Reversal of Lipopolysaccharide-Analog-Induced Antibody Suppression by Anti-Transforming Growth Factor β and Indomethacin

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Monophosphoryl lipid A and a synthetic, nontoxic monosaccharide analog of lipid A, termed GLA 60, both strongly suppressed antibody production when administered 1 to 2 days prior to antigen. Evidence is presented that this suppression was mediated by two cytokines, prostaglandin E and transforming growth factor β , because it was reversed by multiple injections of the cyclooxygenase inhibitor indomethacin and by in vitro addition of antibody to transforming growth factor β .

A variety of microbial compounds not only act as adjuvants to increase the immune response but, under different conditions, also serve to activate the suppressive arm of the immune response. Thus, immunity enhancement occurs when lipopolysaccharide (LPS) or its analogs are given along with protein antigens (11, 13, 17) or 1 to 2 days after antigen in the case of pneumococcus polysaccharide antigens (3). Tomai and Johnson demonstrated that the events that transpire in vivo during the 1 to 2 h after adjuvant injection appear to involve an initial early inducement of gamma interferon, resulting in release of helper cytokines from the macrophage and T-cell populations (29). If macrophages and T cells are sensitized by concomitant injection of protein antigen in this environment, they reap the benefits of this adjuvant-induced increase in release of helper cytokines, resulting in an immune response higher than normal. However, should protein antigen exposure be delayed until 18 to 48 h after adjuvant injection, the helper cytokines have waned in quantity and function and are replaced by suppressive factors designed normally to curtail helper cytokine release. Injection of antigen at this later time (1 to 2 days after adjuvant injection) results in a response that is lower than normal (12, 13, 17–19).

A well-characterized adjuvant compound in these respects is monophosphoryl lipid A (MPLA), a monophosphorylated, beta 1-6-linked glucosamine disaccharide containing acylated fatty acids, isolated from the outer membrane of gram-negative bacteria (30). Recently, synthetic, nontoxic analogs of lipid A also have been characterized with respect to both their immunity-enhancing and -suppressing (10, 13, 15, 18, 22, 28) activity. These compounds, termed glycolipids A (GLA), are acylated glucosamine monosaccharides, representative of the nonreducing subunit of lipid A. In an earlier publication (18), it was established that five of nine GLA analogs were capable, like LPS and MPLA, of suppressing antibody production when given in vivo 1 to 2 days prior to antigen. To determine whether suppression induced by MPLA and one of these analogs, GLA 60, was mediated by one or more endogenous cytokines recognized as immunosuppressive, the experiments described herein were carried out. They document that the suppression

demonstrable late after injection of these two LPS analogs is mediated by the cytokines prostaglandin E (PGE) and transforming growth factor β (TGF- β).

GLA 60 was provided by A.H. Its synthesis and properties have been described previously (15). MPLA was purchased from Ribi Immunochem Research, Inc., Hamilton, Mont. (lots 039-171 and 039-201). MPLA and GLA 60 were solubilized in 0.2% triethylamine in water (1 mg/ml) with sonication and stored at 4°C. To induce suppression of antibody production, 25 to 100 μ g of these compounds was injected into mice intraperitoneally 24 to 48 h prior to sacrifice and culture of the spleen cells in vitro.

BALB/c mice were purchased from Harlan Sprague Dawley, Inc., Indianapolis, Ind. Mice of either sex were used at 7 to 12 weeks of age.

Sheep erythrocytes (SRBCs; Micropure Medical, Inc., Stillwater, Minn.) were used as antigen after being washed three times with Hanks' balanced salt solution. For in vivo experiments, the washed cells were suspended, counted, and diluted in PBS (endotoxin, <0.003 ng/ml [Celox Corp., Minnetonka, Minn.]) to administer 10^8 cells in 0.5 ml intraperitoneally. When added in vitro, the cells were diluted to 4×10^8 cells per ml in Click's medium containing 10% fetal bovine serum and 0.025 ml was added to each culture well (10^7 cells).

Neutralizing polyclonal antibody specific to TGF- β was purchased from R & D Systems, Minneapolis, Minn. It was prepared by injection of highly purified recombinant human TGF- β into either rabbits or chickens. Conservation of the amino acid sequence of TGF is close to 100% in mammals (20a).

Spleen cells were cultured with a modified Mishell-Dutton system (16). Briefly, mice were sacrificed with CO₂, spleens were removed, and single-cell suspensions were prepared in Hanks' balanced salt solution. After washing and counting, cells were cultured in Click's medium–10% fetal bovine serum at 37°C and 5% CO₂. On day 4, cells from duplicate wells were pooled, centrifuged, decanted, suspended in PBS, and added to plates for assay of plaque-forming cells.

Antibody production was measured with a modified hemolytic plaque assay (14). After addition of the test or control spleen cells to poly-L-lysine-coated plates, guinea pig complement was added and the plates were incubated at 37°C and 5% CO₂ for 1 h prior to counting plaques.

Data were analyzed with a single-sample Student's *t* test

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FIG. 1. Injection of indomethacin reverses suppression of antibody production induced by MPLA and GLA 60 in mice. Four mice each were injected intraperitoneally with PBS and MPLA (MPL) (25 to 50 μ g [A]) or GLA 60 (50 to 100 μ g [B]). Two, 8, and 24 h later, indomethacin (120 μ g intraperitoneally) was injected into two PBS-treated and two adjuvant-treated mice, with equal volumes of pH-adjusted NaHCO_3 injected into the remaining mice. The mice were sacrificed 48 h after initial compound injection, and spleen cells were cultured in vitro with antigen. The hemolytic plaque assay was carried out on day 4. Data from individual experiments were first averaged, and then the means were pooled with those of identical groups from other experiments. Results are expressed as means $\pm$ standard errors of three (A) or four (B) experiments. Control values averaged 1,979 (A) and 1,083 (B) plaques per culture. *, $P \leq 0.05$ versus PBS (one tail); **, $P \leq 0.05$ versus MPLA or GLA 60 (two tails).

after determining the $\log_{10}$ ratio of each of the two groups being compared. Statistical significance was assigned to comparisons showing a P value of <0.05 .

Figure 1A demonstrates that injection of indomethacin approximately 2, 8, and 24 h after injection of MPLA reversed the suppression of antibody production compared with that in PBS controls. These times were found to be effective when poly(A)-poly(U) suppression was studied previously (19). Figure 1B shows the same to be true when antibody suppression was induced by the synthetic LPS analog GLA 60.

Figure 2 demonstrates that in vitro addition of antibody to TGF- β , a second suppressive cytokine produced by macrophages, significantly reversed suppression induced in vivo by both MPLA (Fig. 2A) and GLA 60 (Fig. 2B). This antibody also increased the response in control animals, presumably because TGF- β is involved normally in a delayed downregulation of antibody production in response to SRBCs (9).

Accordingly, it is apparent that LPS constituents from gram-

FIG. 2. In vitro addition of TGF- β reverses suppression of antibody production induced by MPLA and GLA 60 in mice. Two or three mice each were injected intraperitoneally with PBS and MPLA (MPL; 25 μ g [A]) or GLA 60 (50 to 100 μ g [B]). Twenty-four hours later, the mice were sacrificed and spleen cells were cultured in vitro with or without 10 μ g of anti-TGF- β (aTGF β) and with SRBCs as antigen. The hemolytic plaque assay was performed on day 4. Data from individual experiments were first averaged, and then means were pooled with those of identical groups from other experiments. Results are expressed as means $\pm$ standard errors of six experiments in panel A and four experiments in panel B. Control values averaged 3,623 (A) and 3,250 (B) plaques per culture. *, $P < 0.05$ versus PBS (one tail); **, $P < 0.05$ versus MPLA or GLA 60 (two tails).

negative bacteria, in addition to their immunity-enhancing activity when given with antigen, can also exert potent suppressive activity if antigenic exposure is delayed. The macrophage products TGF- β and PGE appear to be active in the system described above, and both are known to be immunosuppressive.

Diverse microorganisms have been shown to induce nonspecific suppression of the immune response (24). The findings reported herein provide a lead into defining a responsible component and its endogenous mediators. Recently, Eisenstein and colleagues (1, 2) analyzed the immune suppression induced by attenuated *Salmonella typhimurium* vaccine when given to mice prior to antigens such as tetanus toxoid and SRBCs. A precursor macrophage, via nitric oxide mediation, was implicated by Eisenstein et al. as the cell responsible for inhibition. The suppression was reversible by addition of interleukin 4 (IL-4). Our data, attesting to the involvement of the macrophage secretory products TGF- β and PGE in suppression by glycolipids from gram-negative bacteria, further implicate the macrophage as a participant. A relationship with nitric oxide has not been established in our system.

Various mechanisms for PGE-induced suppression have been reported, including inhibition of both T-cell mitogenesis (7) and release of IL-2 (5) as well as antibody production (25, 27). PGE was hypothesized to bind to a specific receptor which, in turn, induced production of the second messenger cyclic AMP (cAMP), a known immunosuppressive agent (8, 21, 26). Thus, Gold et al. (8) found that PGE₂ and the PGE₁ analog misoprostol inhibited Th₁ human T-cell clones from secreting the helper factors gamma interferon and IL-2 by increasing cAMP. In addition, the cytokine secretory response of Th₀ cells was shifted by PGE₂ and misoprostol from a clone secreting IL-2, gamma interferon, IL-4, and IL-5 to one secreting only the Th₂-dominant cytokines, IL-4 and IL-5. IL-4 is known to inhibit Th₁ helper cytokine release.

TGF- β has been found both to inhibit B- and T-cell proliferation and to suppress immunoglobulin G and immunoglobulin M secretion. It also decreases the number of monocyte TGF receptors and H₂O₂ and O₂⁻ production (31). Although the mechanism by which TGF- β suppresses antibody has yet to be conclusively determined, it has been reported that TGF- β interferes with steps that occur late in the immunological process (6, 20). This is supported by our observation that anti-TGF- β added to spleen cells cultured 24 h after injection of GLA 60 or MPLA reversed the suppression of antibody. In addition, Bogdan et al. (4) found that TGF- β inhibited LPS-induced macrophage production of tumor necrosis factor alpha and IL-1 α and - β 12 to 16 h after culture initiation and that suppression occurred even when TGF- β was added 6 h after LPS.

Little research has considered PGE and TGF- β in the same suppressive environment. However, in one study, Roth and Golub (23) measured suppression by alveolar macrophages of both IL-2-induced proliferation of peripheral blood lymphocytes and suppression of IL-2 induction of lymphokine-activated killer cell activity. These investigators found that two mechanisms were operative, one diffusible and inhibited by indomethacin and one nondiffusible (contact dependent). The nondiffusible suppression was reversed upon addition of both anti-TGF- β and indomethacin. We hypothesize that two independent suppressive mechanisms similarly are functional in the LPS analog system reported herein.

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REFERENCES

1. al-Ramadi, B. K., Y. W. Chen, J. J. Meissler, Jr., and T. K. Eisenstein. 1991. Immunosuppression induced by attenuated Salmonella. Reversal by IL-4. *J. Immunol.* **147**:1954-1961.
2. al-Ramadi, B. K., J. J. Meissler, Jr., D. Huang, and T. K. Eisenstein. 1992. Immunosuppression induced by nitric oxide and its inhibition by interleukin-4. *Eur. J. Immunol.* **22**:2249-2254.
3. Baker, P. J., T. Hrabá, C. E. Taylor, K. R. Myers, K. Takayama, N. Qureshi, P. Stuetz, S. Kusumoto, and A. Hasegawa. 1992. Structural features that influence the ability of lipid A and its analogs to abolish the expression of suppressor T cell activity. *Infect. Immun.* **60**:2694-2701.
4. Bogdan, C., J. Paik, Y. Vodovotz, and C. Nathan. 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor- β and interleukin-10. *J. Biol. Chem.* **267**:23301-23308.
5. Chouaib, S., K. Welte, R. Mertelsmann, and B. Dupont. 1985. Prostaglandin E₂ acts at two distinct pathways of T lymphocyte activation: inhibition of interleukin 2 production and down-regulation of transferrin receptor expression. *J. Immunol.* **135**:1172-1179.
6. Diaz-Meco, M. T., I. Dominguez, L. Sanz, M. M. Municio, E. Berra, M. E. Cornet, A. G. deHerreros, T. Johansen, and J. Moscat. 1992. Phospholipase C-mediated hydrolysis of phosphatidylcholine is a target of transforming growth factor β inhibitory signals. *Mol. Cell. Biol.* **12**:302-308.
7. Ellner, J. J., and P. J. Spagnuolo. 1979. Suppression of antigen and mitogen induced human T lymphocyte DNA synthesis by bacterial lipopolysaccharide: mediation by monocyte activation and production of prostaglandins. *J. Immunol.* **123**:2689-2695.
8. Gold, K. N., C. M. Weyand, and J. J. Goronzy. 1994. Modulation of helper T cell function by prostaglandins. *Arthritis Rheum.* **37**:925-933.
9. Grinwich, K. D., and O. J. Plescia. 1977. Tumor mediated immunosuppression: prevention by inhibitors of prostaglandin synthesis. *Prostaglandins* **14**:1175-1182.
10. Ikeda, S., C. Nishimura, M. Nakatsuka, J. Y. Homma, M. Kiso, and A. Hasegawa. 1988. Antiviral and immunomodulating activities of chemically synthesized lipid A-subunit analogues GLA-27 and GLA-60. *Antivir. Res.* **9**:37-46.
11. Johnson, A. G. 1985. Modulation of antibody synthesis by bacterial endotoxins, p. 174-182. *In* L. J. Berry (ed.), *Endotoxins III. The cellular biology of endotoxins*. Elsevier Science Publishing, Inc., New York.
12. Johnson, A. G. 1985. Regulation of the immune system by nucleic acids and polynucleotides, p. 107-119. *In* P. F. Torrence (ed.), *Biological response modifiers*. Academic Press, New York.
13. Johnson, A. G., M. A. Tomai, Y. Chen, and M. J. Odean. 1991. A comparison of the immunomodulating properties of two forms of monophosphoryl lipid A analogues. *J. Immunother.* **10**:398-404.
14. Kennedy, J., and M. Axelrad. 1971. An improved assay for haemolytic plaque-forming cells. *Immunology* **20**:253-257.
15. Kiso, M., H. Ishida, and A. Hasegawa. 1984. Synthesis of biologically active novel, monosaccharide analogs of lipid A. *Agric. Biol. Chem.* **48**:251-252.
16. Mishell, R., and R. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423-442.
17. Morrison, D. C., and J. L. Ryan. 1980. Bacterial endotoxins and host immune responses. *Adv. Immunol.* **28**:293-450.
18. Odean, M. J., A. G. Johnson, M. Mohrman, A. Hasegawa, and M. Kiso. 1992. Immunosuppression induced by non-reducing acylated monosaccharide subunits of lipid A. *Int. J. Immunopharmacol.* **14**:933-939.
19. Odean, M. J., G. J. Trachte, and A. G. Johnson. 1991. Characterization of immune suppression induced by polyribonucleotides. *Int. J. Immunopharmacol.* **13**:339-348.
20. Pietenpol, J. A., R. W. Stein, E. Moran, P. Yaciuk, R. Schlegel, R. M. Lyons, M. R. Pittlekow, K. Munger, P. M. Howley, and H. L. Moses. 1990. TGF- β 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* **61**:777-785.
- 20a. R & D Systems. Personal communication.
21. Rao, C. V., and S. B. Mitra. 1982. Distribution of PGE and PGF₂ alpha receptor proteins in the intracellular organelles of bovine corpora lutea. *Methods Enzymol.* **86**:192-202.
22. Rietschel, E. T., U. Seydel, U. Zahring, U. Schade, L. Brade, H. Loppnow, W. Feist, M. H. Wang, A. J. Ulmer, H. D. Flad, K. Brandenburg, T. Kirikae, D. Grimmecke, O. Holst, and H. Brade. 1991. Bacterial endotoxin: molecular relationships between structure and activity. *Infect. Dis. Clin. North Am.* **5**:753-779.
23. Roth, M. D., and S. H. Golub. 1993. Human pulmonary macrophages utilize prostaglandins and transforming growth factor β 1 to suppress lymphocyte activation. *J. Leukocyte Biol.* **53**:366-371.
24. Schwab, J. H. 1975. Suppression of the immune response by microorganisms. *Bacteriol. Rev.* **39**:121-143.
25. Simkin, N. J., D. F. Jelinek, and P. E. Lipsky. 1987. Inhibition of human B cell responsiveness by prostaglandin E₂. *J. Immunol.* **138**:1074-1081.
26. Stenson, W. F., and C. W. Parker. 1982. Prostaglandins and the immune response, p. 39-89. *In* J. B. Lee (ed.), *Prostaglandins*. Elsevier/North Holland Publishing Co., Amsterdam.
27. Straite, N. D., and G. S. Panayi. 1982. Regulation of human immunoglobulin production *in vitro* by prostaglandin E₂. *Clin. Exp. Immunol.* **49**:115-122.
28. Stuetz, P. L., H. Aschauer, J. Hildebrandt, C. Lam, H. Loibner, I. Macher, D. Scholz, E. Scheutze, and H. Vypel. 1990. Chemical synthesis of endotoxin analogues and some structure activity relationships, p. 129-144. *In* A. Nowotny, J. J. Spitzer, and E. J. Ziegler (ed.), *Cellular and molecular aspects of endotoxin reactions*, vol. 1. Elsevier Science Publishers, New York.
29. Tomai, M. A., and A. G. Johnson. 1989. Gamma interferon mediates the adjuvant action of a detoxified endotoxin. *J. Biol. Response Modif.* **8**:643-661.
30. Tomai, M. A., L. E. Solem, A. G. Johnson, and E. Ribí. 1987. The adjuvant properties of a nontoxic monophosphoryl lipid A in hyporesponsive and aging mice. *J. Biol. Response Modif.* **6**:99-107.
31. Wahl, S. M., N. McCartney-Francis, J. B. Allen, E. B. Dougherty, and S. F. Dougherty. 1990. Macrophage production of TGF- β and regulation by TGF- β . *Ann. N. Y. Acad. Sci.* **593**:188-196.