

IMMUNE RESPONSE TO A HAPTEN COUPLED TO A NONIMMUNOGENIC CARRIER

INFLUENCE OF LIPOPOLYSACCHARIDE*

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Cooperation between two or more cell types is an essential feature of the immune response to some antigens (1). These cell types are commonly referred to as thymus derived (T) and bone marrow derived (B). Furthermore, in the immune response to a hapten bound to a carrier protein these two cell types perform quite separate and distinct cooperative functions (2-5). The T cell is responsible for recognition of the carrier and has been termed the helper cell (5, 6); the B cell synthesizes antibody to the hapten.

In another series of observations lipopolysaccharide (LPS), endotoxin, from Gram-negative bacteria was shown to alter thymic-deficient mice so that they could respond with antibody formation to thymic-dependent antigens. Mice that were thymectomized, lethally irradiated, and bone marrow reconstituted (TxB) responded to heterologous red cells coated with LPS (7). Also, lethally irradiated, bone marrow reconstituted mice responded to injections of sheep red blood cells (SRBC) when accompanied by LPS (8). LPS alone acted as a mitogen on B cells (9, 10). Thus, when antigen is present LPS might substitute for T cell function by direct stimulation of B cells which are then able to form antibody to the antigen resulting in an immune response.

If a hapten such as trinitrophenyl (TNP) were coupled to a nonimmunogenic carrier such as isologous mouse red blood cells (MRBC), no immune response would be expected from the injected host because the T, or helper, cell would not recognize MRBC as a foreign carrier. However, if LPS did stimulate B cells directly, it might be hypothesized that, in the presence of LPS, an immune response to a hapten on a nonimmunogenic carrier could be induced. In this paper we report such an effect of LPS on the immune response in BALB/c mice to TNP coupled to isologous MRBC.

Materials and Methods

Mice.—BALB/c Dub female mice were used at 10-12 wk of age and obtained from Dublin Farms, Dublin, Va.

Reagents.—Burro red blood cells (BRBC) and SRBC were from Davis Laboratories, Davis,

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Calif., and Colorado Serum Co., Denver, Colo., respectively. Picryl sulfonic acid, 2,4,6-trinitrobenzene sulfonic acid (TNBS), was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. *Serratia marcescens* endotoxin (LPS) lot 557128 was obtained from Difco Laboratories, Detroit, Mich. Bovine serum albumin (BSA), fraction V, was supplied by Armour and Co., Chicago, Ill.

Conjugation.—For plaque assays BRBC and MRBC were conjugated as previously described (11). Briefly, washed RBC (1 ml of packed cells) were added to 7 ml of cacodylate buffer, 0.28 M, pH 6.9, containing 20 mg of TNBS and stirred slowly at 23°–25°C for 10 min. The reaction was stopped by the addition of 15 ml modified barbital buffer (MBB), at pH 7.3, containing 7 mg of glycyl-glycine, and the mixture was centrifuged for 10 min. The cells were washed twice more in MBB and once in balanced salt solution (BSS) before use.

For immunization, MRBC, obtained from isologous BALB/c mice and washed three times, were conjugated with TNBS as previously described (12). Briefly, 3 ml of packed MRBC or SRBC was incubated with 130 mg of TNBS in 7 ml of cacodylate buffer for 30 min at room

TABLE I
Effect of LPS on the Immune Response to TNP-MRBC in BALB/c Mice

Products*	Direct plaque-forming cells on day 4†	
	PFC/10 ⁶ spleen cells‡	PFC/spleen§
TNP-MRBC + LPS¶	25.5	8504
MRBC**	0	0
TNP-MRBC	2.0	75
MRBC, treated‡‡	0.6	300
MRBC, treated + LPS	0.6	219
LPS	0.7	230
TNP-SRBC	38.0	13,880

* Mice, in groups of five, were injected as indicated with 4×10^8 MRBC, 4×10^8 TNP-MRBC, or 4×10^8 treated MRBC in 0.5 ml i.p. with or without 10 μ g LPS on day zero.

† Direct plaque-forming cells were assayed using TNP-burro cells as indicator.

‡ Data are means of five mice; PFC = plaque-forming cells.

|| TNP conjugated to MRBC as described in text.

¶ LPS = lipopolysaccharide, 10 μ g.

** MRBC = mouse red blood cells.

‡‡ MRBC treated with buffers used to conjugate TNP, but no TNP was added.

temperature. The reaction was stopped by centrifugation at 2000 rpm. The cells were washed in MBB during which considerable lysis was noted. The remaining cells and stroma were then washed in BSS immediately before injection. TNBS was conjugated to BSA (17 mol of TNP/mol of BSA) by a modification of the methods of Rittenberg and Amkraut (13).

Antibody Assay.—A modified plaque assay has been previously described (11). TNP conjugated BRBC were used as indicator cells. All mice were assayed on the fourth day after primary immunization.

RESULTS AND DISCUSSION

BALB/c MRBC were obtained from isologous mice of the same sex and age as the prospective recipients of injections. Thus, TNP was coupled to MRBC, BALB/c T cells failed to recognize their own carrier red cells, and no immune response to TNP occurred. Data in Table I show that when 10 μ g of LPS was mixed with 4×10^8 TNP-MRBC and the mixture injected into BALB/c mice a significant response to TNP was obtained. The number of plaque-forming

cells (PFC) was 25.5 PFC/10⁶ spleen cells and 8504 PFC/spleen compared with 2.0 PFC/10⁶ spleen cells and 75 PFC/spleen for the injection of 4 × 10⁸ TNP-MRBC alone. Thus, addition to LPS caused a greater than 10-fold increase in the PFC response to TNP coupled to a nonimmunogenic carrier, isologous MRBC.

It has been suggested that immunological unresponsiveness to self antigens normally might be maintained in T cell populations (14); therefore, a lack of recognition of MRBC as a carrier was expected. Furthermore, no T cells have the capacity to bind LPS (15). Thus, the T cell population of the mouse probably could not respond to either its own red cells or LPS.

The injection of unconjugated MRBC did not result in the appearance of any TNP-specific PFC. If the surface of the MRBC was altered by the buffers used to conjugate TNP to the cell and this change in the surface configuration caused it to cross-react with TNP, then a response to TNP could be expected.

TABLE II
Specificity of TNP-MRBC Response in BALB/c Mice Injected with TNP-MRBC+LPS*

Indicator cell	Direct PFC on day 4	
	PFC/10 ⁶	PFC/spleen
TNP-BRBC‡	25.5	8504
MRBC	0	0
BRBC	2.0	50
TNP-MRBC‡	17.0	2991

* Mice, in groups of five, were treated as described in Table I.

‡ Cells were lightly conjugated as described in Materials and Methods.

However, as in Table I, when buffer-treated MRBC were injected with or without LPS no significant PFC response to TNP in BALB/c mice was noted. Furthermore, the injection of LPS alone did not induce a response in these mice specific for TNP (Table I), suggesting a lack of reactivity.

A previous report indicated that for LPS to convert heterologous red cells into thymus-independent antigens, the LPS had to be fixed on their surfaces (7). However, another report (8) and our results show that free LPS may be injected along with antigen and cause this conversion to occur suggesting that the LPS acts separately from the antigen.

The response of BALB/c mice to TNP coupled to a heterologous carrier, SRBC, was almost equivalent to that obtained when TNP was coupled to MRBC and injected with LPS. A response of 38 PFC/10⁶ spleen cells was noted for TNP-SRBC, whereas the response to TNP-MRBC + LPS was 25.5 PFC/10⁶ spleen cells.

As shown in Table II, the specificity of the PFC for TNP was demonstrated by measuring the number of PFC to TNP conjugated to BRBC. TNP-MRBC could also be used but the number of plaques was not as great, perhaps due to a

lesser susceptibility of MRBC to lysis. The absence of plaques to MRBC and very low number of PFC to BRBC alone further substantiated the specificity of the plaque assay for TNP.

A dose of 10 μg of LPS, when injected with TNP coupled to MRBC, was the most effective in inducing a response to TNP as indicated in Table III. Doses of 1 and 100 μg produced responses somewhat below this optimum. A similar

TABLE III
*Effect of LPS Concentration on Response to TNP-MRBC in BALB/c Mice**

4×10^8 TNP-MRBC +	Direct PFC on day 4	
	PFC/ 10^6	PFC/spleen
1.0 μg LPS	22.8	7871
10.0 μg LPS	30.2	10981
100.0 μg LPS	18.2	8531

* Mice, in groups of five, were given 4×10^8 TNP-MRBC in 0.5 ml i.p. and the amount of LPS indicated above.

TABLE IV
*Inhibition of Anti-TNP Plaque Formation by TNP-BSA from Spleen Cells of Mice Injected with TNP-MRBC+LPS**

Inhibitor \ddagger	Direct PFC on Day 4		% Inhibition \parallel
	PFC/ 10^6 \S	PFC/spleen	
None	21.1	12,225	0
10^{-5} M	1.3	885	94.0
10^{-6} M	4.0	1,937	81.0
$10^{-6.3}$ M	14.3	7,103	32.0

* 4×10^8 TNP-MRBC + 10 μg LPS injected in 0.5 ml i.p.

\ddagger Amount of TNP-BSA added in 100-500 μl of 0.5% agarose in balanced salt solution (BSS) at 45°C. 50 μl of 1:15 (w/v) suspension of TNP-burro cells and 100 μl of immune spleen cells were added. (None = 100 μl of BSS added.) TNP-BSA is trinitrophenyl bovine serum albumin (17 mol TNP/mol BSA) prepared as described in text.

\S Data are means of five mice.

$$\parallel \text{ Per cent inhibition} = \left(\frac{\text{PFC with inhibitor}}{\text{PFC without inhibitor}} \times 100 \right).$$

dose response was observed when addition of LPS increased the number of anti-SRBC PFC in vitro (16).

If the PFC are specific for the hapten TNP, then the plaques should be inhibited by incorporating TNP bound to a carrier, such as BSA, into the agar during the plaquing procedure. Data in Table IV show that indeed this was the case. As the amount of TNP-BSA in the agar increased to a level of 10^{-5} M there was a corresponding increase in the per cent inhibition of PFC specific for TNP. 50% inhibition was achieved between 10^{-6} and $10^{-6.3}$ M.

These data are consistent with the following hypothesis. Lipopolysaccharide acting as a nonspecific mitogen for B cells (9, 10) directly stimulates these cells.

In the presence of a hapten, such as TNP coupled to a nonimmunogenic carrier to which there is presumably no T cell responsiveness nor T cell helper function, some B cells recognize the hapten and, in the presence of LPS, proliferate and differentiate to produce an antibody response to the hapten. This suggested mechanism of action is based on several observations. In TxB mice made immunologically unresponsive to LPS by repeated exposure, antibody formation to horse red blood cells (HRBC) coated with LPS could be induced and even enhanced compared with the response obtained in nonthymectomized mice given LPS-coated HRBC (7). In addition, spleen cells from LPS immunologically unresponsive animals respond with increased numbers of PFC to SRBC after treatment with LPS in vitro to the same extent as do normal spleen cells (16), and in cultures depleted of adherent cells or T cells LPS could substitute for these cell types in inducing a response to SRBC (17). Furthermore, since LPS is a thymus-independent antigen (18) and mice lack T cells capable of binding LPS (15), nonspecific binding to the membranes and subsequent stimulation of B cells may be expected without the need for a T cell function.

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