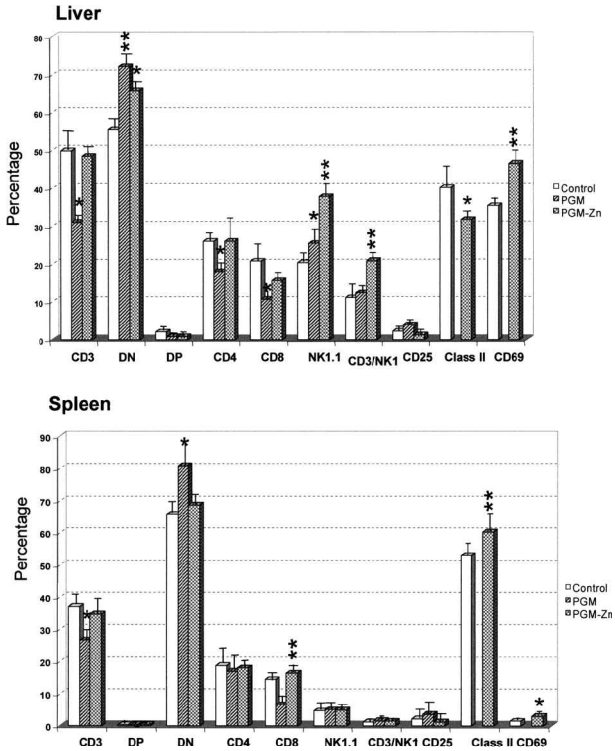

FIG. 1. Phenotypic profiles of hepatic and splenic MNLC obtained from C57BL/6 mice pretreated over 6 days with three doses of PGM-Zn, PGM (10 mg/kg i.p.) or with saline solution ($n = 7$). Mean ± standard errors are presented. *$p < 0.05$, **$p < 0.01$.

Cytotoxicity of hepatic and splenic MNLC activated by PGM-Zn and PGM against YAC-1

To test the ability of PGM-Zn and PGM-activated intrahepatic and splenic MNC to act against the NK-sensitive malignant T-cell line YAC-1, labeled target cells were exposed to killer cells in different killer-to-target ratios (Fig. 4). The data, analyzed after 2 h, showed that PGM-Zn increased, in a dose-dependent manner, the cytotoxicity of both effectors, increasing the destruction of YAC-1 by 70% after addition of hepatic MLNC cells (from 5 to 76% at an effector:target ratio of 50:1, $p < 0.01$) and by 30% from baseline after the addition of splenic cells (from 10 to 40%, $p < 0.05$). Although PGM-activated intrahepatic and splenic cells were more cytotoxic than control MNLC ($p < 0.01$), this cytotoxicity at all ratios was significantly lower than cytotoxicity of PGM-Zn-activated cells ($p < 0.01$). A similar but less expressed stimulatory effect was obtained with PGM-Zn-activated peripheral blood mononuclear cells, which in the control group showed very small cytotoxic activity against YAC-1 (data not shown).

Cytotoxicity of hepatic and splenic MNLC activated by PGM-Zn and PGM against syngeneic thymocytes

To test the possibility that during the treatment of mice with PGM-Zn or PGM autoreactive clones of NKT cells were generated, hepatic and splenic MNLC were incubated also with labeled syngeneic thymocytes (Fig. 5). The data showed that PGM-Zn augmented the cytotoxicity of hepatic MNLC by 10% (from 10 to 20%, at an effector:target ratio of 50:1). Splenic MNLC, obtained from PGM-Zn-treated mice, however, were more cytotoxic for syngeneic thymocytes than control MNLC, even at a very low effector:target ratio (50% at the ratio 6.25:1, $p < 0.01$).

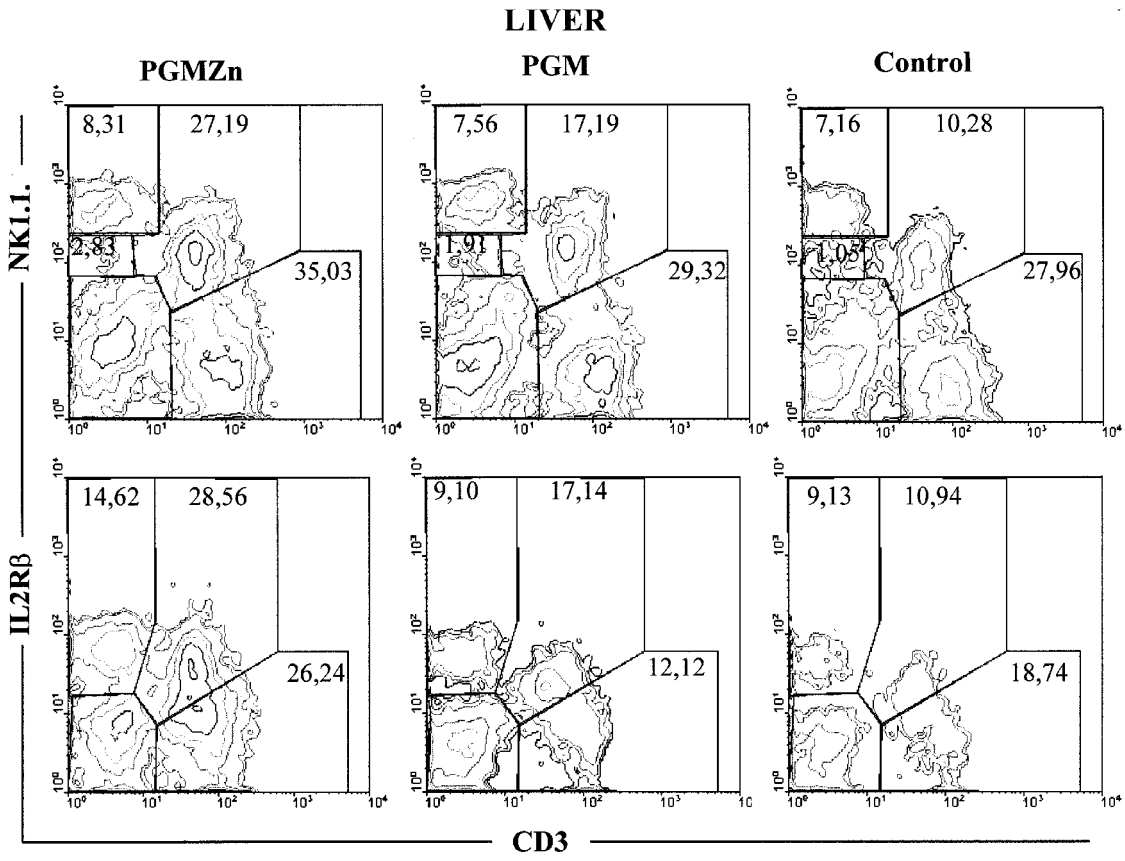

FIG. 2. FACS profiles of two-color immunofluorescence staining of hepatic MNLC after *in vivo* treatment with PGM-Zn, PGM or medium, shown as a representative result of three isolated experiments.

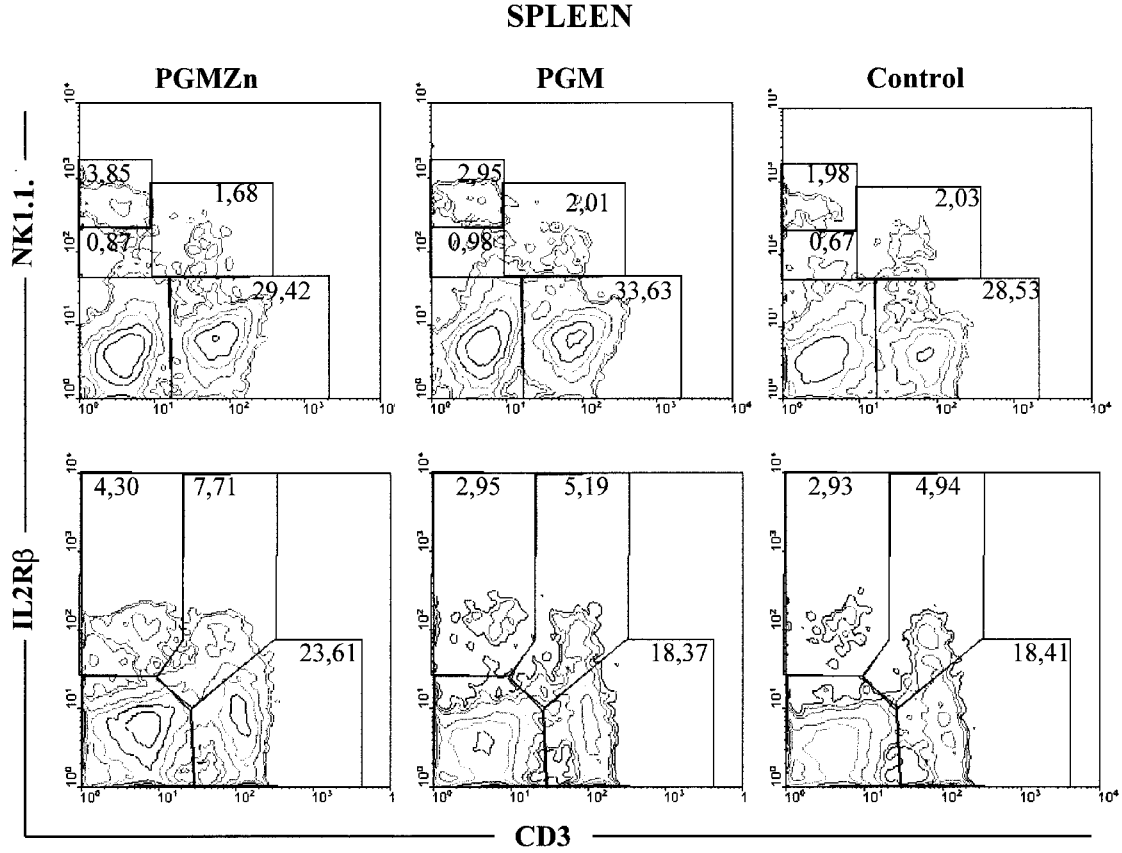

FIG. 3. FACS profiles of two-color immunofluorescence staining of splenic MNLC after *in vivo* treatment with PGM-Zn, PGM or medium, shown as a representative result of three isolated experiments.

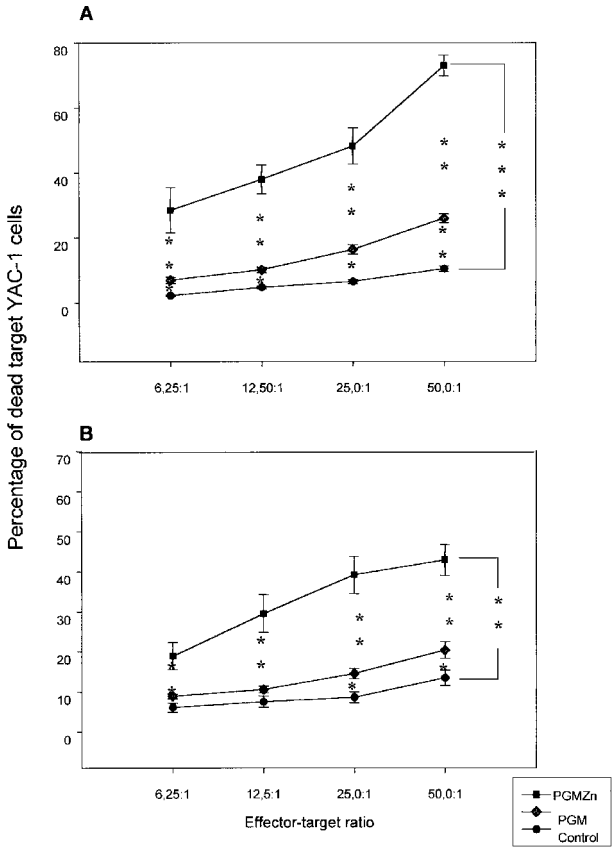

FIG. 4. Destruction of labeled YAC-1 target cells by hepatic (A) and splenic (B) MNLC obtained from mice pretreated with PGM-Zn or saline solution. Results are presented as mean ± standard error ($n = 7$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

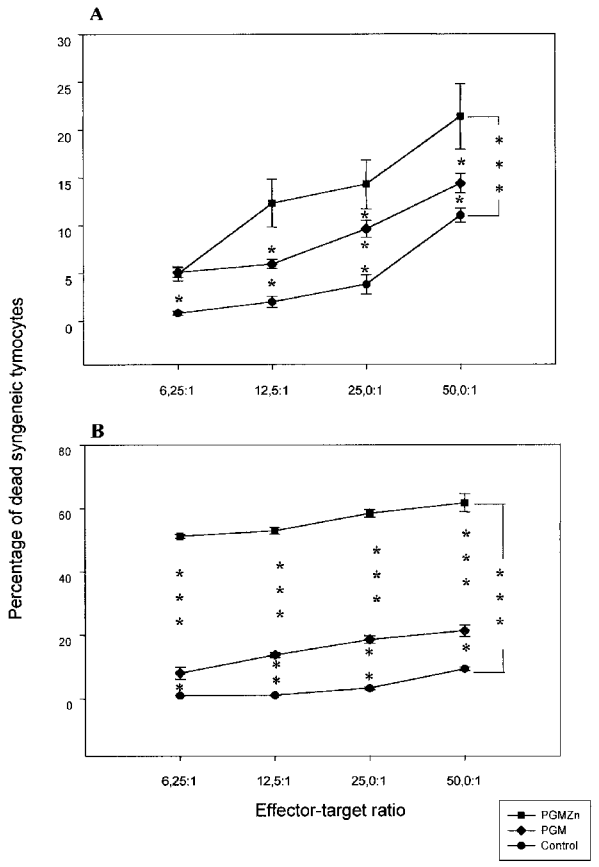

FIG. 5. Destruction of labeled syngeneic target cells by hepatic (A) and splenic (B) MNLC obtained from mice pretreated with PGM-Zn or saline solution. Results are presented as mean ± standard error ($n = 7$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Discussion

The data clearly show that PGM and especially PGM-Zn, given *in vivo*, enhance the antitumor cytotoxicity and autoreactivity of hepatic and splenic MNLC. In the liver, the effect seems to be linked with the increased proportion of activated $CD3^{int}$ $NK1.1^{+}$ cells, which after the treatment with PGM-Zn were present in an almost threefold greater proportion than in the control groups of mice (arose from 10.28% to 27.19%). Almost all of them were also IL-$2R\beta^{+}$ cells, although PGM-Zn increased also the proportion of the $NK1.1^{high+}$ and $CD3^{-}$ cell populations (i.e. NK cells).

The described phenotype is characteristic for the population of intermediate, NKT cells whose link with the liver is particularly emphasized.[19,20] Supportive elements of their differentiation is IL-7, which is produced by hepatocytes, and IL-12, IL-15 and IL-18, which are produced by Kupffer or endothelial cells.[22–24] Although NKT cells phenotypically represent marked heterogeneity, they usually co-express markers that belong to T and NK lineage, and are characterized by a highly restricted TCR repertoire, made of an invariant TCRα chain, Vα14-Jα281, associated with polyclonal Vβ8, Vβ7, and Vβ2 TCR β chains.[22–27] It was hypothesized that they represent primordial T cells, which stay at an intermediate phylogenetic position between NK-derived and thymus-derived T cells.[22,23] The current dogma is that this novel lineage of lymphocytes are selected by the non-classical MHC class I-like molecule, CD1d, which in mice is expressed on dendritic cells, B cells, T cells, macrophages and hepatocytes.[27,28] Unlike the conventional MHC molecules, which bind short peptides in their antigen-binding groove for presentation to either $CD4^{+}$ or $CD8^{+}$ T cells bearing $\alpha\beta$ TCRs, the CD1 molecules are involved in the presentation of lipid and glycolipid antigens.[25–30] The hydrophobic part of these antigens most probably binds in the CD1 ligand-binding groove, whereas the polar headgroup of these antigens appears to make direct contact with the TCR and determines specific recognition.[29,30] In such a way, CD1 molecules also display diverse, covalently attached carbohydrates to T cells, that then help B cells to mount an antibody response to polysaccharide antigens.[31] CD1-restricted NKT and T cells therefore play an important role in host defense against microbial infection, but participate also in the recognition of distinct self-antigens,[28–32] since natural

ligands for CD1 molecules might be the glycosylphosphatidylinositol anchors and phosphoinositol mannosides,[33] various metabolites produced by the cells,[34] as well as stress proteins MICA and MICB,[35] supporting the hypothesis that NKT cells represent autoreactive, forbidden, clones of T cells, which after activation might eliminate abnormal self-cells.[22–26]

Our data showing increased cytotoxicity of hepatic MNLC after *in vivo* treatment of mice with PGM-Zn against NK-sensitive and syngeneic targets are, therefore, in agreement with current knowledge of induction and function of hepatic NKT cells in bacterial infection.[22,28,36] We are, however, among the first to show that addition of Zn to PGM might potentiate these activities. Owing to the relevance of zinc for good functioning of the entire immune system, where Zn is acting as a catalyst, structural (zinc fingers) and regulatory ion,[37–41] the observed effects obviously might be obtained by different mechanisms. Enhanced cytotoxicity probably includes the effect of Zn^{2+} on the level of 'NK cell immune synapse', where Zn modulates specifically the negative signal transmitted to the NK cells, after binding of killer cell immunoglobulin-like receptors to MHC class I proteins.[42,44] However, owing to the possibility that PGM-Zn acts as superantigen, which markedly enhances the activities of T and B lymphocytes, it could be hypothesized that some changes occurred also at the level of MHC class II molecules, outside the peptide-binding groove, since co-ordination of Zn^{2+} is required for high binding of superantigen to this place.[44] Besides numerous other possibilities,[37–41] we would like also to emphasize the crucial role of zinc for the regulation of apoptosis and cell proliferation,[45,46] because this evidence permits one to speculate that PGM-Zn was directly involved in the apoptotic process and CD1 presentation of self-antigens. Namely, during the infection, activated monocytes and other cells undergo spontaneous apoptosis, which involves Fas/Fas ligand interactions.[47–49] Moreover, since binding of bacterial toxins and bacterial lipoproteins through TLR2 signals for apoptosis through the myeloid differentiation factor 88 via a pathway involving Fas-associated death domain protein and caspase 8, as well as for cytokine production,[50] there is a possibility that some of the PGM-Zn-induced modifications of cell-mediated cytotoxicity occurred on the level of Toll-like receptor-2, which is a ligand also for peptidoglycans, released from Gram-positive bacteria.[1–6] Furthermore, since it is known that the intracellular content released from dying cells may stimulate the generation of autoreactive NKT,[24,27,32,33–35] as well as adaptive immune responses,[51] it could be speculated that, in such a way, PGM-Zn has augmented also the cytotoxicity of splenic MNLC to NK-sensitive and syngeneic targets (Fig. 5). The recent finding of a high-speed communication network between the NKT cells, generated *in vivo* after treatment with Alpha-GalCer, and the adaptive immune systems[52] seems to support this conclusion.

Taken together, although the further characteristics of the effects of PGM-Zn on innate immunity remain to be elucidated, our data provide the first evidence that addition of zinc to PGM markedly enhances the generation of hepatic NKT cells and increases the cytotoxic potential of hepatic and splenic MNLC against NK-sensitive and syngeneic targets.

ACKNOWLEDGMENTS. This work was supported by grants from the Croatian Ministry of Science (project No 062002). The authors wish to express their gratitude to the research staff of Pliva Pharmaceutical and Chemical Works for donating the PGM-Zn used in the study.

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Received 7 November 2001
Accepted 15 February 2002