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Immunoprotective Properties of Peptidoglycan Monomer Linked with Zinc in Cholestatic Jaundice

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Key Words
Peptidoglycan monomer linked with zinc · Common bile duct ligation · Jaundice · Blastogenesis

Abstract

Background: Previously it was shown that a new immunostimulator, peptidoglycan monomer linked with zinc (PGM-Zn), might have immunocorrective and hepatotropic effects. Owing to this in the present study we investigated its effects on jaundice-induced immunodysfunction, which might be responsible for serious peri- and postoperative complications in biliary obstruction. ***Methods:*** In vivo effects of PGM-Zn were analyzed in mice subjected to common bile duct ligation (CBDL), where we estimated phenotypic profile and cell cycle of thymocytes, splenocytes and phagocytic function of peritoneal macrophages. In vitro effects of PGM-Zn were evaluated on blastogenesis of human peripheral blood mononuclear cells (PBMNC), obtained from healthy donors and stimulated with anti-CD3 monoclonal antibody and/or PMA, in the presence or absence of jaundice serum obtained from patients with biliary calculosis. ***Results and Discussion:*** Jaundice induced marked disarrangement of lymphatic homeostasis, which at several points might be blocked by PGM-Zn. In mice it delayed the CBDL-induced decline of CD4+ CD8+ thymocytes, decreased the proportion of CD8+ T cells, and increased the percentage of CD4– CD8– thymocytes, augmenting simultaneously the proportion of thymic cells in S and G2 + M phase of cycle. Similar hyperplastic reaction with increased percentage of CD4+, Ig+ and CD5+ cells was noticed in the spleen, together with the enhanced phagocytic ability of peritoneal macrophages. In human PBMNC jaundice reduced the percentages of CD3, CD5, CD4, CD8 and HLA-DR-expressing cells and increased the proportion of CD25 and perforin-positive lymphocytes. PGM-Zn given in vitro was able to abrogate the antiproliferative activity of jaundice serum on PMA and anti-CD3 + PMA-induced blastogenesis.

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Introduction

Peptidoglycans (PGs) isolated from gram-positive bacterial cell walls are well-known immunomodulating agents, capable to influence the immune reaction generated upon antigen or pathogen stimulation [1, 2]. As a minimal structural unit of PG endowed with immunoadjuvant activity muramyl dipeptide (N-acetyl-muramyl-*L*-alanyl-*D*-isoglutamine) was identified [3], but peptidoglycan monomer [PGM, GlcNAc-MurNAc-*L*-Ala-*D*-iso-Gln-meso-diamminopimelic acid (w-NH_2)-*D*-Ala-*D*-Ala] obtained by biosynthesis from culture fluids of penicillin-treated *Brevibacterium divaricatum* NRRL-2311 has also a similar activity [4]. Its immunostimulating and antimetastatic activity were emphasized in many experimental protocols [5, 6]. Recently, however, we reported that PGM linked with Zn (PGM-Zn) had an even better immunocorrective potential than PGM, which possessed a greater enhancing activity on MHC class II expression in the liver and was able to induce the generation of peritoneal exudate cells (PEC) with suppressive activity on blastic proliferation of syngeneic splenocytes [7, 8]. Furthermore, using the model of in vivo T subset cell depletion, we found that stimulating activity of PGM-Zn on humoral immunity might include both polyclonal stimulation of B lymphocytes and generation of costimulatory signals from T cells, implying that it had some characteristic of T-independent-2 antigens [9]. However, since the mechanism of immunoregulatory activity of this new PGM analogue is still unclear, in this study we attempted to clarify its effect on immune dysfunction induced by cholestatic jaundice: (1) in mice subjected to common bile duct ligation (CBDL) and (2) in patients with biliary obstruction. As is well known, both conditions induce marked disarrangement of nonspecific and specific immunity, linked with liver injury and biochemical alterations in jaundiced serum [10, 11], which might result in serious peri- and postoperative complications and a high mortality rate (even up to 13%). Trying to elucidate the immunostimulating activities of PGM-Zn, in this study we investigated its in vivo effects on murine thymus, spleen and peritoneal macrophages, as well as its in vitro ability to modulate the immunosuppressive properties of jaundiced serum, obtained from patients with cholestasis. The data confirmed that PGM-Zn has immunomodulating and hepatotropic effects, which might diminish several toxic effects of cholestatic jaundice.

Material and Methods

Peptidoglycan Monomer Linked with Zn

The immunomodulating agent PGM [GlcNAc-MurNAc-*L*-Ala-*D*-iso-Gln-meso-diamminopimelic acid (w-NH_2)-*D*-Ala-*D*-Ala] linked with zinc (PGM-Zn, 'Pliva'-Zagreb) in the molar ratio 1:1 was prepared by biosynthesis from the culture fluids of *B. divaricatum* NRRL-2311, as an apyrogenic, water-soluble substance, devoid of any toxic effects [4]. The sample used in this study contained less than 0.015 ng endotoxin/mg PGM, according to the limulus amebocyte lysate test (Pyrostat Kit, Millipore).

Subjects

Peripheral blood was collected from 24 patients suffering from biliary calculosis, and directed to the operation and from 24 blood volunteer donors from the Transfusion Center. Both groups were aged from 35 to 60 years.

Mice

In all experiments we used inbred, 2- to 3-month-old, male mice of the BALB/c (H-2^d) strain. They were originally obtained from the Jackson Laboratory and bred in the Central Animal Facility at the Medical Faculty in Rijeka, Croatia. During the experiments, the animals were kept in standard plastic cages, fed with standard mouse food pellets and water ad libitum and exposed to the natural daylight cycle.

Induction of Cholestatic Jaundice in Mice and Administration Protocol for PMG-Zn

Cholestatic jaundice in mice was induced by CBDL after upper midline incision of abdomen in light ether anesthesia. Treatment with PGM-Zn (10 mg/kg of body weight, i.p.) started 6 days before CBDL, giving PGM-Zn dissolved in PBS every 2nd day and immediately after CBDL. Corresponding control groups of mice were identically treated with PBS alone. To elucidate the effects of PGM-Zn on normal and cholestatic animals, the groups of PGM-Zn or PBS-pretreated mice were sacrificed both immediately before CBDL (on day 0), and 1, 2 or 3 days after ligation of biliary duct.

Cell Separation

Human peripheral blood leukocytes (PBL) were separated from heparinized venous blood by Ficoll-Hypaque (Pharmacia Fine Chemicals) density gradient after centrifugation for 20 min at 800 *g*. Cells accumulating at the interface were washed twice in RPMI 1640 and resuspended at a final concentration of 1×10^6 PBL/sample in FACS buffer. Murine spleen and thymuses were aseptically removed from experimental animals and gently pressed through fine stainless steel screens in RPMI 1640. Red blood cells were lysed with Tris-buffered ammonium chloride for 5 min. After washing, the cell suspension was filtered through a fine nylon mesh, resuspended in complete medium and adjusted to the desired final concentration for FACS and cell cycle analysis.

Cytofluorometric Analysis

Surface phenotype of human PBL and murine thymocytes and splenocytes was identified by direct immunofluorescence analysis on FACScan (Becton Dickinson, Immunocytometry Systems, Mountain View, Calif.), using FACScan Research Software, as previously described [7]. Briefly, after rewashing the previously made suspension of cells in RPMI 1640, the cell number was adjusted to $1 \times$

10^6/ml. This suspension was centrifuged and the cells were resuspended in 50 µl of cold FACS medium and incubated with 5 µg of primary monoclonal antibodies (mAb) dissolved in the same volume. For human PBL the following mAbs obtained from Becton Dickinson were used: Leu-4 (anti-CD3), Leu-3a (anti-CD4), Leu-2a (anti-CD8), Leu-11b (anti-CD16) and Leu-19 (anti-CD56). A murine, anti-human perforin mAb δG9 (IgG2b) was purified from Balb/c ascites [12]. For murine thymocytes and splenocytes PE-conjugated goat anti-mouse CD4 and CD8 or FITC-conjugated goat anti-mouse CD5 and Ig (Becton Dickinson) were used. After an incubation of 30 min at +4°C, and removing the unbound antibodies by washing in FACS medium, the cells were finally resuspended in 1 ml of FACS medium. Propidium iodide (PI, Sigma; 1 µg/ml)-stained dead cells were excluded by electronic gating. Relative fluorescence intensities were expressed on a log scale with 2×10^4 cells analyzed.

Cell Cycle Analysis

Cell cycle analysis (G0/G1, S and G2 + M phases) was done on the FACScan flow cytometer (Becton Dickinson). For this purpose thymocytes or splenocytes were washed twice in RPMI 1640, counted and 2×10^6 cells were fixed with ice-cold 70% ethanol. After overnight fixation at 4°C, 70% ethanol was removed and fixed cells were resuspended in PI (Sigma) staining solution (0.05 mg/ml PI; PBS, pH 7.4 with 1% glucose; 0.1 mg/ml RNAse A), and allowed to stain for 1 h at room temperature in the dark. Debris was excluded from thymocyte analysis by selective forward versus side scatter gating.

Determination of Phagocytic Activity of PEC

For evaluation of phagocytosis the modification of Lehrer's method was used [13]. The procedure consists of coincubating the resident peritoneal macrophages of mice with heat-inactivated (at 90°C/ 30 min) *Candida albicans* and microscopic scoring of its ingestion. For this purpose the peritoneal macrophages (obtained by rinsing of peritoneal cavity with 4 ml of RPMI) and *C. albicans* (prepared as suspension of 1 colony of *C. albicans* from Sabouraud's agar slants in 30 ml of sterile saline solution) were cocultured for 30 min in plastic rings. After incubation, the content of the experimental and control chamber was slit out and the remaining glass-adherent cells were fixed with methanol and stained with Giemsa. Under the magnification of 625 × 100 macrophages were counted and the percentage of those containing one or more ingested *C. albicans* was determined.

Proliferative Activity of Human Peripheral Blood Mononuclear Cells in vitro in the Presence of Cholestatic Serum

To elucidate the effect of cholestatic serum on blastic transformation of lymphocytes, human PBL were isolated from fresh defibrinated blood obtained from healthy volunteers by Ficoll-Hypaque (Pharmacia Fine Chemicals) density centrifugation and stimulated by activators of different transmembrane pathways. After washing in RPMI 1640 PBC were finally resuspended at the concentration of 5×10^6 cells/ml in complete culture medium (RPMI 1640, 10% heat-inactivated FCS, *l*-glutamine 2 m*M*, penicillin 100 U/ml, 100 ng/ml streptomycin, 10 m*M* HEPES and 5×10^{-6} *M* 2-mercaptoethanol purchased from Gibco Laboratories, Grand Island, N.Y.). Blastic proliferation of isolated cells was induced by anti-human CD3 mAb (OKT-3, Ortho) in a dose of 0.2 µg/ml, by phorbol myristate acetate (PMA, Sigma Chemical Co.) in a dose of 40 ng/ml, or by combinations of these two stimulators. Simultaneously with mitogens the cholestatic or normal serum was added in different concentrations. The intensity of blastic proliferation was assessed 3 days later. For this purpose, 18–24 h before the end of the culture, 1 µCi of ^{3}H-thymidine (specific activity of 5 Ci/mol, CEA, France) was added in a volume of 50 µl. Cells were collected on fiber-glass strips using a multiple harvester (Titertek, Flow Laboratories) and counts per minute were determined by liquid scintillation counter (Tracor Analytic Delta 300). The activation was calculated from the differences found in the incorporation of ^{3}H-thymidine in stimulated and unstimulated cells.

Statistical Analysis

Data were analyzed using the Sigma Plot Scientific Graphing System, Version 4.03 (Jandel Scientific) and one- or two-way analysis of variance (ANOVA), using the computer program StatSoft. The differences were considered statistically significant for $p < 0.05$.

Results

Immunocorrective Effects of PGM-Zn in Cholestatic Mice

To obtain an insight into the effects of PGM-Zn and cholestasis on lymphatic organs and function of macrophages, the groups of mice treated during 6 days with PGM-Zn or PBS were sacrificed before the CBDL (time 0) and 1, 2 and 3 days after the ligation of bile duct, respectively. Effects on thymic and splenic cell differentiation and proliferation were evaluated by estimation of phenotypic profile of lymphatic cells and cell cycle analysis, while the function of macrophages was tested by estimation of phygocytic abilities of PEC.

Thymus. To elucidate the changes in thymic T cell composition the percentages of CD4– CD8– (double-negative; DN), CD4+ CD8+ (double-positive cells, DP) and committed CD4+ CD8– and CD4– CD8+ (single-positive, SP) T cells were estimated. The data revealed (fig. 1) that short pretreatment with PGM-Zn in unoperated mice (time 0) did not change the phenotypic profile of cells in thymus. CBDL, however, in control, PBS-treated mice markedly reduced the percentage of DP cells (on the 1st and 2nd p.o. day), increasing simultaneously the percentage of DN as well as SP CD4+ CD8– and CD4– CD8+ thymocytes. Pretreatment with PGM-Zn at several time points changed these events. The initial rise in the percentage of SP CD8+ T cells was not seen, while the fall of DP cells was translocated to the later period (on the 3rd day) and followed by a markedly increased proportion of DN and particularly CD4+ T cells. Cell cycle analysis (fig. 2) revealed that jaundice initially diminished (on the 1st and 2nd day) and then augmented (on the 3rd p.o. day) the percentage of thymic cells in the S and G2 + M

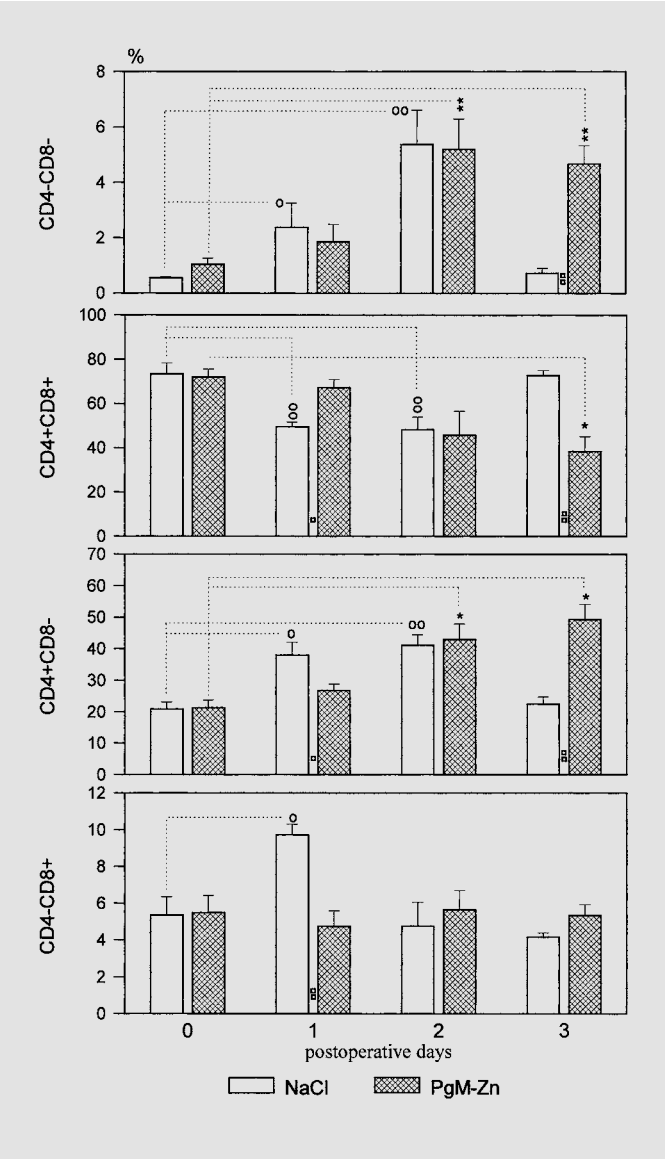

Fig. 1. Phenotypic profiles of thymic cells after CBDL (done on day 0) and pretreatment with saline solution or PGM-Zn (as described in Material and Methods). Results are expressed as mean ± SE (n = 6). O and * = Significance of saline- and PGM-Zn-treated mice versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$.

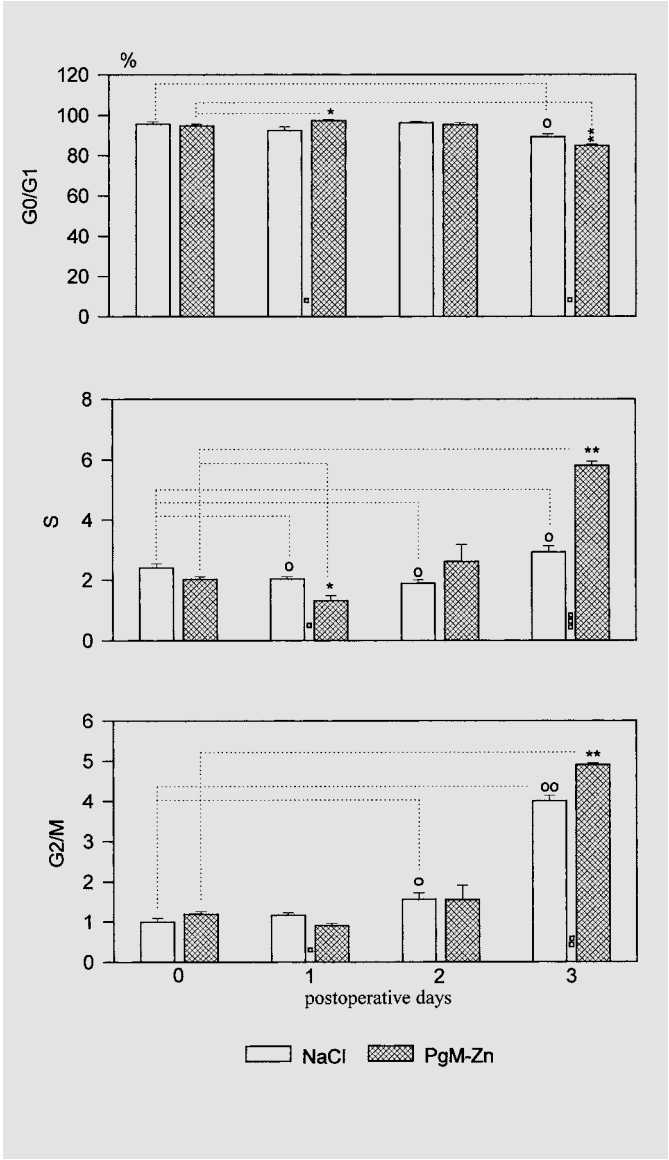

Fig. 2. Cell cycle analysis of thymocytes after CBDL (done on day 0) and pretreatment with saline solution or PGM-Zn. Results are expressed as mean ± SE (n = 6). O and * = Significance of saline- and PGM-Zn-treated mice versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$; three symbols: $p < 0.001$.

phase of the cycle. Pretreatment with PGM-Zn enhanced these changes inducing, however, a significantly greater later hyperplastic answer in the thymus of cholestatic mice.

Spleen. Obstructive jaundice in the spleen decreased the proportion of almost all tested cell types: CD4+ cells in all intervals, CD5+ cells on the 1st and 2nd day, CD8+ cells on the 2nd and Ig+ on the 3rd day (fig. 3). Pretreatment with PGM-Zn, however, increased the starting level of Ig+ cells and abolished the cholestasis-induced declines, minimizing the fall in CD4+, CD5+ and Ig+ cells in the spleen (fig. 3). Simultaneously, jaundice on the 1st

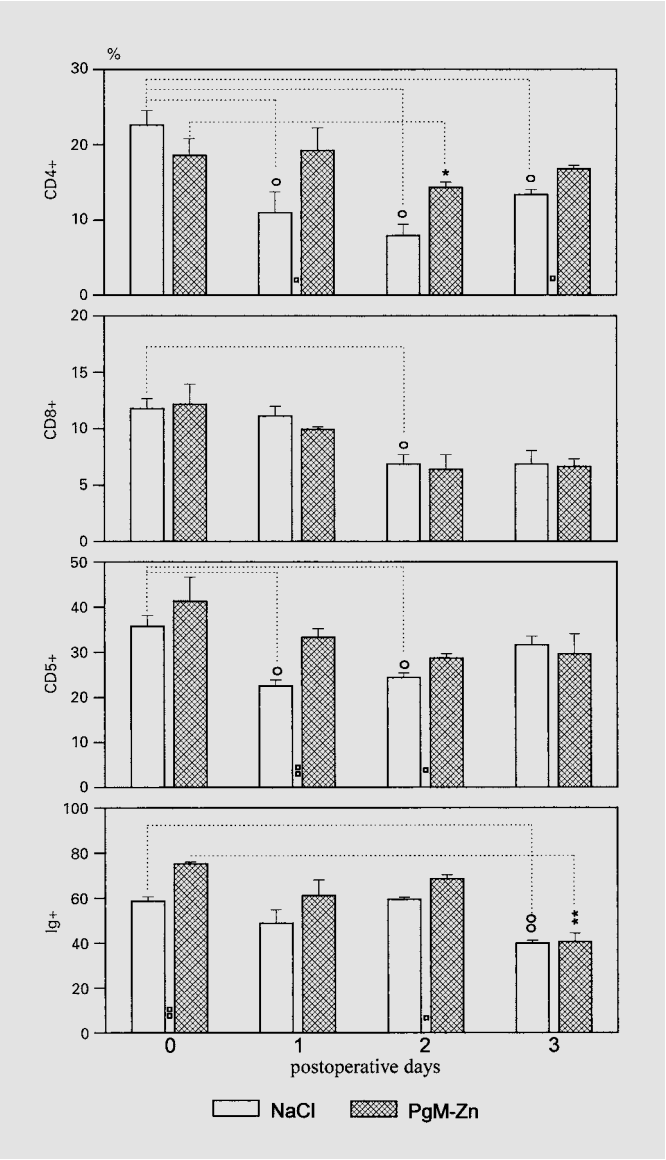

Fig. 3. Phenotypic profiles of splenic cells after CBDL (done on day 0) and pretreatment with saline solution or PGM-Zn. Results are expressed as mean ± SE (n = 6). O and * = Significance of saline- and PGM-Zn-treated mice versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$.

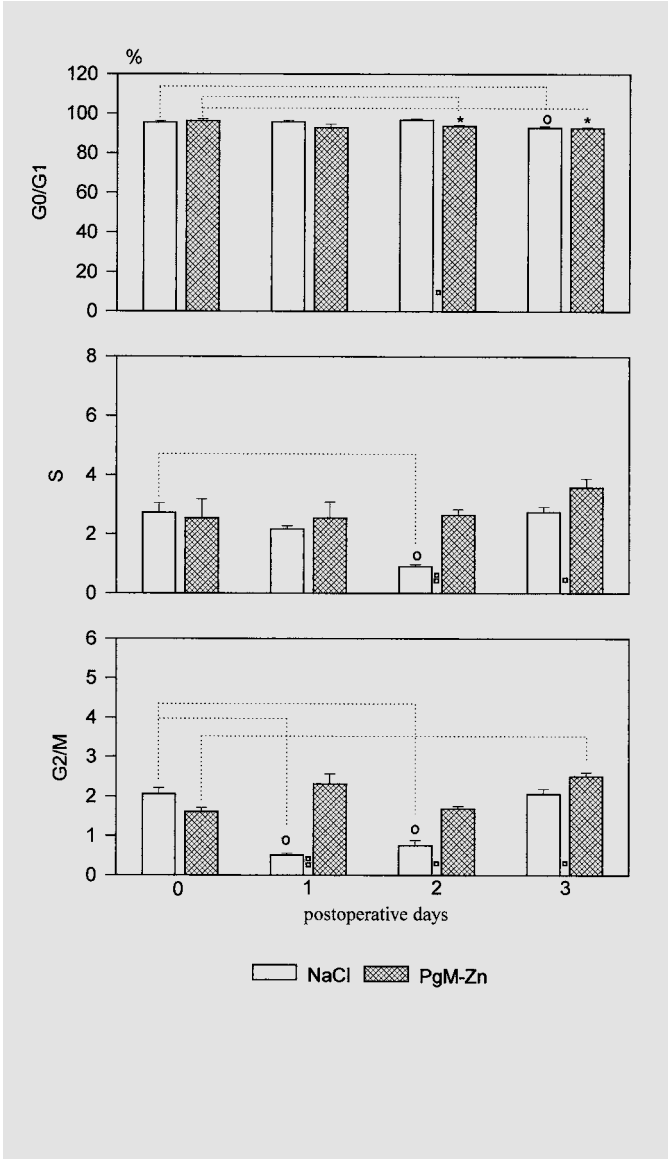

Fig. 4. Cell cycle analysis of splenocytes after CBDL (done on day 0) and pretreatment with saline solution or PGM-Zn. Results are expressed as mean ± SE (n = 6). O and * = Significance of saline- and PGM-Zn-treated mice versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$.

and 2nd p.o. day reduced the proportion of splenic cells in the S and G2 + M phase. PGM-Zn, however, completely prevented this decline, inducing in cholestatic mice an additional hyperplasia on the 3rd p.o. day (fig. 4).

Phagocytic Activity of Peritoneal Macrophages. In contrast to a weak effect of PGM-Zn on T cells in unoperated mice (fig. 1, 3), short pretreatment with PGM-Zn significantly augmented the phagocytic activity of peritoneal macrophages in normal mice at time 0 (fig. 5). Cholestasis suppressed this function, but values found in PGM-Zn-treated mice, at all time points, were significantly higher than in the control, PBS-treated mice.

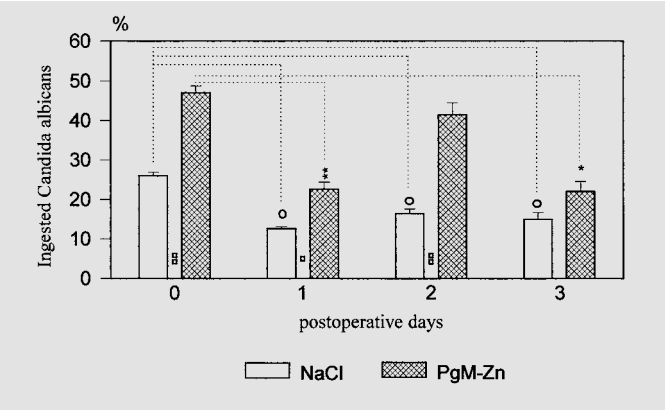

Fig. 5. Phagocytic activity of PEC after CBDL and pretreatment with saline solution or PGM-Zn. Results are expressed as mean ± SE (n = 6). O and * = Significance of saline- and PGM-Zn-treated mice versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$.

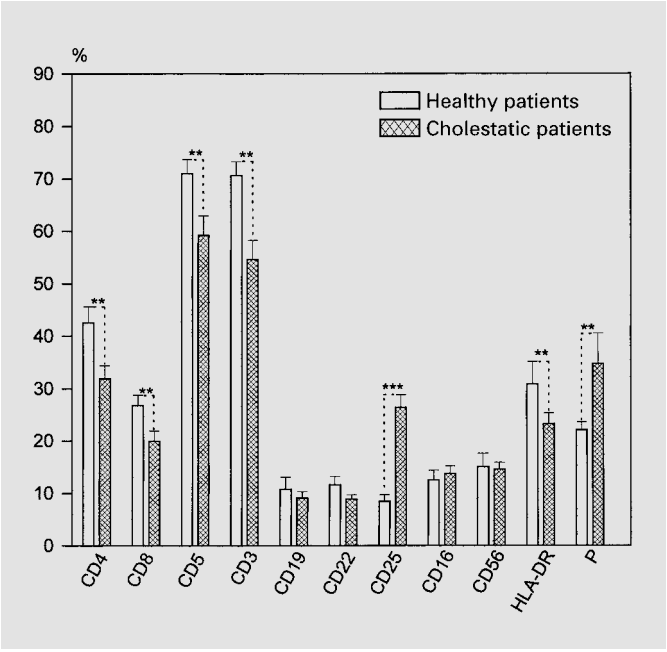

Fig. 6. Comparison of the phenotypic profile of PBMNC obtained from healthy and cholestatic patients suffering from biliary calculosis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Jaundice-Induced Changes in Humans

To elucidate the jaundice-induced changes in the immune system in patients suffering from biliary calculosis and investigate the potential immunocorrective effects of PGM-Zn in these events we (1) compared the phenotypic profile of their peripheral blood mononuclear cells (PBMNC) with those from healthy individuals, and (2) tested in vitro the ability of jaundice serum to affect the reactivity of normal lymphocytes to activators of different transmembrane pathways in the presence and in the absence of PGM-Zn.

Phenotypic Profile of PBMNC in Obstructive Jaundice. As presented in figure 6, in subjects with jaundice reduced percentages of CD4, CD8, CD5, CD3 and HLA-DR-expressing cells were found. In contrast to this, the proportion of CD25 and perforin-positive lymphocytes increased.

Effects of Jaundice Serum and PGM-Zn on Blastic Proliferation of Normal PBMNC Induced by Different Types of Activators. To test the possibility that immunosuppression seen in cholestasis is linked with changes in the composition of jaundice serum, we activated the PBMNC, obtained from healthy donors with anti-CD3 mAb and/or PMA in the presence of an increasing concentration of normal or jaundice serum (fig. 7a, 8a, 9a). The data showed that both types of serum increased the anti-CD3 and particularly PMA and anti-CD3 + PMA-induced blastogenesis. However, the intensity of this stimulation after the addition of jaundice serum in cultures stimulated with anti-CD3 mAb was even greater (fig. 7), in contrast to that stimulated with PMA or anti-CD3 + PMA, where greater concentrations of jaundice serum gave a significantly smaller stimulation in comparison with normal serum (fig. 8, 9).

In further experiments we tried to abrogate the inhibitory effects of jaundice serum on blastogenesis by in vitro addition of PGM-Zn. For this purpose PBMNC stimulated with anti-CD3 and/or PMA were cocultivated with normal or jaundice serum in a concentration of 200 μg/ml, and exposed to an increasing concentration of PGM-Zn (fig. 7b, 8b, 9b). The data showed that greater concentrations of PGM-Zn additionally stimulated all types of blastogenesis, abrogating the difference between the effects of normal and jaundice serum in anti-CD3 and PMA-stimulated blastogenesis (fig. 7, 8), and giving, in cultures stimulated with anti-CD3 + PMA, an even greater answer in the presence of jaundice than normal serum (fig. 9; PGM-Zn in a concentration of 100 and 300 μg/ml).

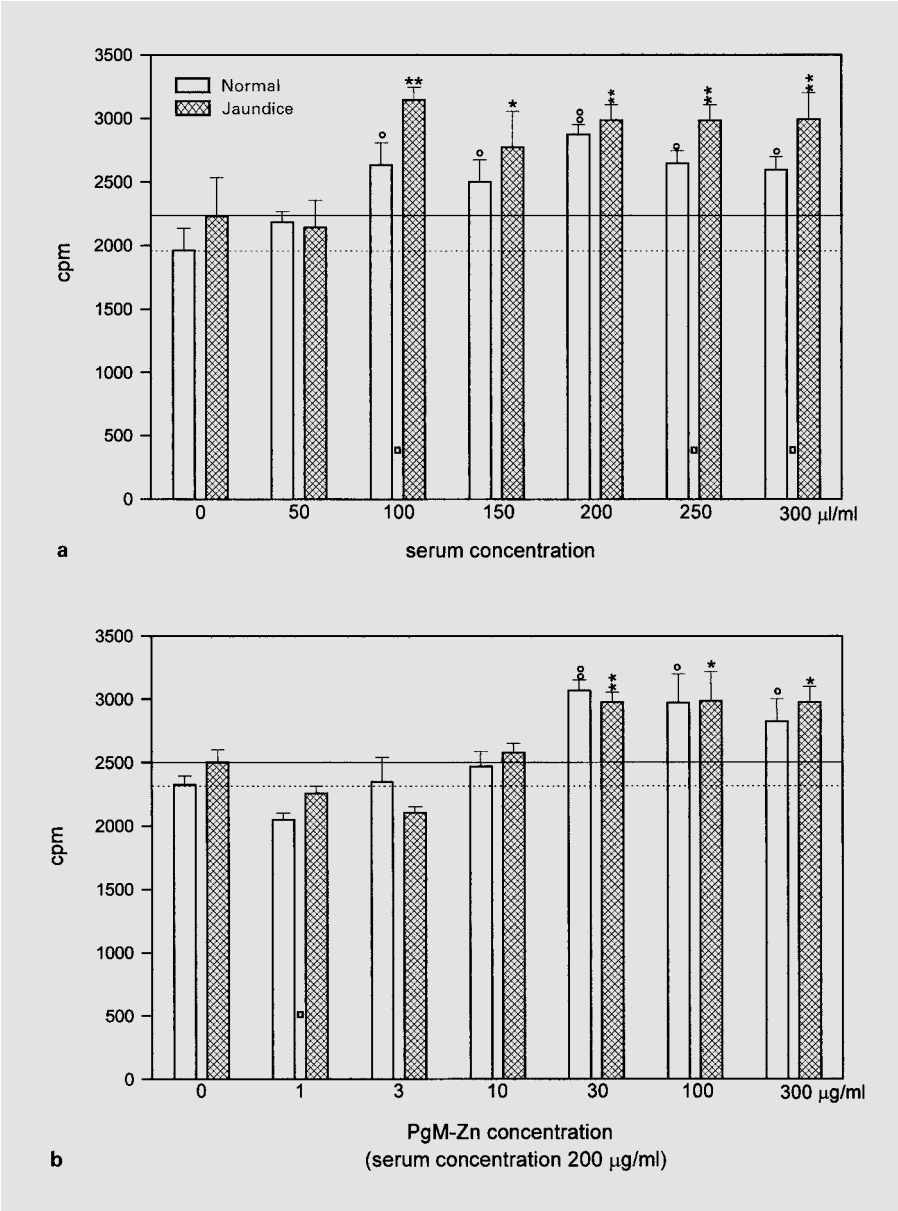

Fig. 7. a Blastic transformation of PBMNC obtained from healthy donors and stimulated with anti-CD3 mAb in the presence of normal and jaundice serum from patients suffering from biliary calculosis. **b** Effects of PGM-Zn on cultures of PBMNC activated with anti-CD3 in the presence of normal and jaundice serum in a concentration of 200 µg/ml. ^{O} and * = Significance of normal and jaundice serum versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$.

Discussion

The main outcome of this study was the finding that PGM-Zn might both in vivo and in vitro affect some aspects of jaundice-related immune dysfunction. Data obtained in mice showed that experimental cholestatic jaundice might induce a marked immunosuppression visible on the level of the thymus (decline of DP and increase of DN, CD4+ CD8- and particularly CD4- CD8+ T cells; fig. 1), spleen (decrease of CD4+ and CD5+ cells; fig. 3) and peritoneal macrophages (reduced phagocytic activity; fig. 5). Similarly, in patients with obstructive jaundice, among the PBMNC we found significantly reduced percentages of CD3, CD5, CD4, CD8 and HLA-DR-expressing lymphatic cells, as well as an increased proportion of CD25 and perforin-positive cells (fig. 6), suggesting that during the jaundice some IL-2R+ T or NK cells are particularly activated with cytotoxic and cytolytic activities. In vitro obtained data also suggest that jaundice serum contains some components which potentiate blastogenesis, induced by anti-CD3 mAb, but markedly suppress the proliferation of PMA and anti-CD3 + PMA-activated

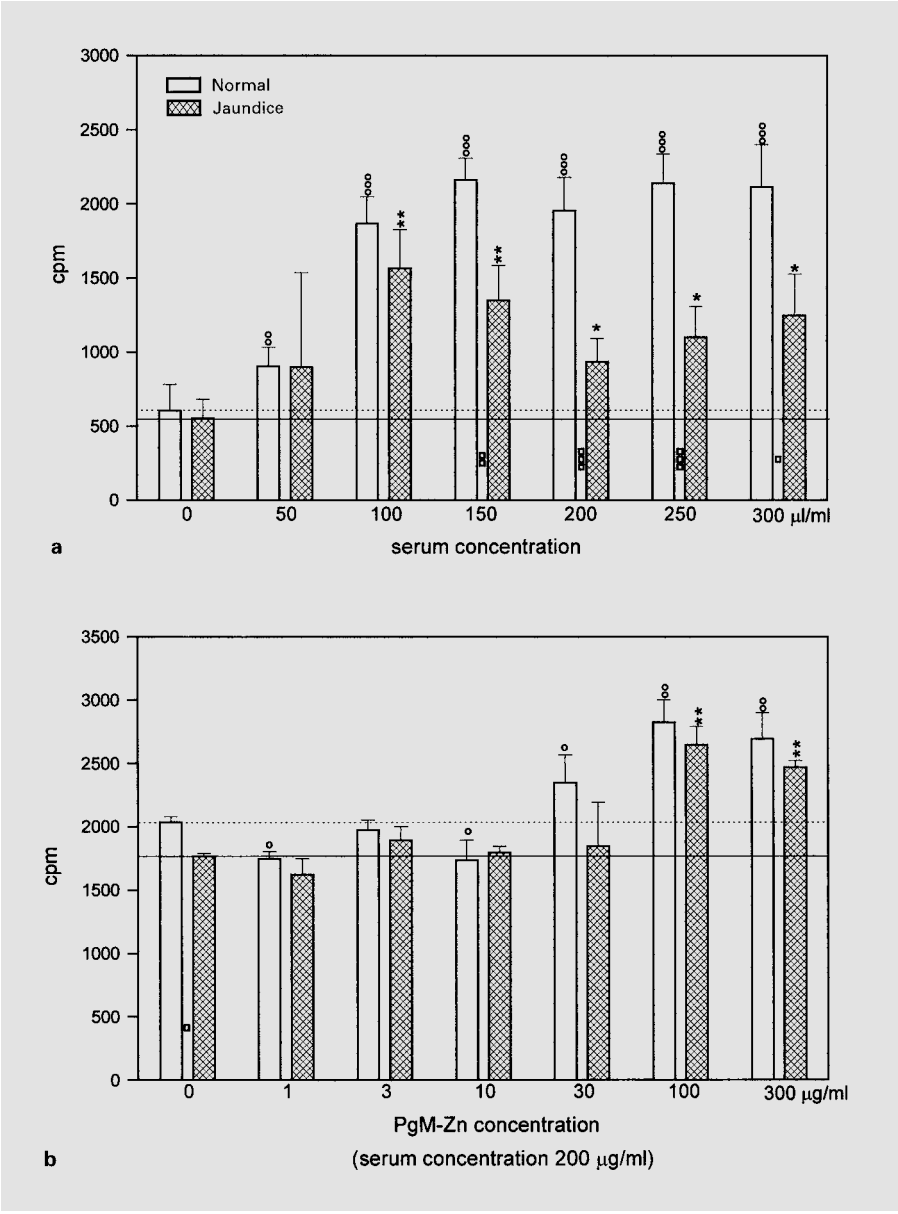

Fig. 8. a Blastic transformation of PBMNC obtained from healthy donors and stimulated with PMA in the presence of normal and jaundice serum from patients suffering from biliary calculosis. **b** Effects of PGM-Zn on cultures of PBMNC activated with PMA in the presence of normal and jaundice serum in a concentration of 200 µg/ml. ^{O} and * = Significance of normal and jaundice serum versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$; three symbols: $p < 0.001$.

human PBMNC (fig. 7–9). Results emphasize that jaundice serum in humans differently affects the events activated by various transmembrane pathways in proliferating lymphocytes and imply that it might block especially some protein kinase C-dependent mechanism activated by PMA. Although the later speculation needs to be proved, the overall data support the widely accepted evidence that obstructive jaundice in mice and patients induces a severe immune dysfunction, related to the accumulation of toxic substances in serum and development of progressive liver failure [10, 11]. The data, however, to our knowledge, also give the first proof that treatment with PGM-Zn may at several levels change these events. Thus, in the thymus of cholestatic mice pretreatment with PGM-Zn delayed the decline of DP T cells, abrogated the early increase of CD8+ T cells (on the 1st p.o. day), and increased the percentage of DN thymocytes (on the 3rd p.o. day), augmenting also the proportion of thymic cells in the S and G2 + M phase of cell cycle (fig. 2). The data point to immunoproliferative effects of PGM-Zn and suggest that it probably delayed the apoptosis, reflected usually as a decreased percentage of DP thymic cells [14].

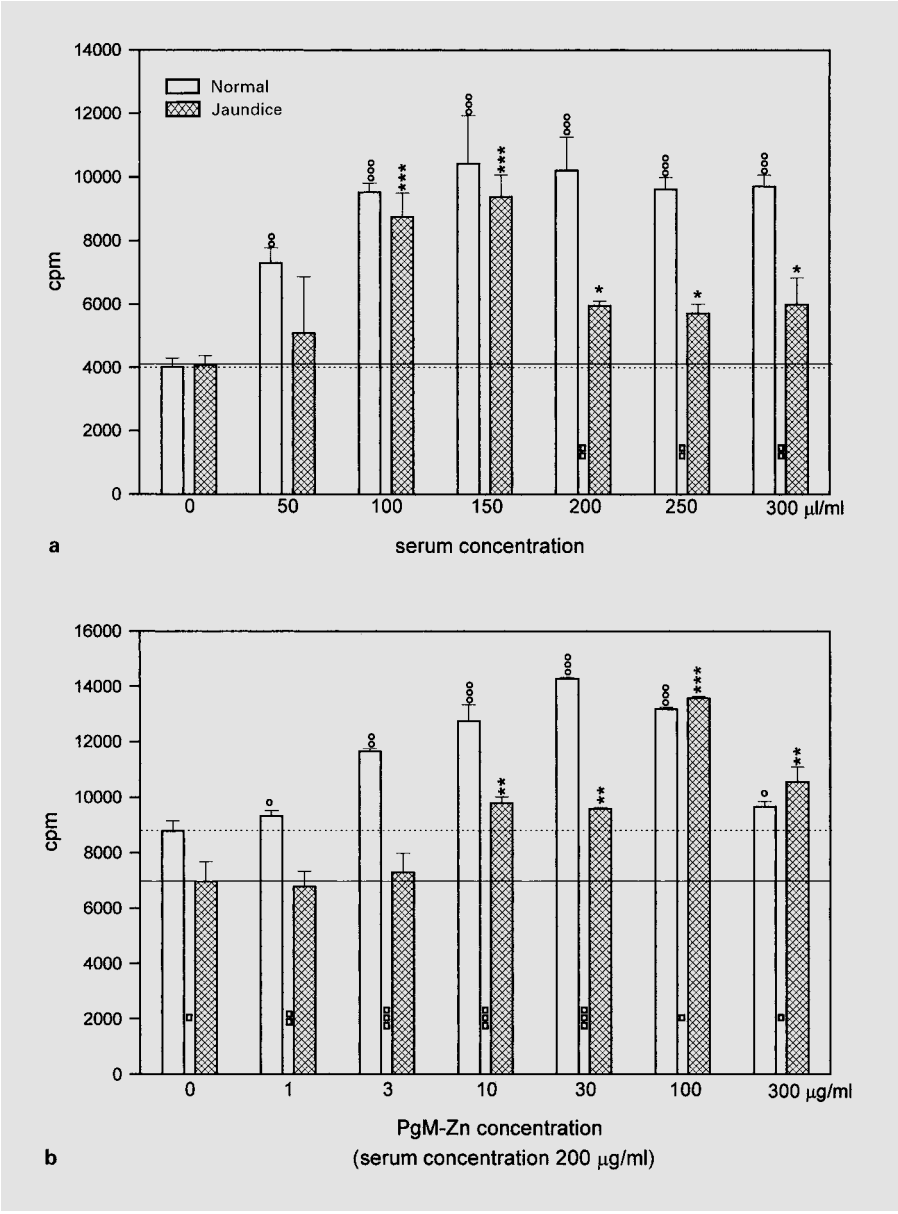

Fig. 9. a Blastic transformation of PBMNC obtained from healthy donors and stimulated with anti-CD3 mAb + PMA in the presence of normal and jaundice serum from patients suffering from biliary calculosis. **b** Effects of PGM-Zn on cultures of PBMNC activated with anti-CD3 + PMA in the presence of normal and jaundice serum in a concentration of 200 µg/ml. ^{O} and * = Significance of normal and jaundice serum versus control values; □ = significance between the experimental and control group. One symbol: $p < 0.05$; two symbols: $p < 0.01$; three symbols: $p < 0.001$.

A similar hyperplastic answer with an increase of CD4+, Ig+ and CD5+ cells was noticed in the spleen (fig. 3, 4), confirming that PGM-Zn may stimulate the proliferation of T lymphocytes and enhance the antibody production, acting as a polyclonal activator of B cells, or as a T cell-independent antigen type 2 [9]. In agreement with our previous findings [7, 8] it also markedly enhanced the expression of MHC class II antigens on hepatic mononuclear lymphatic cells (not shown) and corrected the diminished phagocytic ability of peritoneal macrophages (fig. 5). Furthermore, PGM-Zn given in vitro to cultures of human PBMNC stimulated with PMA or anti-CD3 mAb + PMA abrogated also the inhibitory effects of jaundice serum on lymphatic blastogenesis (fig. 8, 9). Taken together, although the mechanism of its action is still unclear, the data suggest that PGM-Zn has interfered with several pathogenic pathways leading to immunosuppression during the jaundice.

PGM-Zn as a new metal complex of PGM was synthesized in an attempt to combine the immunostimulating properties of PGM [5–9] and Zn [15, 16]. Its enhancing effects on B cells seems to be related with its ability to

potentiate the costimulatory signals coming from activated T cells and enhance the hepatic MHC class II expression [7]. Its effects on isotype switching to IgG1 and IgG2a correlated also with the changes in splenic CD4+, CD8+ and CD5+ cells, pointing to the regulatory role of these cells and/or their cytokines in PGM-Zn-induced immunostimulation [9]. Furthermore, in vitro we noticed that PGM-Zn in a CD14-dependent manner may induce the secretion of IL-1, IL-6 and TNF-α from human PBMNC (unpubl. data). Since most of these data suggested that PGM-Zn is an immunostimulator which shares some properties with the lipopolysaccharide (LPS), but has a different mode of action on lymphatic cells, we speculate that there is the possibility that PGM-Zn has, during jaundice, interfered with some LPS-induced pathways. Namely, in cholestatic jaundice in plasma toxic factors like bilirubin, bile acids, endotoxin, α_2-globulin and abnormal lipoproteins [11] accumulate, which alter Kupffer cell function [17], interfere with the early events at the T cell recognition stage [18] and impair the cell-mediated immunity [19]. This eventually leads to gut barrier failure, systemic endotoxemia and translocation of bacteria and endotoxin to the liver [20], as well as to release of large quantities of mainly proinflammatory cytokines (TNF-α, IL-1, IL-6) from various host cells [21]. Moreover, most of these participants after bile duct ligation show a greater expression of CD14 [22, 23], which is the receptor not only for LPS, but also for gram-positive microbial components (soluble PGs, lipoteichoic and teichoic acid), and use the same CD14-dependent pathways, sharing the same membranous CD14 receptors on target tissue and the same lipid transfer protein (LBP) to accelerate the binding to soluble CD14 and enhance the responses to LPS and soluble PG [2]. Supporting the possible inference of PGM-Zn with LPS-induced events at the level of CD14, which represent a pattern recognition receptor for a diverse array of bacterial constituents [24], the comparison based on several aspects of bindings of LPS and soluble PG on CD14 revealed the existence of partially identical and partially different conformation binding sites for both bacterial products [25, 26]. Our finding that PGM-Zn given in vitro may abrogate the jaundice induced blockade of some protein C-related event during activation of human PBMNC by PMA and anti-CD3 + PMA (fig. 8, 9) also points to interference at the postreceptor level.

There is, however, also the possibility that PGM-Zn has influenced the hepatic production of LBP, and other aspects of LBP function, since PGM might transiently inhibit the activity of UDP-glucuronyltransferase and cytochrome P 450-related enzymes in the liver [27]. The supplementation with Zn probably also had important effects on the liver, since it was reported that patients with chronic liver diseases had a reduced serum level of zinc related with endotoxemia [16]. Additionally, since Zn deficiency also induces the TH_1/TH_2 imbalance with high levels of TH_2 cytokines IL-4, IL-6, IL-10, leukotriene B_4 and prostaglandin E_2 [15, 28], there is the possibility that pretreatment with PGM-Zn has also enhanced the TH_1 function. In this case the greater production of IL-2, IFNγ as well as nitrogen monoxide might explain the increased appearance of PEC with suppressive abilities [7, 8, 29], as well as the greater effects of PGM-Zn than PGM alone on hepatic MHC class II expression in cholestatic liver [7], and its enhancing effects on phagocytosis (fig. 5).

It has also been suggested that Zn^{2+} plays a central and diverse role in the structure and function of several superantigens [30], implying that PGM-Zn might also act as a superantigen. New evidence shows that Zn^{2+} is required to coordinate the high affinity binding to the β chain of the class II molecule and is involved in stabilizing the homodimeric structure of the superantigen [31, 32]. Trimolecular complexes that directly bridge T cells and antigen-presenting cells, in that case, probably elicited a more powerful stimulation of the immune response in our cholestatic mice, affecting the antigen-presenting-cell-derived cytokines as well as certain Vβ subsets of T cells.

Besides, Zn^{2+} has a crucial role in basic cellular functions [33], in other proteins that have zinc-binding motifs [34], and in the maintenance of neuroendocrine homeostasis [35, 36]. Since most of these factors might be seriously disturbed in septic shock [1], PGM-Zn obviously represents an interesting new immunomodulator, that is able to correct some jaundice-related events.

In conclusion, although the mechanism of action of PGM-Zn needs further clarification, the data presented in this work are the first pointing to the immunocorrective potential of PGM-Zn in experimental jaundice, and its ability to abrogate the jaundice-induced blockade of blastogenesis of human PBMNC in vitro.

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