INFECTION AND IMMUNITY, Apr. 1996, p. 1461–1466 Vol. 64, No. 4
0019-9567/96/$04.00+0

In Vivo Induction of Apoptosis and Immune Responses in Mice by Administration of Lipopolysaccharide from *Porphyromonas gingivalis*

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Received 24 July 1995/Returned for modification 21 November 1995/Accepted 31 January 1996

In vivo administration of *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS) to mice induced apoptosis before a specific immune response. Apoptosis was associated with the expression of immunoglobulin and Ia on B cells and of CD5 and several markers on T cells. Apoptosis peaked in the spleen and lymph nodes on day 2, and the second peak occurred in the thymus on day 9. Tumor necrosis factor alpha (TNF-α) could mediate apoptosis, because the serum TNF-α levels were significantly higher than those of controls at 1 day before apoptosis and recombinant murine TNF-α induced apoptosis. The apoptosis induced by Pg-LPS was similar to that induced by *Escherichia coli* LPS in its basic manner, but it was unique in the response of thymus T cells. It was suggested that Pg-LPS could induce apoptosis for the elimination of early nonspecific activated lymphocytes.

It is generally accepted that with periodontal disease, there is a change in the microflora of the gingival pocket from a predominantly gram-positive flora to a more pathogenic and anaerobic gram-negative flora (3, 5, 8). *Porphyromonas gingivalis* is a gram-negative anaerobic rod that is frequently associated with adult periodontitis (5, 18).

P. gingivalis lipopolysaccharide (Pg-LPS) exhibits a variety of biological activities (19). Pg-LPS has a similar (or even greater) number of responses in host immune cells (6, 7, 10, 13, 19). In contrast, Pg-LPS has much less potency than does enterobacterial LPS in some endotoxic activities, such as pyrogenicity, chicken embryo lethality, and lethal toxicity to mice (14, 19). We reported that a considerable reduction of Thy-1-positive lymphocytes was observed in peripheral areas of the spleen (10). It was considered that lesions in the spleens of mice inoculated with Pg-LPS may be similar to necrosis, but it may be another type of cell death.

It has been reported that Pg-LPS acts as a mitogen and a polyclonal activator of B cells (4, 10, 13). If polyclonal activation of B cells was to occur in vivo, a number of processes could be set in motion, leading ultimately to tissue destruction. Therefore, it is of interest to know whether Pg-LPS can induce nonspecific B-cell activation in vivo and how to eliminate this response. In the present study, we studied in vivo induction of apoptosis and immune responses in mice after Pg-LPS administration.

P. gingivalis 381 was grown anaerobically in GAM broth (Nissui Co., Tokyo, Japan) supplemented with hemin and menadione at 37°C for 24 h. Bacterial cells were collected by centrifugation and washed three times with pyrogen-free saline solution. Pg-LPS was extracted by the hot-phenol method and purified by repeated ultracentrifugation (100,000 × *g*, 3 h, six times) and the treatment of RNase A by the method previously described (10). The final product was lyophilized. In the final sample, 32% neutral sugar, 17% fatty acid, and a small amount of protein (less than 1%) were detected.

A total of 176 C3H/HeN mice (male, 5 to 6 weeks of age, and specific pathogen free) was used for these experiments. Lyophilized Pg-LPS was dissolved in Hanks balanced saline solution (pH 7.4). Mice received intravenous injections of Pg-LPS (100 μg per mouse [1 mg/ml; 100 μl]). They were sacrificed at 2, 4, 9, and 16 days after Pg-LPS injection and examined for apoptosis and the antibody response against Pg-LPS by flow cytometry (FCM) analysis. For estimations of tumor necrosis factor alpha (TNF-α) and interleukin-2 (IL-2) levels, serum samples were obtained from mice at 1, 3, and 6 h after Pg-LPS injection. Similarly, mice were immunized with Pg-LPS (10 and 1 μg per mouse). Each group contained five animals. Five mice not injected with Pg-LPS (untreated mice) were used as controls. The LPS preparation extracted by the hot-phenol method from *Escherichia coli* O111:B4 (E-LPS) was obtained from Difco Laboratories (Detroit, Mich.) and used as an additional control. E-LPS was injected intravenously (100 μg per mouse) as was Pg-LPS. Because TNF-α may be an important cytokine in the induction of the cell death observed in these experiments, we used recombinant murine TNF-α (R & D Systems Europe Ltd., Abingdon, Oxon, England) for intravenous injections (10 ng per mouse).

Apoptosis was examined by using an enzyme-linked immunosorbent assay (ELISA) kit (cell death detection ELISA; Boehringer, Mannheim, Germany). Briefly, this assay is based on a quantitative sandwich ELISA, with mouse monoclonal antibodies directed against DNA and histones, respectively. This allows specific determinations of the mono- and oligonucleosomes in cell lysates. Lymphocytes from the thymus, spleen, and lymph nodes were prepared by Ficoll-Conray centrifugation. The cell suspension was washed and adjusted to a concentration of 10^6 and 10^5/ml in CMRL medium (Gibco). After cell lysis, the cytoplasmic fraction was obtained and used for the ELISA.

To confirm the cell death detection ELISA results, the Apo Tag Plus in situ apoptosis detection kit (Oncor Inc., Gaithers-

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burg, Md.) was used. The labeling target of this kit is the multitude of new 3′-OH DNA ends generated by DNA fragmentation and typically localized in morphologically identifiable nuclei and apoptotic bodies. Residues of digoxigenin nucleotide are catalytically added to the DNA by terminal deoxynucleotidyl transferase, an enzyme which catalyzes a template-independent addition of nucleotide triphosphate to the 3′-OH ends of DNA. The incorporated nucleotides form a random heteropolymer in a ratio that has been optimized for anti-digoxigenin antibody binding. The anti-digoxigenin antibody fragment carries a conjugated reporter enzyme (peroxidase) to the reaction site. The localized peroxidase enzyme then catalytically generates an intense signal from the chromogenic substrate. Lymphocytes from the thymus and spleen were fixed in neutral buffered formalin for 10 min at room temperature. After washing and quenching endogenous peroxidase with 2% hydrogen peroxide, the steps discussed above were performed.

Antibodies to Pg-LPS were measured by a modified ELISA previously described (11). In this ELISA, the antigen used was Pg-LPS instead of fimbriae. Wells of microtiter plates were coated with 1.5 μg of Pg-LPS per well. After blocking, test sera were diluted at 1:100 and 100 μl was added to each well. A standard curve was generated by diluting a positive pool from mice, which was arbitrarily assigned a value of 100 ELISA units. Peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) (heavy + light chain) (Zymed Laboratories, San Francisco, Calif.) were diluted 1:1000, 100 μl of these dilutions was added to each well, and the plates were again incubated for 1 h. The plates were washed, and then 100 μl of substrate solution was added to each well. The reaction was allowed to proceed for 15 min, at which time 100 μl of 1 N H_2SO_4 was added to stop the reaction. Data were expressed in ELISA units by the method of Mouton et al. (15).

C3H/HeN mice were immunized intravenously with Pg-LPS, and their lymphocytes were monitored by FCM analysis, as described previously (9). After Pg-LPS immunization of C3H/HeN mice, the change in the Ig-bearing B-cell population was monitored by fluorescent-antibody staining by FCM analysis. A suspension of spleen cells from mice was prepared in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco). B cells which adhered to a nylon wool column were washed and pelleted in a 1.5-ml conical test tube. After treatment with anti-Thy-1.2 serum (Cedarlane, Hornby, Ontario, Canada) plus 1:10 rabbit complement (Low Tox; Cedarlane), B cells were washed in staining medium (phosphate-buffered saline with 1% bovine serum albumin) at 4°C. Cells (10^7/ml; 1 ml) were resuspended in 50 μl of fluorescein-labeled rat anti-mouse IgG monoclonal antibody (Zymed Laboratories) diluted in staining medium or in 50 μl of anti-mouse Ia monoclonal antibody (Cedarlane) diluted in staining medium and incubated at 37°C for 1 h. The latter B-cell samples were washed three times with staining medium and suspended in fluorescein-labeled rat anti-mouse IgG monoclonal antibody (Zymed Laboratories) before analysis. T cells collected by passage over a nyron wool column were pelleted in a 1.5-ml conical test tube and washed once in staining medium. Cells (10^7/ml; 1 ml) were resuspended in 50 μl of anti-CD5 monoclonal antibody (Cedarlane), diluted in staining medium, and incubated at 37°C for 1 h. Similarly, the expression of T-cell markers (CD8, Thy-1, and Ia) was examined with each monoclonal antibody (Cedarlane). Cells were washed three times with staining medium and suspended in fluorescein-labeled rat anti-mouse IgG monoclonal antibody (Zymed Laboratories) before 10,000 cells were analyzed by FCM on an EPICS 752 (Coulter Electronics, Hialeah, Fla.).

To confirm the results of FCM, histological examination was done, as described previously (10). Spleens and lymph nodes were fixed in periodate-lysin-paraformaldehyde solution. Paraffin sections were made and stained with hematoxylin and eosin. The other sections were used in immunohistochemical examinations for the detection of cells positive for Pg-LPS (detected by rabbit anti-Pg-LPS serum), Thy-1 (detected by mouse monoclonal antibody anti-Thy-1.2 [Cedarlane]), and CD5 (detected by mouse monoclonal antibody anti-CD5 [Lyt-1.1; Cedarlane]).

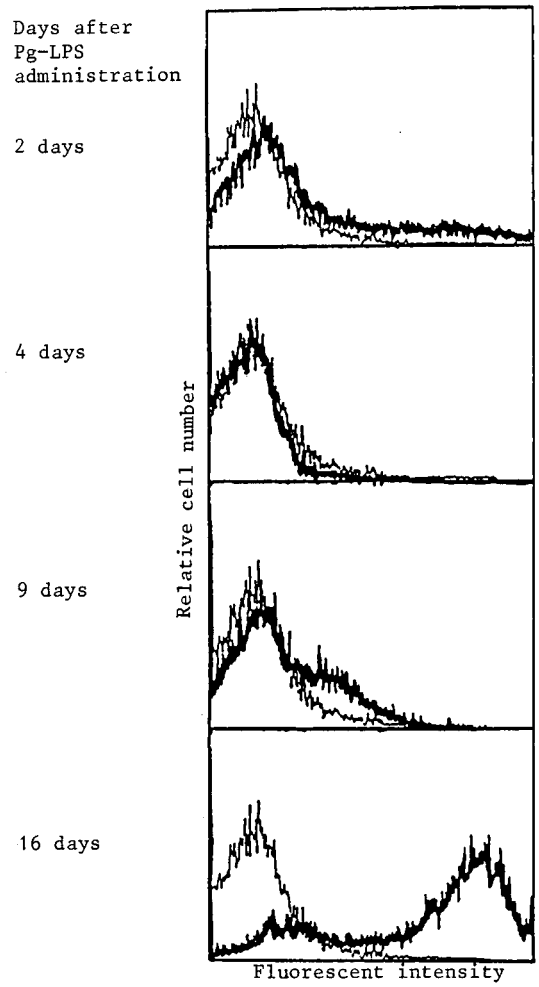

FIG. 1. FCM profiles of spleen B cells from mice treated with Pg-LPS. Lightface lines, untreated control mice; boldface line, Pg-LPS-treated mice.

Serum TNF-α and IL-2 levels were quantitated by ELISA (Genzyme, Cambridge, Mass.). The detection limit for both cytokines was 15 pg/ml.

An unpaired two-sample *t* test was used for comparisons between controls and Pg-LPS-inoculated mice. A simple correlation coefficient and regression analysis were used to examine the relation of T- and B-cell activation in vivo.

To investigate the in vivo activation of B cells in response to Pg-LPS, FCM analysis was done. Figure 1 indicates that the Ig expression of spleen B cells changed after intravenous inoculation of Pg-LPS. The percentage increased in two phases. In the first phase, the percentage of Ig-positive cells increased from 5% (untreated controls) to 24.8% (2 days after Pg-LPS inoculation). However, the peak of fluorescence intensity was unclear for Pg-LPS-inoculated mice after 2 days. The second

TABLE 1. Changes in surface markers of spleen lymphocytes after Pg-LPS administration[a]

Cell type	Surface marker	% of cells positive on postinoculation day					Peak of fluorescence intensity on postinoculation day				
		0	2	4	9	16	0	2	4	9	16
B	Ig	5.0 ± 0.4	24.8 ± 0.7[b]	4.6 ± 0.3	24.5 ± 1.4[b]	65.3 ± 1.8[b]	Unclear	Unclear	Unclear	106.4 ± 13.6	208.3 ± 5.5
	Ia	26.1 ± 1.3	48.9 ± 1.2[b]	34.2 ± 1.8[b]	25.2 ± 0.8	61.9 ± 1.5[b]	Unclear	Unclear	152.3 ± 4.1	189.3 ± 4.2	227.7 ± 2.5
T	CD5	63.4 ± 1.5	89.5 ± 1.8[b]	59.5 ± 2.0	66.9 ± 1.0	84.6 ± 2.6[b]	181.3 ± 0.6	208.3 ± 2.5[b]	186.0 ± 3.2	186.3 ± 5.5	201.0 ± 10.4[b]
	CD8	18.3 ± 0.7	29.7 ± 8.5	15.4 ± 1.9	18.4 ± 0.8	26.1 ± 0.3[b]	108.3 ± 4.3	205.7 ± 2.5[b]	108.0 ± 5.2	121.7 ± 3.1	208.7 ± 5.7[b]
	Thy-1	62.1 ± 5.8	83.7 ± 3.7[b]	65.2 ± 0.2	69.2 ± 0.9	83.2 ± 4.3[b]	Unclear	Unclear	Unclear	Unclear	Unclear
	Ia	4.0 ± 2.2	28.9 ± 1.3[b]	6.1 ± 0.8	5.6 ± 1.3	16.4 ± 2.0[b]	Unclear	Unclear	Unclear	Unclear	Unclear

[a] Data are means ± standard deviations ($n = 5$).
[b] Significantly higher than the value for control mice on day 0 (before Pg-LPS inoculation).

phase was recognized by day 16 after Pg-LPS inoculation. The Ig-positive cell percent and fluorescence intensity were higher than those of untreated controls ($P < 0.01$) (Table 1). Pg-LPS inoculation also resulted in Ia surface marker expression in a systemic response equivalent to that observed for the Ig surface marker (Table 1). For Ia, however, an intermediate phase, between days 4 and 9, was observed. Ig expression showed more drastic change than did Ia expression.

The CD5 expression of spleen T cells changed after intravenous inoculation of Pg-LPS. The percentage and fluorescence intensity increased in two phases. They increased sharply within 16 days of Pg-LPS inoculation. In vivo stimulation by Pg-LPS also resulted in CD8, Thy-1, and Ia expression in a systemic response equivalent to that observed for CD5 expression (Table 1).

A comparison of two regression parameters in lymphocyte activation was done. There were a significant correlation between the percentage of positive cells and the CD5 expression of T cells and Ig expression of B cells ($r = 0.88$; $P < 0.01$). Similarly, the CD5 expression of T cells correlated with the Ia expression of B cells ($r = 0.67$; $P < 0.01$). The Ig expression and Ia expression of B cells were positively associated each other ($r = 0.83$; $P < 0.01$).

Histopathological changes developed 1 day after Pg-LPS administration. Immunohistochemically, Pg-LPS was detected in macrophages of both spleens and lymph nodes between observation periods. To confirm the results of FCM, we examined the changes of lymphocytes with T-cell markers. A considerable reduction in the number of Thy-1- and/or CD5-positive lymphocytes was observed in the peripheral areas of spleens and lymph nodes 1 day after Pg-LPS administration. The percentage of positive cells increased extremely within 2 days but decreased within 4 days. Then the percentage increased gradually in the spleen and lymph nodes.

Whether the administration of Pg-LPS to mice induced apoptosis in the thymus, spleen, and lymph nodes was tested. Mice were injected intravenously with 100 μg of Pg-LPS, and fragmented DNA was extracted after cell lysis. The cytoplasmic fractions of samples were examined by ELISA for the detection of apoptosis. Apoptosis was detected in Pg-LPS-administered mice (Fig. 2). The spleen and lymph nodes showed similar patterns. The time course of the appearance of apoptosis in the spleen and lymph nodes was monitored. Apoptosis reached its maximum between 1 and 2 days after Pg-LPS administration. A similar peak was detected in mice injected with E-LPS or murine TNF-α (data not shown). The time course of the appearance of apoptosis in the thymus was different from that in the spleen and lymph nodes. Early and late apoptosis peaks were recognized in mice injected with Pg-LPS, but not in mice injected with E-LPS or murine TNF-α.

To confirm the cell death detection ELISA results, in situ apoptosis detection after stimulation with Pg-LPS was performed. These data were compared with those for E-LPS and TNF-α stimulation (Table 2). Apoptosis was detected in both the spleen and thymus in mice stimulated with Pg-LPS, a result similar to that observed in mice stimulated with E-LPS or TNF-α. The number of apoptosis-positive lymphocytes significantly increased between days 1 and 2 ($P < 0.01$) but decreased at day 4 after stimulation. Pg-LPS was more effective in lymphocytes from the spleen, whereas E-LPS was more effective in lymphocytes from the thymus.

As shown in Table 3, all of the LPS-injected mice showed marked elevations in TNF-α levels at 1 h. At that time, the level of TNF-α in the sera of mice injected with E-LPS was significantly higher than that of mice injected with Pg-LPS ($P < 0.01$). The elevation in TNF-α level continued to 6 h. In mice injected with murine TNF-α, this cytokine was detectable at 1 and 3 h. IL-2 was also detected at 6 h and 9 days in the sera of mice injected with Pg-LPS, at 6 h in the sera of mice injected with E-LPS, and at 3 and 6 h in the sera of mice injected with murine TNF-α.

Antibody responses against Pg-LPS were recognized only at 16 days after Pg-LPS injections of 100 μg per mouse ($P < 0.01$) (Fig. 3). Mice injected with 10 μg of Pg-LPS showed slight increases in the numbers of ELISA units, but there were no significant differences between mice treated with 10 μg of Pg-LPS and untreated mice. Mice inoculated with 1 μg of Pg-LPS showed no antibody responses. The FCM profiles of B and T cells from mice treated with 1 or 10 μg of Pg-LPS were similar to those of controls. In contrast, the profiles changed drastically after inoculation with 100 μg of Pg-LPS.

In the present study, we have demonstrated that the administration of Pg-LPS to mice induced apoptosis in the thymus, spleen, and lymph nodes. Previously, we reported decreases in the numbers of lymphocytes in spleens and lymph nodes injected with Pg-LPS. However, apoptosis was not fully clarified at that time; we did not show clear evidence supporting necrotic cell death. Therefore, apoptosis here clearly supports decreases in the numbers of lymphocytes in the thymus, spleen, and lymph nodes.

It was reported that immature thymocytes were sensitive to apoptosis caused by the injection of enterobacterial LPS (16, 20). DNA fragmentation was predominantly detected in the thymus, while it was detectable in the spleen and lymph nodes (20). Recently, in C3H/HeN mice, Norimatsu et al. detected enterobacterial-LPS-induced apoptosis in lymphocytes not only in the thymus but also in the spleen and lymph nodes (16). In this mouse strain, apoptotic changes in the thymus were more severe than those in the spleen and lymph nodes. Our data support those results. In our study, Pg-LPS also induced apo-

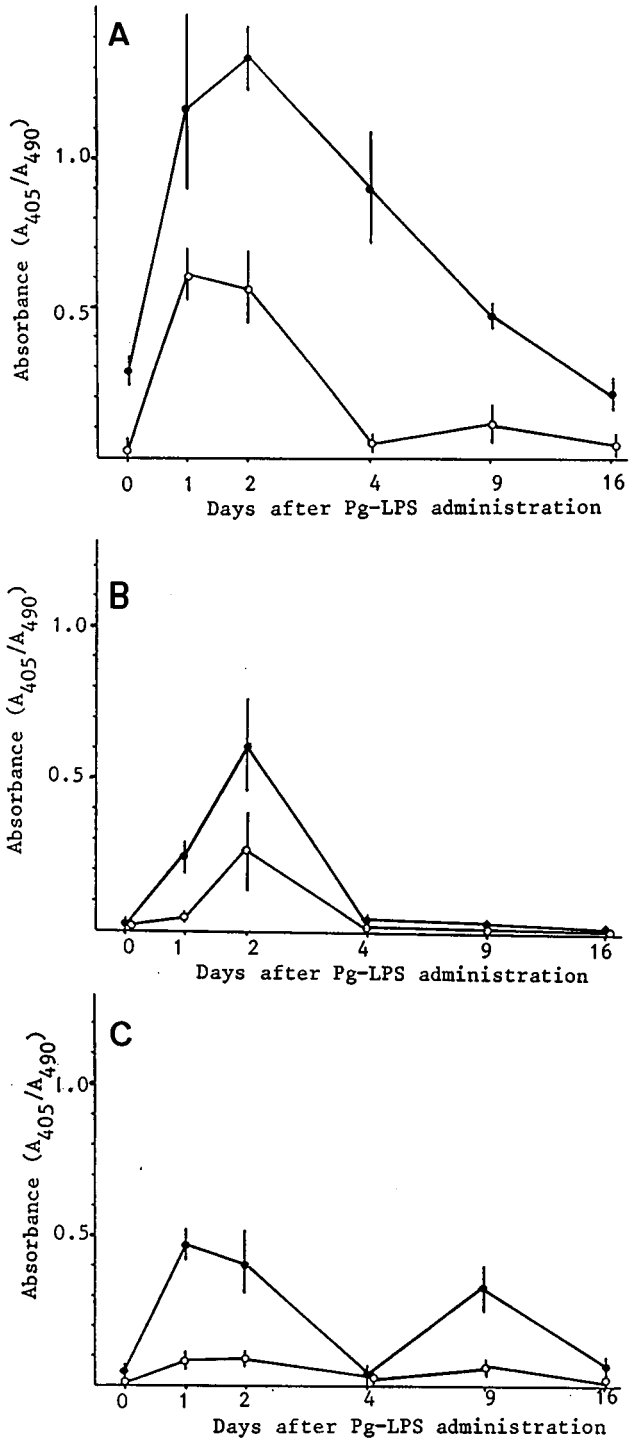

FIG. 2. Time course of apoptosis in the spleen (A), lymph nodes (B), and thymus (C) after Pg-LPS administration. Cytoplasmic histone-associated DNA fragments were obtained from 10^6 (●) or 10^5 (○) cells per ml.

ptosis in lymphocytes not only in the thymus but also in the spleen and lymph nodes. It was shown that Pg-LPS induced more severe apoptosis in the spleen that enterobacterial LPS did, while enterobacterial LPS induced more severe apoptosis in the thymus than Pg-LPS did. Pg-LPS can be a unique inducer of apoptosis.

DNA fragmentation is not a single primary event in apoptotic death. Indeed, Fas-mediated death appears to be regulated at several levels; the apoptotic response of lymphocytes to anti-Fas is regulated at least at two levels (1). Fas, also known as APO-1, is a member of the TNF receptor superfamily. Early apoptotic response can be mediated by TNF-α, because a similar pattern was recognized in mice stimulated with E-LPS or murine recombinant TNF-α. Immediate TNF-α responses were also observed in the sera of mice stimulated with Pg-LPS or E-LPS. In the thymus, Pg-LPS induced two-phase apoptosis. This was not observed in the E-LPS-induced apoptosis pattern in mice. The apoptotic response to Pg-LPS in the thymus may be complex. Simultaneous signaling via the TNF receptor would be important for apoptosis in mice injected with Pg-LPS. The balance between the TNF receptor and the other signaling receptor may regulate thymocyte selection. Further study will be needed.

Our results demonstrate that E-LPS induced both polyclonal B-cell activation and a specific antibody response in vivo. In vivo polyclonal activation without a specific antibody response is similar to the in vitro polyclonal activation induced by Pg-LPS. The mitogenic response of mouse spleen cells (5×10^6 cells per ml) to Pg-LPS (25 μg/ml) was recognized at 48 h in vitro (10).

It has been reported that Pg-LPS reacts with B cells (4, 10, 13). This also appears to apply to E-LPS, since the results of many investigations have shown that Pg-LPS does not stimulate proliferation of T cells in vitro (13). In our FCM analysis, B-cell activation and T-cell activation in vivo were recognized at same time in both early and late responses. Pg-LPS was a potent stimulator of macrophages (6, 7, 10, 13), and macrophages released various multifunctional cytokines, such as IL-1 and TNF-α (6, 10, 13). Therefore, we considered that T cell activation can occur via macrophage activation. Indeed, both cytokines, TNF-α and IL-2, were detected in the sera of mice injected with Pg-LPS. T cells may contribute to the immune regulation associated with the response against Pg-LPS.

The data in the present study show that B-cell activation was related to T-cell activation in mice inoculated with Pg-LPS. However, it does not mean that rises in the percentages of Ig- and Ia-positive B cells resulted in T-cell activation. It seems that in a given individual, a combination of many factors may induce changes in surface expression at the same time. A single factor or another combination may cause the emergence of prolonged polyclonal activation in B cells. The polyclonal activation of B cells in vivo could be set in motion to eliminate inadequate responses. Apoptosis may be one immune regulation system used before aggressive immune responses. It was

TABLE 2. In situ apoptosis detection after stimulation with Pg-LPS, E-LPS, and murine recombinant TNF-α

Stimulation with:	Day(s) after stimulation	% of lymphocytes apoptosis positive	
		Spleen	Thymus
Pg-LPS	1	62.1 ± 1.7[a]	35.3 ± 2.9[a]
	2	23.5 ± 4.4[a]	33.5 ± 4.4
	4	6.2 ± 2.3	5.5 ± 1.1
E-LPS	1	43.9 ± 9.8[a]	61.8 ± 13.8[a]
	2	35.6 ± 2.7[a]	50.4 ± 4.0[a]
	4	6.3 ± 5.2	7.7 ± 1.6
TNF-α	1	41.9 ± 6.9[a]	20.8 ± 3.3[a]
	2	20.9 ± 3.3[a]	30.0 ± 9.1[a]
	4	4.9 ± 0.4	6.4 ± 1.9
Control		4.1 ± 2.5	3.8 ± 1.9

[a] Significantly higher than the percentage in controls (without stimulation).

TABLE 3. TNF-α and IL-2 levels in the sera of mice after injection with Pg-LPS, E-LPS, or murine TNF-α

Time postinjection	Level (pg/ml) in serum					
	TNF-α			IL-2		
	Pg-LPS	E-LPS	Murine TNF-α	Pg-LPS	E-LPS	Murine TNF-α
1 h	1,377 ± 174[a]	4,372 ± 451[a]	130 ± 8[a]	ND[b]	ND	ND
3 h	136 ± 28[a]	641 ± 74[a]	84 ± 13[a]	ND	ND	33 ± 11
6 h	102 ± 4[a]	160 ± 26[a]	ND	28 ± 2[a]	32 ± 10	28 ± 12
1 day	ND	ND	ND	ND	ND	ND
2 days	ND	ND	ND	ND	ND	ND
4 days	ND	ND	ND	ND	ND	ND
9 days	67 ± 16[a]	NT[c]	NT	22 ± 5[a]	NT	NT
16 days	49 ± 8[a]	NT	NT	ND	NT	NT

[a] Significantly higher than the level in controls (less than 15 pg/ml; $P < 0.01$).
[b] ND, not detected (less than 15 pg/ml).
[c] NT, not tested.

observed that the process continued for only a short time and subsequently specific immune response could be induced in mice inoculated with Pg-LPS.

We showed that surface Ig expression is markedly decreased on spleen B cells undergoing apoptosis in vivo. Paramithiotis et al. demonstrated that the loss of B-cell surface Ig expression precedes death by apoptosis in the bursa (17). They suggested that maintenance of a threshold level of surface Ig is a requirement for the continued progression of chicken B-cell development in the bursa. Therefore, we conclude that progression of the B-cell activation associated with antibody production requires continued surface Ig expression and that those cells which lose surface Ig expression are eliminated by apoptosis.

C3H/HeN mice immunized with 100 μg of Pg-LPS per mouse produced corresponding IgG antibodies in their sera. However, immunization with 10 or 1 μg of Pg-LPS per mouse failed to produce serum antibodies and to activate spleen B cells in vivo. Similarly, Chen et al. reported that BALB/c mice immunized with 10 μg per mouse failed to produce serum antibodies (2). Kesavalu et al. showed that protein-poor Pg-LPS (25 μg per mouse) elicited IgG and IgM responses, but these responses were significantly lower than those elicited by whole-cell antigen (12), suggesting that serum antibody production was dependent on the inoculum dose of Pg-LPS.

Immunization with protein-rich Pg-LPS elicited LPS-reactive antibodies, but immunization with the same dose of protein-poor Pg-LPS did not (2). These findings suggest not only that a humoral immune response to Pg-LPS is dependent on the presence of associated proteins but also that it may induce an antigen-specific unresponsive state. Our Pg-LPS was protein-poor LPS and induced both polyclonal and antigen-specific responsive states in experimental mice. LPS is present in a number forms as macromolecules. With regard to physical state, LPS (as generally prepared in a laboratory) has low-level solubility and hence low-level bioavailability. Our Pg-LPS was suspended in HBSS. It contained various ions. The results of this study suggest that the two stages of B-cell activation depend on not only LPS-associated protein but also physical states.

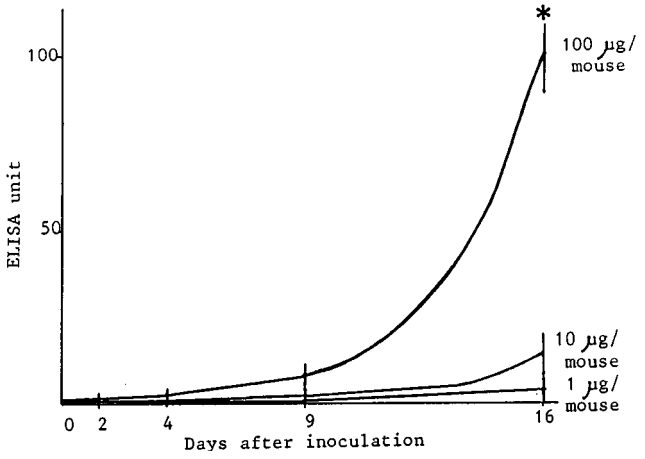

FIG. 3. Antibody response against Pg-LPS. Data are the means ± standard deviations for five mice. *, the number of ELISA units significantly increased after Pg-LPS inoculation, ($P < 0.01$).

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Editor: J. R. McGhee