

Group B streptococcus (GBS) modifies macrophage phosphatidylserine metabolism during induction of apoptosis

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Abstract Group B streptococcus (GBS) induced macrophage apoptosis by which it could avoid host defence mechanisms. Macrophages, which constitutively express phosphatidylserine (PtdSer) on the outer leaflet of plasma membrane, increased PtdSer exposure during GBS-induced apoptosis. Induction of apoptosis decreased PtdSer radioactivity of macrophages incubated with [³H]serine. The effect appeared not due to increasing conversion of PtdSer to phosphatidylethanolamine or phosphatidylcholine nor to the release of radioactive membrane vesicles. The radioactivity in lysoPtdSer was also reduced. These results confirm that induction of apoptosis involves a modification of PtdSer metabolism and point out the typical features of the GBS-induced apoptosis with respect to other models of apoptosis. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Phosphatidylserine; Apoptosis; Macrophage; Group B streptococcus

1. Introduction

Group B streptococcus (GBS), a pathogen that causes serious neonatal infections, induces macrophage apoptosis [1], which may be important for the initiation of infection, bacterial survival and escape from the host immune response. In fact, since apoptotic cells are engulfed by phagocytes, apoptosis does not usually lead to inflammation [2], and this may be an advantage for the pathogen, which might avoid the triggering and recruitment of host defences. GBS-induced apoptosis is characterised by early plasma membrane permeability alterations and involves the modulation of cytosolic calcium level and of protein kinase C activity [1].

Several morphological and biochemical events characterise apoptosis [2], including the loss of plasma membrane lipid asymmetry with exposure of phosphatidylserine (PtdSer) to the exterior of the cell [3]. PtdSer exposure is mainly attributed to the inhibition of the aminophospholipid translocase

and activation of the scramblase [4], but several aspects need to be clarified.

In Jurkat cells incubated with radioactive serine, CD95-induced apoptosis causes a rapid and transient increase of PtdSer radioactivity, mainly due to the strong inhibition exerted on PtdSer decarboxylation to phosphatidylethanolamine (PtdEtn) [5]. In thymocytes, incubated with the same radioactive precursor, the apoptotic stimulus dexamethasone (Dex) increases PtdSer radioactivity without significant changes in PtdSer decarboxylation. Stimulation of PtdSer synthesis by Dex is confirmed by the assay of serine base exchange enzyme (SBEE) in the thymocyte lysate [6].

Newly synthesised [³H]PtdSer is translocated to the external leaflet of the plasma membrane in different models of apoptosis [5,7] and, in Jurkat cells, stimulation or inhibition of PtdSer synthesis enhances or decreases PtdSer exposure, respectively [8]. An increase of membrane PtdSer may contribute to its exposure during apoptosis and/or play a role in signal transduction. In fact, all protein kinase C isoforms require PtdSer for their activity [9].

In this study we verified whether or not PtdSer metabolism is modified during GBS-induced macrophage apoptosis, measuring in murine macrophages infected with GBS the incorporation of [³H]serine into PtdSer and the conversion of newly synthesised PtdSer into metabolically related glycerophospholipids.

This study demonstrates that macrophage PtdSer metabolism is modified by GBS and that this effect is strongly related to macrophage apoptosis. This also suggests that GBS-induced macrophage apoptosis influences PtdSer metabolism in a different manner with respect to other models of apoptosis.

2. Materials and methods

2.1. Incubation of GBS and gGBS with [³H]serine

GBS type III, strain COH31 r/s, was kindly provided by Dr M. Wessel (Channing Laboratory, Boston, MA, USA). GBS was grown in Todd–Hewitt broth (THB; Unipath, Milan, Italy) at 37°C and aliquots were stored at –70°C until used. For assays, GBS was grown in THB overnight and prepared as described elsewhere [1]. For some experiments GBS was grown for 18 h in THB in the presence of 10 mg/ml glucose (gGBS), conditions not allowing haemolysin expression and apoptosis [1].

GBS (600 × 10⁶ colony forming units, CFU) and 20 μCi [³H]serine (specific radioactivity (S.R.) 25.8 Ci/mmol; NEN Life Science Products, Boston, MA, USA) were added to each well of a 6-well plate in 2 ml RPMI 1640 with 10% foetal calf serum (complete medium). The

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Abbreviations: PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; lysoPtdSer, lysophosphatidylserine; GBS, group B streptococcus; gGBS, group B streptococcus grown in the presence of glucose; SBEE, serine base exchange enzyme

incubation was performed at 37°C in 5% CO₂ for 1 or 2 h. Bacteria suspensions were recovered and centrifuged (1000×g, 15 min). Lipids were extracted as described below.

2.2. Incubation of peritoneal macrophages with [³H]serine in the absence or presence of GBS

Outbred CD-1 mice of both sexes, 8–10 weeks old, were obtained from Charles River Breeding, Calco, Milan, Italy. Murine peritoneal macrophages were elicited by intraperitoneal injection of 1 ml of a 10% solution of thioglycollate broth (Difco, Detroit, MI, USA) and the cells were recovered 4 days later as previously described [1]. Macrophages (3×10⁶/ml in 2 ml of RPMI) were allowed to adhere in 6-well plate.

After 90 min of preincubation at 37°C in 5% CO₂ macrophages were incubated with 20 μCi [³H]serine (S.R. 25.8 Ci/mmol) in 2 ml for 1 or 2 h. The radioactive precursor was added to macrophage monolayers directly without washing (*procedure a*), or after removal of non-adherent cells and washing of monolayers (*procedure b*). In some experiments, cells were treated as in *procedure b* but [³H]serine was added 30 min before the end of the incubation (*procedure c*). The effect of GBS on macrophage PtdSer metabolism was studied in parallel samples by adding GBS at the beginning of the incubation at macrophage:GBS ratios of 1:50 or 1:200. The effect of gGBS was studied in a similar manner.

After 1 or 2 h of incubation, supernatants were removed and monolayers were recovered in phosphate buffer saline by scraping. Macrophages were centrifuged and lipids were extracted and analysed for lipid radioactivity (see below). In some experiments done according to *procedure b*, culture supernatants were recovered and, after removal of bacteria, lipids were extracted and analysed for phospholipid radioactivity. A complete removal of bacteria was achieved by centrifugation (1000×g, 15 min), followed by filtration of the supernatant through MINISART filters (0.22 μm).

2.3. Measurement of apoptosis and PtdSer exposure

At 24 h, macrophages infected for 1 or 2 h and controls were recovered for detection of apoptosis. The centrifuged cell pellets were re-suspended in 1 ml of hypotonic fluorochrome solution (propidium iodide (PI) 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma). Samples were placed overnight in the dark at 4°C, and the PI fluorescence of individual nuclei measured using FACScan cytofluorometry [10].

PtdSer externalisation was detected by staining macrophages infected for 1 or 2 h and controls with annexin V-FITC using the Annexin-V-FLUOS staining kit (Roche) in accordance with the manufacturer's instructions. The FITC fluorescence of samples was measured using FACScan cytofluorometry [2].

The data of PI and annexin V-FITC staining were processed by a Hewlett-Packard computer and analysed with Lysis software (BD).

2.4. Evaluation of radioactivity in lipids

Lipids were extracted according to Folch [11]. Phospholipids were separated by two-dimensional thin layer chromatography (TLC) and the radioactivity in PtdSer, PtdEtn and phosphatidylcholine (PtdCho) was measured with a Packard 1600 CA Tri-Carb liquid scintillation analyser, as previously described [6]. The radioactivity in lysophosphatidylserine (lysoPtdSer) was measured after separation of phospholipids by one-dimensional TLC according to Renooij and Snyder [12].

2.5. Statistical analysis

Statistical analysis was performed by ANOVA followed by multiple comparison (Scheffe's test).

3. Results

A preliminary study demonstrated that macrophages are able to synthesise PtdSer by base exchange. SBEE activity was assayed in cell lysate from macrophage suspension at pH 7.4 and at pH 8.2, as in previous studies [6,13]. Macrophage SBEE activity was higher (four times) at pH 8.2 (data not shown), indicating a difference with respect to the thymocyte enzyme.

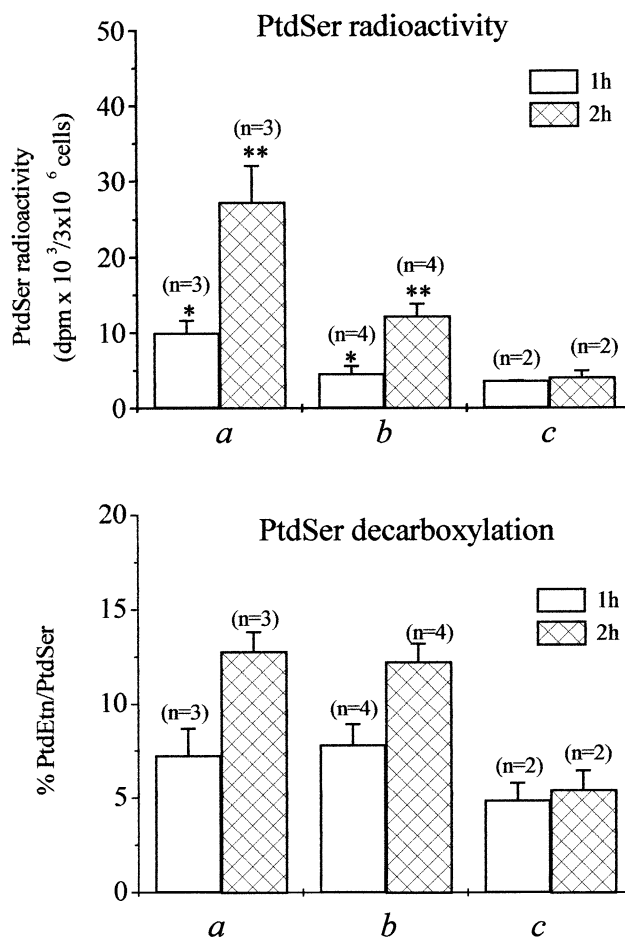


Fig. 1. Macrophage PtdSer synthesis and decarboxylation. Macrophages were allowed to adhere and incubated with [³H]serine for 1 and 2 h, according to *procedures a, b* and *c* as described in Section 2. PtdSer radioactivity is expressed as dpm × 10³ / 3 × 10⁶ cells. PtdSer decarboxylation is expressed as a ratio between radioactivity found in PtdSer and that found in PtdEtn. Results are the mean ± S.D. of experiments done on different days, whose number is reported in parentheses. **P* < 0.05 (1 h: *procedure a* vs. *procedure b*); ***P* < 0.05 (2 h: *procedure a* vs. *procedure b*).

In further experiments, macrophages were incubated with [³H]serine and the radioactivity in phospholipids was measured. Macrophage PtdSer radioactivity increased from 1 to 2 h, in a time-dependent manner, when [³H]serine was added at the beginning of the incubation (*procedures a* and *b*). PtdSer radioactivity was lower with *procedure b* with respect to *procedure a* (Fig. 1). Newly synthesised PtdSer was decarboxylated to PtdEtn; the percentage of radioactive PtdEtn with respect to PtdSer increased in the time without any significant differences between *procedures a* and *b* (Fig. 1). The levels of PtdSer radioactivity and PtdSer decarboxylation were not modified from 1 to 2 h when [³H]serine was added only 30 min before the end of the incubation (*procedure c*; Fig. 1). The greater percentage of radioactive PtdEtn with respect to PtdSer observed after 2 h of incubation with *procedures a* and *b* may reflect the movement of [³H]PtdSer to reach the mitochondrial PtdSer decarboxylase [14].

The effect of GBS on macrophage PtdSer metabolism was studied by simultaneously adding GBS and [³H]serine at the beginning of the incubation, using macrophage:GBS ratios of 1:50 or 1:200. GBS reduced the radioactivity in macrophage

PtdSer in a ratio- and time-dependent manner when using both *procedures a* and *b* (Fig. 2). gGBS that has lost haemolysin expression and does not induce macrophage apoptosis [1] had almost no effect because the lowering of PtdSer radioactivity observed at 2 h of incubation with 1:200 macrophage:gGBS ratio was much lower than that exerted by GBS (Fig. 2). The reduction of PtdSer radioactivity by GBS was also observed when [3 H]serine was added 30 min before the end of the incubation (data not shown).

The reduction of macrophage PtdSer radioactivity by GBS was not due to a decrease in cell numbers in GBS-infected macrophage monolayers, which was evaluated by trypan blue assay at all times examined (data not shown).

The percentage of apoptotic cells was measured in parallel samples by cytofluorometry at 24 h after the different times of incubation in the presence or absence of GBS. At 24 h no significant percentage (about 4%) was found in control macrophage incubated for 1 or 2 h with *procedures b* and *c*, while about 13% of apoptosis was observed in samples incubated according to *procedure a* (data not shown). As shown in Fig. 3, GBS caused apoptosis which was ratio- and time-dependent

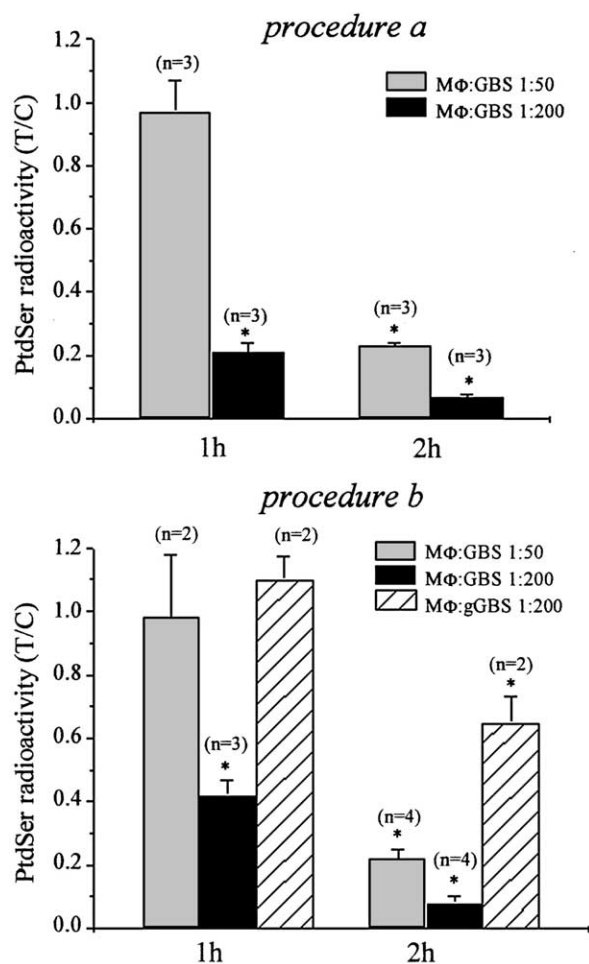


Fig. 2. Effect of GBS on macrophage PtdSer radioactivity. Macrophages (MΦ) were incubated with [3 H]serine according to *procedures a* and *b* as described in Section 2. GBS and gGBS were added simultaneously to [3 H]serine at the macrophage:bacteria ratios indicated. Results are reported as ratios between PtdSer radioactivity of GBS-infected macrophages and that of controls. * $P < 0.05$ (PtdSer radioactivity of GBS-infected macrophages vs. that of control).

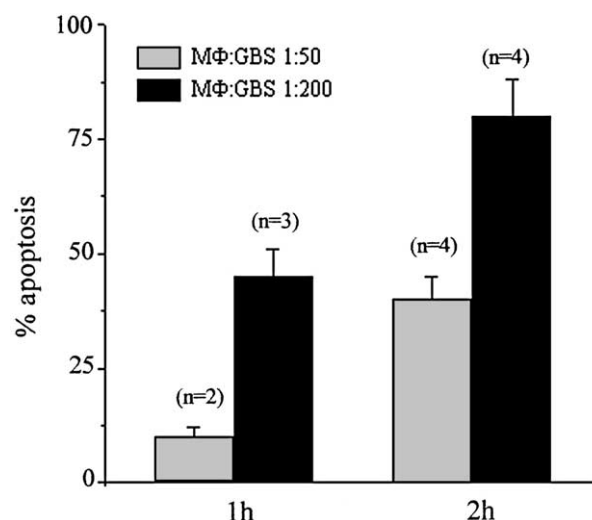


Fig. 3. Effect of GBS on macrophage apoptosis. Apoptosis after 24 h was evaluated by cytofluorometry as described in Section 2, in macrophages infected with GBS at the indicated ratios for 1 or 2 h. Results are the mean \pm S.D. of different experiments, whose number is reported in parentheses.

and this effect was then like that observed for PtdSer radioactivity (see Fig. 2). In fact, no significant percentage of apoptotic cells was observed in macrophages infected with GBS for 1 h at a cell:GBS ratio of 1:50, whereas 45% apoptosis was found in macrophages infected for 1 h at a cell:GBS ratio of 1:200. Apoptosis of macrophages infected for 2 h at a ratio of 1:50 and 1:200 increased by 40% and 80%, respectively. Fig. 3 refers to experiments done with *procedure b*, but similar values were obtained using *procedure a*. The gGBS, which has lost haemolytic activity [1] did not cause significant apoptosis (data not shown).

The analysis of annexin V binding demonstrated that macrophages constitutively express PtdSer on the surface (about 60% of annexin V positive cells at 1 and 2 h) and PtdSer

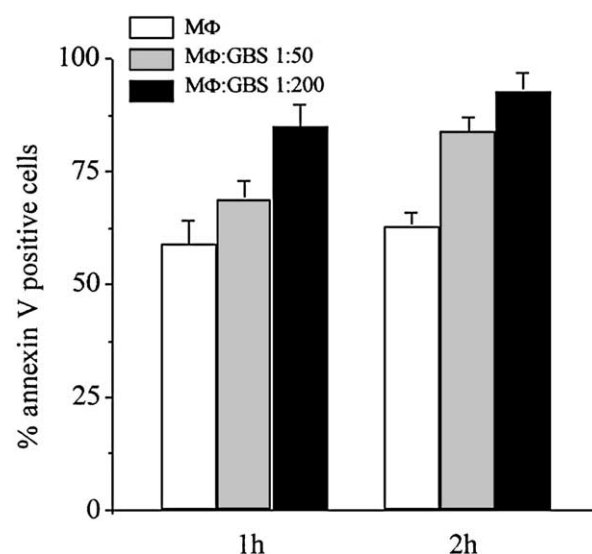


Fig. 4. Effect of GBS on macrophage PtdSer exposure. PtdSer externalisation was measured by cytofluorometry as described in Section 2 in controls and macrophages infected with GBS at the indicated ratio for 1 and 2 h. Results are the mean \pm S.D. of two experiments.

exposure increased during GBS-induced apoptosis (Fig. 4). Macrophage infected with gGBS did not change the PtdSer exposure at all times examined (data not shown).

The strong correlation between the decrease of PtdSer radioactivity and macrophage apoptosis is clearly visible in Fig. 5 ($r = -0.9299$; Pearson correlation test), which reports data from all the experiments, independent of the procedure and time of incubation and the cell:GBS ratios used.

Since GBS is a living organism, we measured the incorporation of [^3H]serine into GBS phospholipids. As other bacteria [15,16], GBS and gGBS utilised [^3H]serine mainly for the synthesis of PtdEtn by PtdSer decarboxylation. Interestingly, methylation of PtdEtn to PtdCho was greater in GBS than in gGBS (Fig. 6). The utilisation of [^3H]serine by bacteria did not greatly modify the amount of precursor available for macrophage PtdSer synthesis. In fact, in a representative experiment the [^3H]serine radioactivity found in the supernatants after 2 h of incubation was 16 μCi in non-infected macrophages and 14.5 μCi in GBS-infected macrophages at a ratio of 1:200 (data not shown).

GBS may reduce macrophage PtdSer radioactivity, stimulating its conversion to PtdEtn and PtdCho. The real extent of this conversion in infected macrophages was difficult to evaluate. In fact, as evident from a comparison between Fig. 1 and Fig. 6, macrophage PtdEtn radioactivity could be overestimated even in the presence of the lower concentration of bacteria which could remain in the monolayer at the end of the incubation. However, we can exclude this possibility since the total PtdSer+PtdEtn+PtdCho radioactivity was also reduced in GBS-induced macrophage apoptosis (data not shown). The lowering of PtdSer radioactivity was not accompanied by an increase of macrophage lysoPtdSer radioactivity, which represented about 30% of radioactive PtdSer as in control macrophages (data not shown).

To verify whether apoptotic cells release radioactive membrane vesicles, in two experiments we measured phospholipid

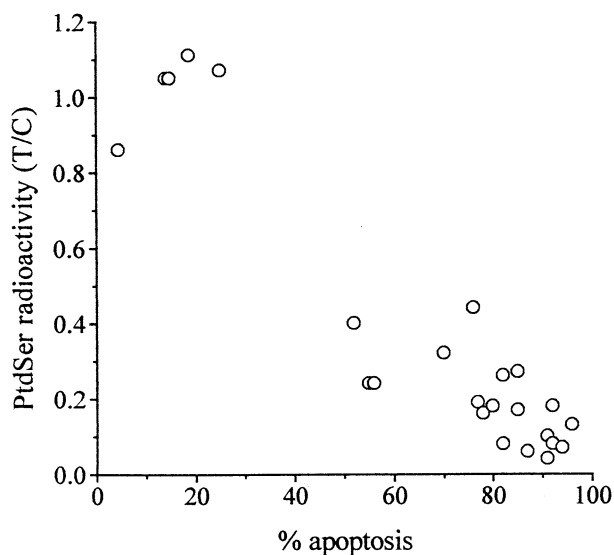


Fig. 5. Correlation between GBS-induced macrophage apoptosis and PtdSer radioactivity. The percentage of GBS-induced apoptosis and the decrease of PtdSer radioactivity with respect to controls were measured as described in Section 2. Results refer to all the experiments, independent of the macrophage:GBS ratio and/or the time of incubation.

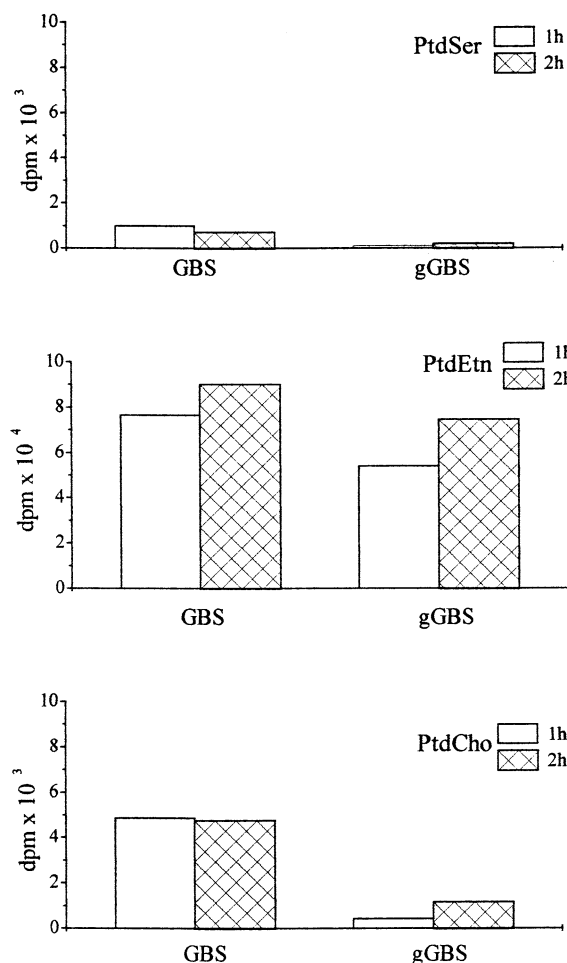


Fig. 6. Distribution of the radioactivity in glycerophospholipid of GBS and gGBS incubated with [^3H]serine. Bacteria (600×10^6 CFU) were incubated with [^3H]serine for 1 or 2 h as described in Section 2 and the radioactivity in glycerophospholipids was measured. Results refer to one experiment representative of others.

radioactivity in the supernatants recovered at the end of incubation. Bacteria were eliminated before lipid extraction by centrifugation at $1000 \times g$ for 15 min. After 1 h of incubation with GBS, the supernatant PtdSer+PtdEtn+PtdCho radioactivity was 2.4 nCi in the first experiment and 2 nCi in the second experiment. These values were similar to the corresponding decrease of total PtdSer+PtdEtn+PtdCho radioactivity in GBS-infected macrophage monolayers. However, the supernatant phospholipid radioactivity was mainly associated with PtdEtn (about 45%) and PtdCho (about 16%), thus indicating the presence of residual bacterial contamination (see Fig. 6). After 2 h of incubation, the radioactivity found in supernatant phospholipids represented only 30% of the corresponding decrease of macrophage PtdSer radioactivity due to GBS infection. Furthermore, it was mainly associated with PtdEtn and PtdCho. Filtration of centrifuged supernatants through a 0.22- μm membrane completely eliminated bacteria, and no radioactivity in phospholipids was found (data not shown).

4. Discussion

This study demonstrates that GBS modifies macrophage

PtdSer metabolism, and this effect is strongly related to the GBS-induced macrophage apoptosis. PtdSer radioactivity of macrophages incubated with [^3H]serine was decreased by GBS. The dependence of this effect on the time of incubation and on the macrophage:GBS ratio was similar to that observed for GBS-induced macrophage apoptosis. The correlation between the two effects was confirmed by experiments with gGBS, which does not induce apoptosis [1]. In fact, a slight reduction of PtdSer radioactivity was observed only after 2 h of incubation with the macrophage:gGBS ratio of 1:200.

The GBS-induced decrease of macrophage PtdSer radioactivity was not due to conversion of newly synthesised PtdSer to PtdEtn and PtdCho nor was it accompanied by an increase of lysoPtdSer radioactivity. Furthermore it appears not due to the release of radioactive membrane vesicles, an event demonstrated in other models of apoptosis [5,7]. We could conclude that PtdSer synthesis is inhibited in GBS-induced macrophage apoptosis. At the moment we cannot explain the opposite effect with respect to other models of apoptosis [5–7], which may reflect peculiarities of macrophage PtdSer metabolism and/or of the macrophage apoptosis induced by GBS. This possibility is suggested by the observation that GBS does not induce apoptosis in Jurkat cells [1].

Contrary to other cell types, macrophages constitutively expose PtdSer on the outer leaflet of plasma membrane [17]. The exposure of PtdSer in macrophage monolayers was 60% in controls and increased to 90% in GBS-infected macrophages concomitant to apoptosis. Thus the decrease of [^3H]PtdSer in apoptotic macrophages did not prevent PtdSer exposure. This finding appears to contrast with the relation between modulation of PtdSer synthesis and PtdSer exposure reported in Jurkat cells [8]. The mechanisms that regulate PtdSer exposure in macrophages may be different from those used by other apoptotic cells.

The lowering of PtdSer radioactivity by GBS and the level of GBS-induced apoptosis in the macrophages were not affected by the use of different experimental procedures for macrophage incubation. Interesting differences were observed when comparing control macrophages incubated with the two experimental procedures. In fact, incubation of macrophages with *procedure b* caused negligible apoptosis. A slight but significant increase of apoptosis (13%) was observed in macrophages when incubated according to *procedure a*. Incubation with *procedure a* also resulted in a greater macrophage PtdSer radioactivity with respect to *procedure b*. Further studies will verify the possible relevance of this finding, which correlates an increase of macrophage apoptosis to an increase of PtdSer radioactivity, as observed in Jurkat cells and thymocytes [5,6].

Incubation of cells with radioactive serine and determining the radioactivity in lipids is a widely used procedure for studying the effect of various stimuli on PtdSer metabolism. However, in evaluating results one has to take into account the metabolism of newly synthesised PtdSer. Different enzyme

isoforms are involved and several aspects concerning SBEE(s), phospholipid methyltransferases and phospholipases have been not completely elucidated so far. The final effect on PtdSer radioactivity may depend on the peculiar enzyme expression in different cell types. Interestingly, in Jurkat cells the apoptosis-induced increase of PtdSer radioactivity is mainly due to the inhibition of PtdSer decarboxylase [5], whereas in apoptotic thymocyte stimulation of PtdSer synthesis by base exchange is not accompanied by inhibition of PtdSer decarboxylation [6].

In conclusion, these results demonstrate that GBS modifies macrophage PtdSer metabolism and that this event is correlated with the GBS-induced apoptosis. The GBS-induced modification of PtdSer metabolism appears atypical with respect to other models of apoptosis. This finding could be relevant in elucidating the mechanism underlying the GBS-induced macrophage apoptosis, whose peculiarities have been already reported [1].

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