Volume 242, number 2, 357-362 FEB 06640 January 1989

Induction of expression of protein disulphide-isomerase during lymphocyte maturation stimulated by bacterial lipopolysaccharide

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Received 23 October 1988

Protein disulphide-isomerase (PDI) activity, and the level of immunodetectable PDI protein, were monitored in splenic lymphocytes and in BCL_1 cells during culture in the presence of various activating factors. Bacterial lipopolysaccharide stimulated induction of PDI in splenic B cells and BCL_1 cells. The time-course and specificity of induction indicated that the increase in expression of PDI is closely coupled to the final stages of B cell differentiation into antibody-producing plasma cells. The system will prove valuable in studies on the control of expression of PDI.

Protein disulfide-isomerase; Lymphocyte maturation; Endoplasmic reticulum; Enzyme induction

1. INTRODUCTION

Protein disulphide-isomerase (PDI), an enzyme found within the e.r. lumen of a wide range of eukaryotic cells, catalyzes thiol-disulphide interchange in proteins in vitro and is believed to be responsible for the formation of the native set of disulphide bonds during secretory protein folding at biosynthesis [1,2]. The evidence for this biological role includes the finding, in several systems, that the level of PDI activity undergoes developmental changes in parallel with changes in the rate of synthesis of a major disulphide-bonded protein [3,4] and the observation, both in lymphoma cell lines and fibroblast lines in culture, of a quantitative correlation between PDI activity and the rate of synthesis of immunoglobulins and procollagens, respectively [5,6]. This correlation suggests that the expression of PDI is carefully regulated, but the mechanism of regulation is unknown.

In the process of differentiation and maturation to Ig-secreting plasma cells, B lymphocytes increase the rate of synthesis of immunoglobulins by more than an order of magnitude. This is a promising system in which to study the regulation of PDI expression, since PDI can catalyze in vitro the formation of disulphide bonds in immunoglobulin assembly [5,7,8], and in mouse lymphoma cell lines, there is a 40-fold variation in PDI activity with the highest levels of activity being seen in highly secretory lines such as MOPC 104E (IgM) and P1 (IgG_{2a}) [5]. The process of lymphocyte differentiation and maturation has been defined in detail, so that a variety of stages, and factors required for progress to each stage, can be distinguished [9].

No direct study of PDI activity through lymphocyte maturation has been reported. We demonstrate here that the enzyme is induced by the polyclonal B cell activator, bacterial lipopolysaccharide (LPS), and that the specificity and time-course of this induction suggest that the expression of PDI is closely coupled to the final stages of differentiation to the secreting plasma cell. A preliminary account of this work has been presented [10].

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00145793/89/$3.50

2. MATERIALS AND METHODS

Bovine protein disulphide-isomerase was purified to homogeneity [11]. A rabbit was injected a total of 7 times with 0.5 mg of purified enzyme mixed 1:1 with Freund's complete adjuvant. The resultant antiserum (J3B) was stored in 1 ml aliquots at −20°C. For immunoblotting, SDS-PAGE gels were electroeluted onto nitrocellulose filters either at 100 mA overnight, or at 250 mA for 3–4 h, in 25 mM Tris, 0.2 M glycine containing 20% (v/v) methanol. Blots were developed using J3B at a dilution of 1:500 in 10 mM Tris-HCl, pH 7.4, 0.82% (w/v) NaCl and 0.1% (v/v) Tween 20, followed by anti-rabbit IgG horseradish peroxidase conjugate (Sigma) at a dilution of 1:750. Foetal calf serum was used to block excess sites; see [12] for details.

For ELISA, homogeneous bovine PDI was dissolved in sodium carbonate buffer (0.05 M, pH 9.6) to a concentration of 10 μg/ml and 0.1 ml aliquots were applied in duplicate to wells of a micro-ELISA plate, followed by doubling dilutions to generate a standard curve. The concentration of PDI in cell sonicates was directly assayed by sensitizing the microtitre plates with aliquots of sonicate in the same buffer, by overnight incubation at 4°C or by incubation for 3–4 h at 37°C in a moist airtight box. Wells were washed twice with 1% (w/v) BSA and 0.5% (v/v) Tween 20 in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.2) and blocked by incubation for 1 h at 37°C with 0.2 ml per well of 1% (w/v) BSA in PBS. A 200 μl aliquot of antiserum (J3B) diluted 1:500 was added to each well. After a two further washes with the above washing buffer, 0.1 ml of second antibody (as for immunoblotting) was added per well and incubated for 1 h at 37°C. Wells were washed once, 0.1 ml of substrate solution was added and after 10–30 min 0.05 ml of 12.5% H_2SO_4 was added to terminate reaction, and plates were read with a Dynatech automatic plate reader with a 490 nm filter. Substrate solution comprised 40 mg of *o*-phenylene-diamine dissolved in pH 5 buffer (26 ml 0.2 M Na_2HPO_4, 24 ml 0.1 M citric acid and distilled water to 100 ml) plus 0.02 ml H_2O_2 added immediately before use.

For cell culture, a cell suspension was prepared from the spleens of 15 BALB/c mice by standard methods, cells were centrifuged at 200 × *g* for 6 min and resuspended in 30 ml 0.83% (w/v) NH_4Cl for 5 min at room temperature. Cells were recentrifuged, resuspended in PBS, counted and recentrifuged. For stimulation of T cells with Con A, the cells were resuspended (2×10^6 cells/ml) in standard culture medium (RPMI 1640 supplemented with 5% (v/v) foetal calf serum, amino acids, penicillin (0.06 mg/ml), streptomycin (0.1 mg/ml), glutamine (0.3 mg/ml) and β-mercaptoethanol (5×10^{-5} M)) plus Con A (2 μg/ml). For B cell stimulation, the washed, red cell-depleted spleen cells were resuspended (10^7 cells/ml) in the standard culture medium above. To 50 ml of cell suspension, 5 ml of guinea pig serum (a source of lytic complement activity) and 0.15 ml of the mouse monoclonal anti-Thy-1 antibody NIM-R1 [13] were added and this suspension was incubated at 37°C for 45 min. The cells were centrifuged, washed in PBS as above and the final T cell-depleted B cells were resuspended in the tissue culture medium at 10^6 cells/ml. For LPS stimulation, LPS was added to 0.05 mg/ml and the cells were cultured in 50 ml aliquots as above. Other batches of splenic B cells were stimulated with IL-4 with or without submitogenic concentrations (2 μg/ml) of affinity-purified goat anti-mouse Ig. The IL-4 preparation was an IL-4 enriched tissue culture supernatant obtained from the T cell hybridoma T-Ova (O'Garra, A. and Sanderson, C., unpublished). The B-cell lymphoma BCL_1 was maintained in vivo and T cell-depleted cell suspensions were stimulated in vitro with LPS or with IL-4 from T cell hybridoma T-Ova [14].

All cultures were divided into 50 ml aliquots in individual flasks which were incubated at 37°C for 0–4 days. At each time point a flask was removed and rapidly frozen by immersion in a bed of solid CO_2. For subsequent analysis the cell suspensions were thawed, diluted in 3 vols of TKM buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$) and sonicated in an ice-bath using an MSE 150 probe sonicator at peak-to-peak amplitude of 5–7 μm, for 3 bursts of 15 s with 15 s cooling intervals. The sonicates were assayed for protein [15], for PDI protein by ELISA (see above) and for PDI activity by the standard assay involving reactivation of incorrectly oxidised (scrambled) bovine pancreatic ribonuclease [16,17].

3. RESULTS

The polyclonal rabbit antiserum raised against homogeneous bovine PDI (J3B) was found to detect this antigen at levels down to 1 ng and to cross-react with PDI from a wide range of higher eukaryotes [3]. It detected a single protein band on Western blots of microsomal membranes from rat liver and dog pancreas, and was used in a detailed study of the distribution of several proteins between various endo-membranes in the dog pancreas [18]. The anti-bovine PDI antiserum cross-reacted with a protein of 59 kDa in preparations of 'reticuloplasm' [19] (i.e., e.r. lumenal content) from MOPC 315 cells, and was used to demonstrate the e.r. location of this protein by confocal laser immunofluorescence (Macer, D.R.J. and Koch, G.L.E., personal communication). With an antiserum provided by Dr M. Green (St. Louis University School of Medicine) and raised against a major MOPC 315 e.r. protein termed ERp59 [20], we observed similar specificity to that of our anti-PDI antiserum (fig.1) demonstrating that ERp59 is PDI. The antiserum (J3B) therefore cross-reacted well with mouse PDI, which enabled both PDI activity and PDI protein to be quantitated in response to various treatments.

Mouse spleen lymphocytes were cultured in the presence and absence of various activating factors. Preliminary results indicated that 3-day B cell cultures stimulated with bacterial lipopolysaccharide (LPS) showed a 1.4- to 2.2-fold increase in

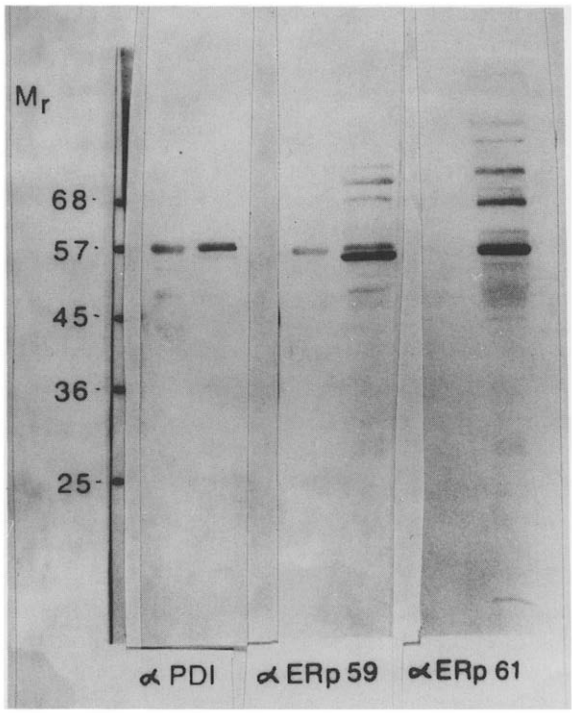
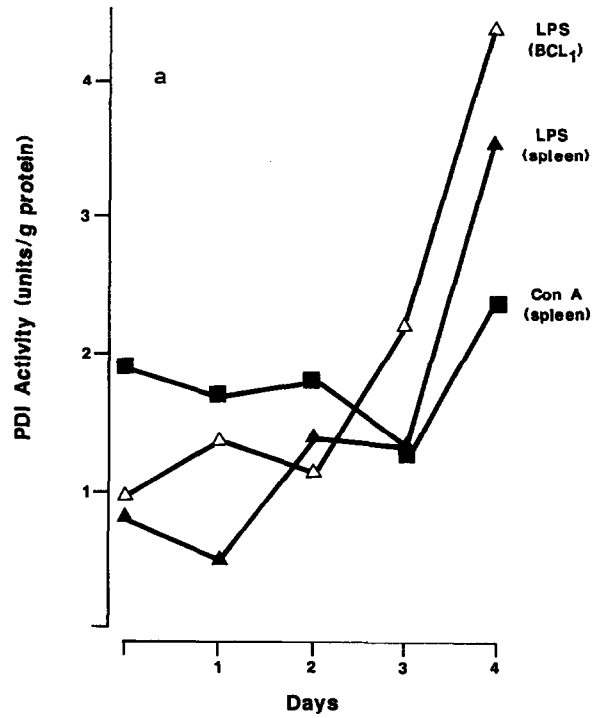


Fig.1. Cross-reactivity between anti-bovine PDI antiserum and murine ERp59. Samples of 5 μg purified bovine PDI (lanes 1,3,5) and 50 μg rat liver microsomal membranes (lanes 2,4,6) were resolved by SDS-PAGE and blotted onto nitrocellulose. Blots were then developed, as described in section 2, using anti-bovine PDI antiserum J3B (lanes 1,2) and antisera raised against specific endoplasmic reticulum proteins ERp59 (lanes 3,4) and ERp61 (lanes 5,6) purified from murine plasmacytoma (MOPC-315) cells. The latter antisera [22] were kindly supplied by Dr M. Green of St Louis University School of Medicine, and were used at a dilution of 1:250.



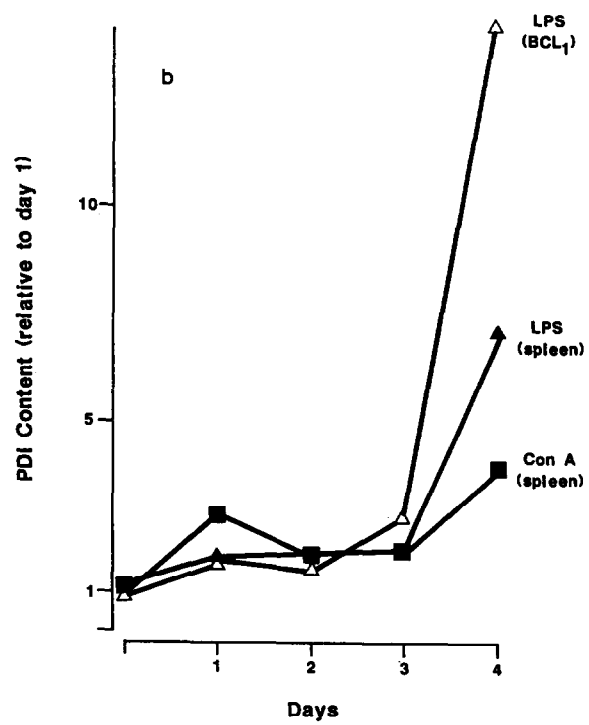


Fig.2. Time course of induction of protein disulphide-isomerase by LPS. Splenic B cells and BCl_1 cells were isolated and cultured in the presence either of LPS or of Con A for 0–4 days, frozen, thawed and sonicated, as described in section 2. The sonicated samples were assayed for total protein, for PDI activity, and for PDI antigen by ELISA. (a) PDI specific activity, and (b) relative PDI antigen concentration, as a function of time of culture. PDI activity data are means of activities determined on duplicate cell samples from the same time point, ELISA data are means of duplicate dilutions of the same cell samples. (■) Spleen cells in presence of Con A; (▲) splenic B cells in presence of LPS; (△) BCl_1 cells in presence of LPS.

PDI activity not observed in control or in Con A-stimulated T cells. The results of a time-course study over 4 days are shown in fig.2a. Both splenic B cells and BCL_1 cells show significant increases in PDI activity when cultured with LPS (4.3- and 4.6-fold, respectively) with most of the increase occurring between day 3 and day 4. Con A-treated splenic T cells showed little increase (1.3-fold): BCL_1 cells treated with the IL-4 enriched T cell supernatant showed an intermediate response (2.3-fold increase, not shown). Parallel ELISA studies on the same samples showed that the LPS treatment produced an increase in PDI content in both B lymphocytes and BCL_1 cells which could account for the increase in PDI activity; again the major effect was noted between days 3 and 4 of culture (fig.2b).

By contrast to the effect of LPS, PDI was not induced by factors which cause B cell excitation and proliferation, but without producing full differentiation into Ig-secreting plasma cells. Thus goat anti-mouse μ-chain antibodies and IL-4 enriched T cell supernatants had no significant effect on spleen cell PDI levels when included separately and together in cell cultures (fig.3).

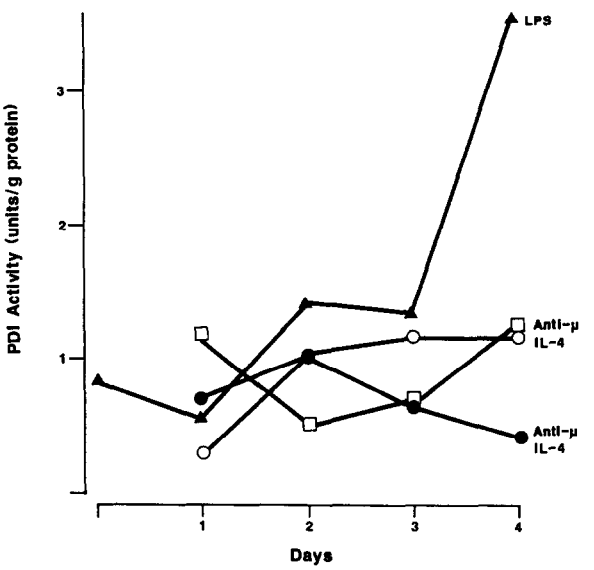


Fig.3. Effects of various treatments on PDI activity in cultured lymphocytes. Isolated splenic B cells were cultured in presence of IL-4 enriched supernatant from T-Ova cells (○), or of goat anti-mouse μ-chain antibodies (□) or of both of these agents (■). Cells were cultured, isolated, sonicated and assayed for PDI activity as described in section 2. For comparison, data on LPS treatment of splenic B cells from fig.2a are also included (▲).

4. DISCUSSION

The process of activation of B lymphocytes is complex and involves interaction between cell types through the production of various specific activating factors. In the physiological activation process, three successive stages of activation, proliferation and differentiation to Ig-secreting cells can conveniently be distinguished [21] and lymphokines and other factors capable of stimulating each of these steps can be identified [9].

Two models of B-cell activation to secretion were tested. The better known is stimulation of resting splenic B lymphocytes by bacterial lipopolysaccharide (LPS) [22], which yields 10–20% of Ig-secreting cells by day 4 (d4) of culture. A particular advantage of this system is that at earlier times, when activation (d1) and proliferation (d2–d3) can be demonstrated, there are few Ig-secreting cells. The observation of a major increase in PDI at d4 of LPS-activated B cells, and not d4 of Con A-activated T cells, thus correlates with the appearance of Ig-secreting cells in culture. The necessary development of secretion as well as activation and proliferation was implicated by the insignificant change in PDI activity in B cells activated under conditions that do not yield Ig secretion. Stimulation of B cells with IL-4 alone results in activation (enlargement and increased surface expression of Class II antigen), whereas the simultaneous presence of IL-4 and sub-mitogenic amounts of anti-Ig causes activation and proliferation, but does not drive the cells into Ig secretion [9]. As demonstrated, B cells stimulated with IL-4, with or without goat anti-mouse μ-chain, did not develop the elevated PDI levels noted in the LPS-activated cultures, even when cultured for 4 days.

Our second model of B cell stimulation was provided by the murine B cell lymphoma BCL_1. This cell line will proliferate in response to both IL-4 and LPS and, with LPS, will also differentiate into Ig-secreting cells [23,24]. In total agreement with the results obtained with the first system, a major increase in PDI activity was observed in BCL_1 cells stimulated under conditions giving rise to immunoglobulin secretion but not when only proliferation was induced.

Hence, both the specificity and the time-course of induction of PDI by LPS-treatment, link the increase in PDI activity to the final stages of B cell differentiation when immunoglobulin synthesis and secretion are increased. As noted above, there is already abundant evidence that PDI can act in vitro to catalyse disulphide bond formation in Ig folding and assembly, and that PDI is present at high levels in lymphoma-derived cell lines actively secreting immunoglobulins. PDI is located within the lumen of the endoplasmic reticulum [25] the site of immunoglobulin folding and assembly [26]. Furthermore, PDI can be chemically cross-linked to nascent immunoglobulins in hybridoma cells [27]. All these results imply that PDI acts as catalyst of immunoglobulin folding and assembly in vivo, and the specific induction of PDI demonstrated here is consistent with that proposal.

Another lumenal protein has been implicated in facilitating immunoglobulin assembly, namely BiP (heavy-chain binding protein, or glucose-regulated protein 78) [28]. With an anti-BiP antiserum kindly provided by Dr Hugh Pelham (Laboratory of Molecular Biology, Cambridge) we have demonstrated by Western blotting that BiP levels increase in parallel with those of PDI during culture of spleen cells in presence of LPS (not shown). One of the major morphological consequences of differentiation to the plasma cell and induction of Ig-secretion is extensive proliferation of the e.r. and secretory apparatus. Green and colleagues [20,29] described several major e.r. proteins whose synthesis was induced by 48-h culture in presence of LPS, including one Erp59, which we have now identified as PDI. They studied the biosynthesis and accumulation of these proteins (ERp99, ERp72, ERp60, ERp59 and ERp49) and noted that their accumulation was matched by a correspondingly increased rate of biosynthesis, implying that all control was exerted at synthesis. Taken together with our findings on increases in PDI (and BiP) levels over 4 days of LPS induction, these results imply that a family of e.r. proteins are coinduced by LPS. This family appears to correspond to the 'reticuloplasmins' defined by Koch [19] i.e. the permanent resident proteins of the e.r. lumen.

The LPS-stimulated lymphocyte is clearly a specialised system, but nevertheless the data presented here show that it will be an extremely useful model in which to study the control of expression of PDI, and the manner in which the level of PDI in cells is controlled in parallel to their rate of synthesis of disulphide-bonded proteins.

Acknowledgements: We are grateful to Dr Tony Bradshaw and Shell Research Sittingbourne for their support of J.P. through a CASE studentship and to Drs Mike Green (St. Louis) and Hugh Pelham (Cambridge) for the provision of antisera. We thank the SERC for support (CASE studentship and project grant GR/D/85310) and the Nuffield Foundation for a small grant.

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