GENETICAL CONTROL OF B-CELL RESPONSES

III. Requirement for Functional Mitogenicity of the Antigen in Thymus-Independent Specific Responses*

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We and others have recently described the selective unresponsiveness of C3H/HeJ mice to the B-cell mitogen lipopolysaccharide from *Escherichia coli:* 055:B5 (LPS)[1] (1–3). We have also characterized this unresponsiveness as a pure B-cell defect, since it does not depend upon the inactivation of the mitogen or other trivial in vivo or in vitro conditions particular to these mice, nor is it caused by the activity of non-B cells (T cells or macrophages).[2] LPS is known to induce a fraction of all B cells in high-responder strains into proliferation and selectively increased synthesis and secretion of immunoglobulin (4). B cells from C3H/HeJ mice fail to develop either type of response when in the presence of LPS concentrations which are optimal for high-responder strains.

We have recently proposed a hypothesis for B-cell activation in thymus-independent (TI)-specific immune responses which ascribes to the Ig surface receptors on B cells the passive role of focusing molecules with nonspecific triggering properties onto the membrane of the specific cells. Activation is thought to be the result of "one nonspecific signal", delivered to the cell by the polyclonal B-cell activator (PBA) properties of the TI antigen (4, 5). The LPS unresponsiveness of C3H/HeJ mice offered a suitable system to critically test this hypothesis. The prediction would be, according to the model, that C3H/HeJ mice would also fail to mount TI-specific immune responses to LPS (6) or to hapten-LPS conjugates, which are also known to be TI in conventional strains (7). On the other hand, since the genetical B-cell defect in these mice is selective for LPS-induced responses and normal induction is obtained with other PBAs (3, footnote 2), these mice should be expected to mount normal responses to other TI antigens.

On the contrary, if the mechanism of TI B-cell induction were the pattern of

[1] *Abbreviations used in this paper:* AECM, aminoethyl carbamylmethyl; FCS, fetal calf serum; LPS, lipopolysaccharide from *E. coli* 055:B5; NNP, (4-hydroxy-3,5-dinitrophenyl) acetyl; PBA, polyclonal B-cell activator; PFC, plaque-forming cells; PPD, purified protein of tuberculin; TD, thymus-dependent; TI, thymus-independent

[2] Coutinho, A., and E. Gronowicz. 1975. Genetical control of B-cell responses. II. Characterization of the B-cell unresponsiveness to lipopolysaccharide in C3H/HeJ mice. Manuscript submitted for publication.

antigen presentation, inducing a suitable degree of cross-linkage of the Ig surface receptors (8), C3H/HeJ mice should be expected to respond to LPS or hapten-LPS conjugates as the high-responder strains. In the present paper we report our initial experiments performed along these lines, namely the first clear conclusion: C3H/HeJ mice fail to mount specific TI responses to LPS or to hapten-LPS conjugates.

Materials and methods

C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and bred in our colony for the last 3 yr. In some experiments, adult mice obtained from the same source were used. The high-responder strain used in the present experiments was B10.5M (H-2^b). Mice of the same age and sex were selected for each experiment. LPS was extracted from $E.$ $coli$ 055:B5 by the phenol-water method (9), and obtained from Dr. T. Holme, Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden. Purified protein derivative (PPD) from tuberculin RT 32 was obtained from Statens Seruminstitutet, Copenhagen, Denmark. (4-hydroxy-3,5-DNP)acetyl-LPS (NNP-LPS) conjugates were prepared as previously described (7). DNP-AECM-Ficoll (a kind gift from Dr. W. E. Paul, NIH) was prepared by Dr. John Inman, National Institute of Allergy and Infectious Diseases, Bethesda, Md., and the conjugate had a substitution ratio of 56 DNP and 83 aminoethyl carbamylmethyl (AECM) groups per 400,000 daltons of Ficoll. $E.$ $coli$ bacteria used for in vivo immunization were inactivated by boiling them for 15 min, and 10^8 bacteria were injected i.v. to each mouse.

Culture conditions for activation by mitogens and antigens were as previously described (7), as well as the hemolytic plaque assay for detecting high and low avidity anti-NNP plaque-forming cells (PFC). For detecting anti-DNP-AECM-Ficoll responses, TNP-substituted red cells were used in the plaque assay (10), which were prepared according to Rittenberg and Pratt (11), using 15 mg of TNBS (Sigma Chemical Co., St. Louis, Mo.) for 1 ml packed red cells in 10 ml of a cacodylate buffer pH 6.8. For detecting anti-LPS PFC, the coupling of red cells to the polysaccharide was done as described by Möller (12). Indirect (IgG) PFC were detected by incorporating in the agar a suitable dilution of a polyvalent rabbit antimouse Ig serum.

Results

The selective unresponsiveness of C3H/HeJ spleen cells to LPS is shown in Fig. 1, as compared to normal responses obtained with cells from a high-responder strain (B10.5M). The mitogen-induced responses were measured in this particular experiment by detecting activation for polyclonal antibody production, by means of a sensitive plaque assay which employs highly NNP-substituted red cells, and can detect around 1% of all high-rate antibody-secreting cells (4). High-responder spleen cells show significant increases in numbers of PFC after stimulation by LPS concentrations as low as 0.01 μg/ml, whereas C3H/HeJ spleen cells barely mount any response at all. Only at very high concentrations of LPS (100 and 1,000 μg/ml) does a response of very low magnitude appear.

Fig. 1. Spleen cells from these two strains of mice were then tested for their ability to mount specific antibody responses to the TI antigen NNP-LPS (7). As shown in Fig. 2A, NNP-LPS, over a wide range of concentration, is not competent to induce an antihapten-specific response in C3H/HeJ cells, whereas the same concentrations elicit in B10.5M cells the typical bell-shaped dose-response curve, as previously described (7). In a large number of experiments the same results were invariably obtained, namely the clear failure to induce anti-NNP-specific responses with NNP-LPS conjugates. In parallel with the pattern of unresponsiveness to LPS as a polyclonal mitogen, a very low but

definite antihapten response was observed with very high concentrations of NNP-LPS, which were completely paralytogenic for high-responder cell cultures (results not shown).

Fig. 2. Serum-free cultures are known to be suitable for the induction of TI antibody responses (7, 13, 14, 15). Therefore, the failure of NNP-LPS to induce a
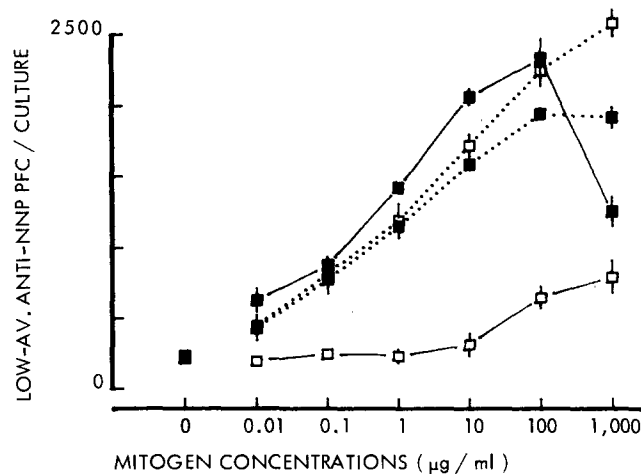

FIG. 1. Spleen cells from B10.5M (■) or C3H/HeJ (□) mice were cultured in the presence of the indicated concentrations of either LPS (———) or PPD (· · ·), at the cell concentration of 10⁷ cells/ml/culture, in serum-free medium. Responses were measured 2 days later, by detecting numbers of PFC against SRBC coated with a high density of NNP determinants. Results are shown as the mean ± 1 SE of triplicate cultures.
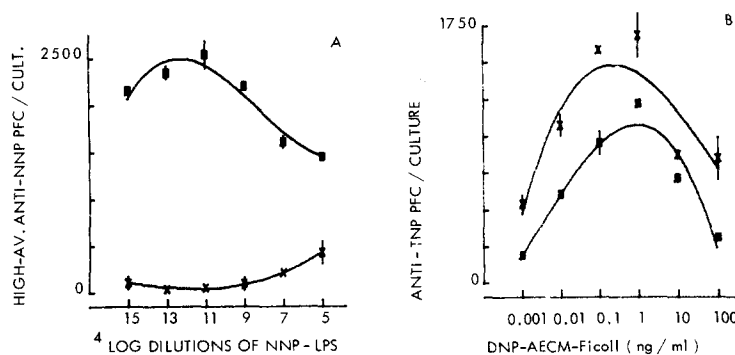

FIG. 2. Spleen cells from B10.5M (■—■) or C3H/HeJ (x—x) mice were cultured (10⁷ cells/ml/culture) in serum-free medium in the presence of the indicated concentrations of either NNP-LPS (A), or DNP-AECM-Ficoll (B). Responses were measured after 3 days in culture by detecting high-avidity PFC against NNP or TNP, in A and B, respectively. Symbols represent the means ± 1 SE of PFC responses observed in triplicate cultures, after subtracting from each experimental value the average number of PFC detected in control, unstimulated cultures. These were 40 ± 10, and 27 ± 13 anti-NNP PFC, in B10.5M and C3H/HeJ cell cultures, and 33 ± 3, and 62 ± 7 anti-TNP PFC, in B10.5M and C3H/HeJ cell cultures, respectively. Curves are the least squares fit for the experimental values, plotted in a Hewlett-Packard 9820A calculator, using the appropriate program. The biological characterization of the NNP-LPS batch used in this experiment has been given elsewhere (6).

specific response in C3H/HeJ spleen cells indicates that this antigen lacks TI characteristics for LPS nonresponder cells. To test whether this unresponsiveness to NNP-LPS conjugates was selective for the carrier or whether it was due to other defects of C3H/HeJ cells when stimulated in vitro for TI responses, parallel cultures from those two strains were challenged with another TI hapten conjugate, DNP-AECM-Ficoll (10). As shown in Fig. 2 B, C3H/HeJ spleen cells responded as well or better than B10.5M cells to this antigen. This excludes trivial reasons as the explanation for the unresponsiveness of C3H/HeJ spleen cells to NNP-LPS.

The high-low responder system was further investigated in vivo. We had found before that the unresponsiveness of C3H/HeJ mice to LPS is also observed in vivo.[2] Thus, high-responder strains mount a very sharp polyclonal PFC response to mitogenic doses of LPS in vivo (30-fold increases over the values in control mice) which peaks 3 days after injection of the mitogen (16, footnote 2). C3H/HeJ mice barely display a very faint response at supra-optimal doses of LPS.[2] We have now investigated the capacity of high and low responder strains to develop specific anti-LPS antibody responses in vivo. C3H/HeJ or B10.5M mice were challenged with doses of LPS suitable for the induction of a specific antibody response (17). Anti-LPS plaque responses were measured 6 days later. As can be seen in Table I, low responder mice do not mount a specific antibody response to LPS, which is known to be TI in conventional strains (6). To critically exclude other reasons for unresponsiveness, such as nonspecific mechanisms of elimination of the antigen, V gene defects or suppressor cell activity, we tried to obtain specific anti-LPS responses where the mitogenicity of the antigen should be irrelevant, according to our model of B-cell activation. This could presumably be

TABLE I

Specific Anti-LPS Antibody Responses in Vivo in C3H/HeJ and B10.5M Mice

| Antigen | PFC/spleen | | | |
| | Direct | | Indirect | |
	C3H/HeJ	B10.5M	C3H/HeJ	B10.5M
—	0	0	0	0
0.01 μg LPS	0.8 ± 0.1 (6)	2.85 ± 0.069 (707)	0	0
1 μg LPS	1.38 ± 0.23 (24)	3.31 ± 0.17 (2,062)	0	0
10⁸ E. coli bacteria	4.43 ± 0.06 (27,169)	4.33 ± 0.01 (21,402)	4.77 ± 0.03 (58,253)	4.51 ± 0.02 (32,400)

Groups of three mice were injected i.v. with the indicated doses of LPS or E. coli bacteria. 6 days later spleens were removed and assayed for specific anti-LPS PFC. Values of PFC obtained against uncoated red cells were subtracted from the values obtained against LPS-coated red cells. No PFC specific for LPS were detected in control, untreated mice in this experiment. Results are presented as the geometrical mean ± 1 SE of the numbers of PFC detected in each experimental group. The antilog values are also shown in parentheses.

achieved by using a TD form of the immunogen. In this case, triggering signals for the specific B cells would be provided by the cooperative cell system, rather than by the antigen itself (4). We had found before that when mice are challenged by mildly heat-inactivated *E. coli* bacteria, an IgM and IgG antibody response develops, which is dependent on the presence of thymus-derived cells, and is superimposed on an IgM TI response, comparable to the responses induced by soluble LPS (17, and A. C. and G. Möller, unpublished). This finding has been interpreted in terms of the stimulation of the acessory cell system by protein antigens present in these mildly inactivated bacteria. Whatever the mechanism, since this response is thymus-dependent (TD), LPS mitogenicity should not be expected to play a role in its development. As shown in Table I, C3H/HeJ mice, when challenged with this TD form of the immunogen, mount a very good specific plaque response against LPS, of both IgM and IgG classes.

Discussion

Both NNP-LPS and LPS are known to induce TI-specific responses (6, 7). TI antigens are competent to induce specific in vitro responses in serum-free media, whereas TD responses require further addition of well-defined B- or T-cell mitogens, or else, of a "good" batch of fetal calf serum (FCS) (13, 14, 15, 18). Therefore, the failure of NNP-LPS to be immunogenic for C3H/HeJ spleen cells under these conditions might reflect that it is a TD antigen for low responder cells or else that it is not at all immunogenic. Nonmitogenic TNP-LPS conjugates were recently shown to be nonimmunogenic both in vitro and in vivo (19), suggesting that the immune response to the hapten-LPS conjugates necessarily requires functional mitogenicity. This could be due to the fact that T cells seem to "ignore" some structures of this type (20) and it has been actually impossible to detect LPS-binding T cells (21) in conventional strains. The lack of T-cell involvement in the responses to LPS would make the B-cell mitogenicity of this ligand a strict requirement not only for thymus independence but also for immunogenicity. Our present results demonstrating unresponsiveness of C3H/HeJ mice to LPS in vivo are in support of that concept. However, two other sets of observations have recently reported a low but definite response of C3H/HeJ mice to in vivo challenge with LPS (22, 23). The differences most probably depend on the purity of the LPS preparations used for immunization, as well as in the doses of antigen injected, since, as pointed out above, C3H/HeJ mice do respond to very high concentrations of LPS.

The specific defect in C3H/HeJ mice to mount TI responses to LPS or to NNP-LPS is clearly not due to a V gene defect in these mice. Thus, (a) Anti-NNP PFC develop in spleen cell cultures from these mice at normal values, when polyclonally activated by another B-cell mitogen, PPD (Figs. 1 and 2) (b) Spleen cell cultures from these mice mount good specific TI responses to a structurally related antigen (Fig. 2B), and (c) C3H/HeJ mice develop normal anti-LPS responses when challenged with a TD form of the antigen (Table I). The latter experiments also make the possibility very unlikely that the unresponsiveness is due to suppressor cell activity induced by LPS determinants in these mice. Trivial reasons for explaining the in vitro unresponsiveness to NNP-LPS can be

excluded in terms of the normal responses obtained to DNP-AECM-Ficoll in parallel cultures. All these results are further supported by our previous demonstration that the unresponsiveness of C3H/HeJ mice to LPS as a polyclonal mitogen is due to a pure B-cell defect.[2]

The present results, as well as the recent findings that C3H/HeJ mice are low responders to an antigenic challenge of LPS in vivo (22, 23) can only be interpreted in terms of the strict requirement for functional mitogenicity of the antigen for the induction of TI-specific immune responses (4, 5). This requirement might be considered in two different ways, discussing the mechanisms of B-cell activation. Either it is assumed that a nonspecific signal is the only one relevant for activation (5), or else, it might be postulated that, though a nonspecific signal is strictly required, immune activation also requires one specific, Ig-mediated signal. This would be the "two-signal" concept as applied to TI responses (24, 25), though the basic "two-signal" hypothesis actually denies the existence of such type of induction (24). Experiments to argue either way are available, and quite clearly suggest that immune activation is the result of "one nonspecific signal" (4).

It should be clearly pointed out that these observations, as other results obtained in the low responder system (2, 22, 23), definitely exclude another model of B-cell activation, fashionable a few years ago, namely the "pattern of determinant presentation" (8, 26). The nature of the B-cell defect in C3H/HeJ mice is still unclear, and a number of parallel efforts are being developed in several laboratories for its elucidation. However, it is already clearly established that the unresponsiveness to LPS at the polyclonal level segregates together with the unresponsiveness to LPS as an antigen (23). Also in the set of observations reported in the present paper, genetical analysis was carried out, and it was observed that unresponsiveness to LPS was linked to unresponsiveness to NNP-LPS in F_2 and backcrosses between C3H/HeJ and B10.5M mice. These observations are critical for the above conclusions and they will be published elsewhere (A. Coutinho and E. Gronowicz, to be published).

One last point should be commented upon, namely the B-cell mitogenicity of Ficoll. Though the structure of this substance is very similar to other well known B-cell mitogens, it does not display sharp mitogenicity for spleen B cells, as pointed out already when the immunogenicity of DNP-Ficoll conjugates was originally described (10). However, it is still possible to demonstrate nonspecific B-cell activating properties in this molecule, using sensitive test systems for polyclonal antibody production. We also have strong evidence, from hot-pulse experiments, that the B cells responding to NNP-LPS participate in the polyclonal response to LPS, whereas B cells responding to DNP-AECM-Ficoll do not. This provides further insight to the understanding of the results reported here.

Summary

Spleen cells from C3H/HeJ mice fail to respond with polyclonal antibody synthesis to mitogenic concentrations of lipopolysaccharide (LPS) which are optimal for activating spleen cells from a high-responder strain (B10.5M). This

unresponsiveness is selective for LPS, since C3H/HeJ cells respond as normals to another B-cell mitogen, purified protein derivative of tuberculin.

Spleen cells from low-responder mice also fail to mount a specific anti-NNP plaque-forming cell (PFC) response, when challenged in vitro by NNP-LPS. However, C3H/HeJ cells develop normal responses to another thymus-independent hapten conjugate, DNP-AECM-Ficoll.

C3H/HeJ mice fail to mount a specific anti-LPS antibody response, when challenged in vivo with doses of soluble LPS which are fully immunogenic for the high-responder strain. However, C3H/HeJ mice develop normal direct and indirect PFC responses to LPS, when challenged with a thymus-dependent form of the immunogen. These results are interpreted as indicating an absolute requirement for functional mitogenicity of the antigen, in the induction of specific thymus-independent antibody responses.

References

1. Sultzer, B. M., and B. S. Nilsson. 1972. PPD tuberculin—a B cell mitogen, *Nat. New Biol.* **240:** 198.
2. Chiller, J. M., J. A. Louis, B. J. Skidmore, and W. O. Weigle. 1974. Manipulation of a tolerant state: cells and signals. *In* The Immune System: Genes, Receptors, Signals. E. E. Sercarz, A. R. Williamson, and C. F. Fox, editors. Academic Press, Inc., New York. 553.
3. Coutinho, A., E. Gronowicz, and B. M. Sultzer. 1974. Genetical control of B cell responses: selective unresponsiveness to lipopolysaccharide. *Scand. J. Immunol.* In press.
4. Coutinho, A., and G. Möller. 1975. Thymus-independent B cell induction and paralysis. *Adv. Immunol.* In press.
5. Coutinho, A., and G. Möller. 1974. Immune activation of B cells: evidence for "one nonspecific triggering signal" not delivered by the Ig receptors. *Scand. J. Immunol.* **3:** 133.
6. Möller, G., and G. Michael. 1971. Frequency of antigen-sensitive cells to thymus-independent antigens. *Cell. Immunol.* **2:** 309.
7. Coutinho, A., E. Gronowicz, W. W. Bullock, and G. Möller. 1974. Mechanism of thymus-independent immunocyte triggering: mitogenic activation of B cells results in specific immune responses. *J. Exp. Med.* **139:** 74.
8. Greaves, M. F., G. Janossy, M. Feldman, and H. Doenhoff. 1974. Polyclonal mitogens and the nature of B lymphocyte activation mechanisms. *In* The Immune System: Genes, Receptors, Signals. E. E. Sercarz, A. R. Williamson, and C. F. Fox editors. Academic Press, Inc., New York. 271.
9. Westphal, O., O. Luderitz, and F. Bister. 1952. Uber die extraktion von bakterium mit phenolwasser. *Z. Naturforsch.* **76:** 148.
10. Mosier, D. E., B. M. Johnson, W. E. Paul, and P. R. B. McMaster. 1974. Cellular requirements for the primary in vitro antibody response to DNP-Ficoll. *J. Exp. Med.* **139:** 1354.
11. Rittenberg, M. B., and C. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132:** 575.

12. Möller, G. 1965. 19S antibody production against soluble lipopolysaccharide antigens by individual lymphoid cells in vitro. *Nature (Lond).* **207:** 1166.

13. Coutinho, A., and G. Möller. 1973. In vitro induction of specific immune responses in the absence of serum: requirement for nonspecific T or B cell mitogens. *Eur. J. Immunol.* **3:** 521.

14. Schrader, J. W. 1974. Evidence for the presence in unimmunized mice of two populations of bone marrow derived B lymphocytes, defined by differences in adherence properties. *Cell. Immunol.* **10:** 380.

15. Feldman, M., and M.B . Pepys. 1974. Role of C3 in lymphocyte co-operation in vitro. *Nature (Lond.).* **249:** 159.

16. Gronowicz, E., and A. Coutinho. 1974. Selective triggering of B cell subpopulations by mitogens. *Eur. J. Immunol.* **4:** 771.

17. Coutinho, A. 1974. Immune activation of B cells: an analysis of the triggering mechanisms. Ph. D. Thesis. Karolinska Institutet, Stockholm, Sweden.

18. Watson, J., R. Epstein, I. Nakoinz, and P. Ralph. 1973. The role of humoral factors in the initiation of in vitro immune responses. II. Effect of lymphocyte mitogens. *J. Immunol.* **110:** 43.

19. Jacobs, D. M., and D. C. Morrison. 1975. Stimulation of a T-independent primary anti-hapten response *in vitro* by TNP-Lipopolysaccharide (TNP-LPS). *J. Immunol.* In press.

20. McDevitt, H. O., and M. Landy, editors. 1972. Genetical Control of Immune Responsiveness. Academic Press, Inc., New York.

21. Sjöberg, O., 1971. Antigen-binding cells in mice immune or tolerant to Escherichia coli polysaccharide. *J. Exp. Med.* **133:** 1015.

22. Skidmore, B. J., J. M. Chiller, D. C. Morrison, and W. O. Weigle. 1975. Immunological properties of bacterial lipopolysaccharides (LPS). Correlation between the mitogenic adjuvant and immunogenic activities. *J. Immunol.* In press.

23. Watson, J., and R. Riblet. 1974. Genetical control of responses to bacterial lipopolysaccharides in mice. I. Evidence for a single gene that influences mitogenic and immunogenic responses to LPS. *J. Exp. Med.* **140:** 1147.

24. Cohn, M. 1972. Conference evaluation and commentary. *In* Genetical Control of Immune Responsiveness. H. O. McDevitt, and M. Landy, editors. Academic Press, Inc. New York. 370.

25. Watson, J., E. Trenkner, and M. Cohn. 1973. The use of bacterial lipopolysaccharides to show that two signals are required for the induction of antibody synthesis. *J. Exp. Med.* **138:** 699.

26. Feldman, M., and G. J. V. Nossal. 1972. Tolerance, enhancement and the regulation of interactions between T cells, B cells and macrophages. *Transplant. Rev.* **13:** 3.