

**LACK OF ORAL TOLERANCE IN C3H/HeJ MICE\***

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Oral tolerance, or systemic unresponsiveness to ingested antigen, was first described at the turn of the century; however, the underlying mechanisms involved have only recently been studied in detail (1). Several investigations (2-4) have shown that oral administration of sheep erythrocytes (SRBC) to mice for prolonged periods results in systemic unresponsiveness to this antigen. The lack of responsiveness has been ascribed to T suppressor ( $T_s$ ) cells (5), suppressor factors (3, 5), and immune complexes (2) in serum. Oral tolerance to soluble antigen has also been attributed to the induction of  $T_s$  cells in the gut-associated lymphoreticular tissue (GALT), e.g., Peyer's patches (6). The primary site for induction of IgA precursor cells to orally administered antigen is in GALT, e.g., Peyer's patches (PP), and the selective migration of these sensitized cells to secretory tissues constitutes a major pathway for immune responses at mucosal surfaces (7). The relationship between induction of IgA responses and oral tolerance is not clear; however, recent studies (8) have provided convincing evidence that oral administration of soluble or particulate antigen leads to concurrent induction of systemic unresponsiveness and secretory immunity. Our laboratory has shown (9) that high IgA immune responses to orally administered, thymic-dependent (TD) antigen occur in lipopolysaccharide (LPS)-nonresponsive C3H/HeJ mice, and this response is due to enhanced production of T helper ( $T_h$ ) cells in GALT. If systemic unresponsiveness (oral tolerance) is mediated by  $T_s$  cells, which are induced in GALT, then the C3H/HeJ mouse may lack this cell type and thus offer a model for discriminating between T cell regulation of IgA immune responses and oral tolerance.

We report that prolonged oral administration of SRBC to LPS-nonresponsive C3H/HeJ mice leads to heightened splenic responses to systemically administered antigen, whereas similar treatment of LPS-responsive mice results in oral tolerance. Tolerance was due to the induction of  $T_s$  cells, which occurred in both PP and spleen; however, this T cell effector pathway was not induced in C3H/HeJ mice.

**Materials and Methods**

*Mice and Immunization.* C3H/HeJ, C3H/HeN, BALB/c, and Swiss mouse strains were bred and maintained in the Core Facility for Immunocompromised Mice, The Comprehensive Cancer Center at the University of Alabama in Birmingham, Ala. Mice were 8-15 wk of age. SRBC (Colorado Serum Company, Denver, Colo.) were washed extensively and used for

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gastric intubation (GI) of mice, as previously described (9). For peripheral immunization of mice, SRBC (a 20% suspension) were administered by the intraperitoneal route (0.1 ml).

*Purification of PP and Splenic T Lymphocytes and In Vitro Assays.* Mice were killed, spleens and PP were removed, and single spleen cell and enzyme-dissociated PP cell suspensions were prepared (10). T cell-enriched fractions were prepared by first passing spleen or PP cells through two consecutive Sephadex G-10 or glass wool columns, respectively (11). The eluted T and B cells were then treated twice with anti-mouse immunoglobulin serum and rabbit complement (C). Portions of the T cell-enriched fractions were then treated with either anti-Lyt-1.1 or anti-Lyt-2.1 (both hybridoma reagents were kindly provided by Dr. John F. Kearney, the University of Alabama in Birmingham) and C. The specificity of the anti-Lyt reagents was established by demonstrating that treatment of Con A-stimulated T cell cultures with anti-Lyt-2.1 and C abrogated their ability to suppress in vitro anti-SRBC plaque-forming cell (PFC) responses of spleen cell cultures (without treatment, responses were completely suppressed); T cell preparations from spleens of SRBC-primed mice that had been treated with anti-Lyt-1.1 and C did not support in vitro immune responses to SRBC in T cell-depleted spleen cell cultures.

For assessment of  $T_h$  cell activity, the various T cell preparations ( $1 \times 10^6$ ) were added to C3H/HeN splenic B cell cultures ( $5 \times 10^6$ ) and incubated with heavily-conjugated trinitrophenyl (TNP)-SRBC (9). For assessment of  $T_s$  cell activity, the various T cell preparations ( $1 \times 10^6$ ) were added to C3H/HeN spleen cell cultures ( $5 \times 10^6$ ) and incubated with SRBC. In another series of experiments, a portion of spleen and PP cell preparations from C3H/HeN mice were treated with anti-Lyt-2.1 and C, and then both treated and untreated cell preparations were cultured ( $5 \times 10^6$ ) with SRBC. All cultures were assessed on day 5 for numbers of anti-SRBC or anti-TNP PFC responses.

*PFC Assay.* Anti-SRBC or anti-TNP PFC responses were assayed using the slide modification (9-11) of the Jerne hemolytic plaque assay. Anti-TNP PFC were assessed using lightly-conjugated TNP-SRBC (9, 11), and specific anti-TNP PFC were calculated by subtracting background anti-SRBC PFC. Monospecific anti- $\gamma$  and anti- $\alpha$  sera (9) were used for IgG and IgA isotype-specific PFC responses. Plaques were developed by incubation with C.

## Results and Discussion

In the first experimental series, the effect of daily GI with SRBC for 2 wk followed by intraperitoneal administration of SRBC to LPS-responsive (C3H/HeN, BALB/c, and Swiss) and LPS-nonresponsive C3H/HeJ mice was evaluated (Fig. 1). A divergent immune response pattern was noted; the three LPS-responsive mouse strains tested did not respond to SRBC, whereas C3H/HeJ mice elicited IgM, IgG, and IgA anti-SRBC PFC responses (Fig. 1). These results indicate that oral administration of SRBC for 2 wk to LPS-responsive mice results in oral tolerance, whereas similar treatment of C3H/HeJ mice primes these animals for anamnestic responses. In data not shown, GI of C3H/HeJ mice with SRBC for 28, 45, or even 60 consecutive days did not result in oral tolerance. Furthermore, when spleens from mice given SRBC for 2 wk were assessed directly or cultured in vitro with SRBC, C3H/HeJ mice gave IgM, IgG, and IgA anti-SRBC PFC responses, whereas C3H/HeN mice exhibited no responses (see below).

The cellular basis for oral tolerance in C3H/HeN mice and for heightened responsiveness in C3H/HeJ mice given SRBC orally was primarily mediated by regulatory T cells. Splenic and PP T cell-enriched fractions from mice given SRBC by GI for 2 wk were assessed for  $T_h$  and  $T_s$  cell activity. Low anti-TNP PFC responses were seen in B cell cultures containing T cells from C3H/HeN spleen or PP (Fig. 2, top panel a); good responses were seen in those cultures containing purified spleen or PP T cells from C3H/HeJ mice. Treatment of C3H/HeJ splenic or PP T cells with anti-Lyt-1.1 and C completely abrogated in vitro B cell responses (Fig. 2, top panel b), whereas similar treatment of T cells with anti-Lyt-2.1 and C gave heightened anti-

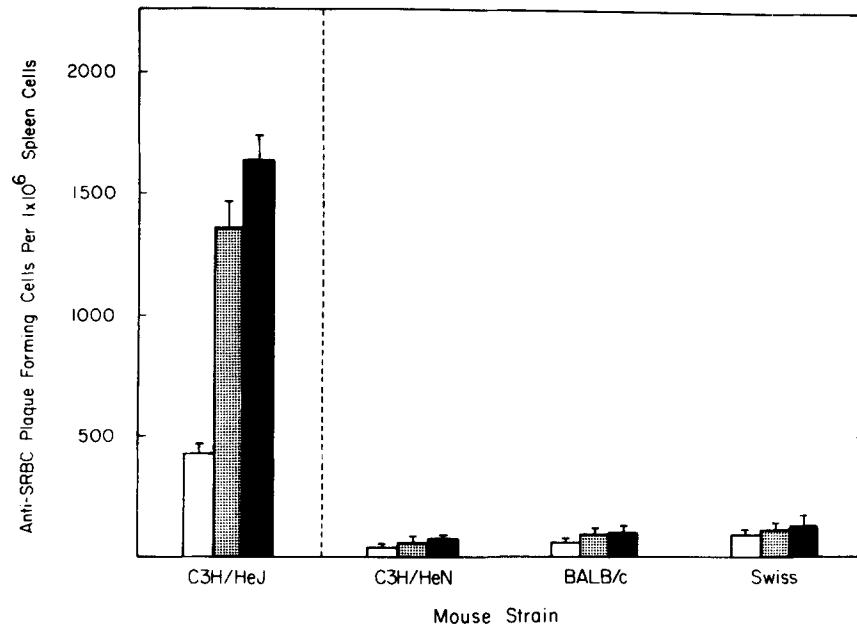


FIG. 1. Effect of daily GI of SRBC on systemic responses to antigen of LPS-nonresponsive C3H/HeJ and LPS-responsive C3H/HeN, BALB/c, and Swiss mice. Data expressed as the mean  $\pm$  SEM of splenic IgM (□), IgG (▨), and IgA (■) class-specific anti-SRBC PFC responses obtained from triplicate determinations per spleen (20 mice per group).

TNP PFC responses (Fig. 2, top panel c). Treatment of T cells from the spleen or PP of C3H/HeN mice with anti-Lyt-2.1 and C enhanced anti-TNP PFC responses in B cell cultures; however, these responses were lower than those seen in cultures containing identically treated C3H/HeJ T cells (Fig. 2, top panel c). These results demonstrate that significant numbers of  $T_h$  cells are present in the PP and spleen of orally treated C3H/HeJ mice, but less  $T_h$  cell activity is seen in identically treated C3H/HeN mice.

$T_s$  cells accounted for the observed tolerance in LPS-responsive mice because T cells from the spleen or PP of C3H/HeN mice, previously given SRBC orally for 2 wk, suppressed anti-SRBC PFC responses of normal spleen cell cultures (Fig. 2, bottom panel a). This suppression was abrogated by prior treatment of T cells with anti-Lyt-2.1 and C (Fig. 2, bottom panel c). T cells from the spleen or PP of SRBC orally primed (14 d) C3H/HeJ mice exhibited little or no  $T_s$  cell activity, as evidenced by the observation that prior treatment of T cells with anti-Lyt-1.1 and C (to remove  $T_h$  cells) did not significantly reduce normal in vitro responses (Fig. 2, bottom panel b). In data not shown, the addition of purified B cells or  $M\phi$  from the spleen or PP of C3H/HeN mice given SRBC orally for 2 wk to normal spleen cell cultures immunized with SRBC did not suppress in vitro immune responses.

Evidence that  $T_s$  cells probably originate in PP and mediate lack of systemic responses in lymphoid tissues such as spleen was provided by experiments demonstrating that prior treatment of dissociated PP or spleen cells from C3H/HeN mice given SRBC by GI for 14 d with anti-Lyt-2.1 and C abrogated unresponsiveness (Table I). It should be noted that higher IgA responses occurred in PP cultures treated with anti-Lyt-2.1, a finding that is consistent with our other studies that demonstrated that enzyme-dissociated PP cells from orally primed mice elicit principally IgA PFC responses (10). In this regard, both IgG and IgA splenic PFC responses were obtained

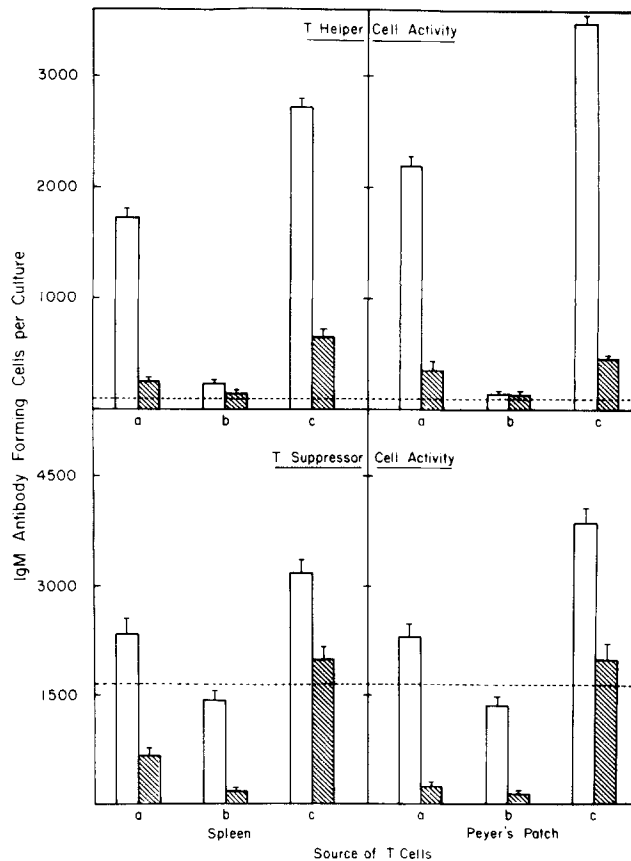


FIG. 2. Effect of daily GI of SRBC for 2 wk on the induction of  $T_h$  (upper panels) or  $T_s$  (lower panels) cell activity in spleens (left panels) and PP (right panels) of C3H/HeJ (open bar) and C3H/HeN (hatched bar) mice. T cell-enriched fractions added to cultures were untreated (a) or treated with either anti-Lyt-1.1 (b) or anti-Lyt-2.1 (c). For assessment of  $T_h$  cell activity, T cells were added to C3H/HeN splenic B cell cultures and immunized with TNP-SRBC, whereas  $T_s$  cell activity was determined by addition of T cells to normal C3H/HeN spleen cultures immunized with SRBC. Data are expressed as the mean  $\pm$  SEM of anti-TNP (for  $T_h$  cell activity) or anti-SRBC (for  $T_s$  cell activity) PFC from triplicate determinations per experiment and four separate experiments. Dashed lines indicate PFC responses in cultures without added T cells.

that clearly suggest that prolonged oral administration of SRBC primed the B cells for immune responses. In data not shown, similar results were obtained when either PP or spleen cells from BALB/c mice given SRBC by GI for prolonged periods were treated with an anti-Lyt-2 antibody to remove the  $Lyt-2,3^+$  T cell population.

Our studies indicate that significant numbers of  $T_s$  cells are induced in PP of LPS-responsive mice and suggest that the migration of these cells to peripheral lymphoid tissue such as the spleen probably accounts for systemic unresponsiveness. This  $T_s$  cell induction pathway is either absent or defective in C3H/HeJ mice. C3H/HeJ mice may lack the necessary  $Lyt-1^+$  cell required for induction of  $Lyt-2,3^+$  effector cells in GALT. The net result of this would be that C3H/HeJ GALT  $T_h$  cells continue to be induced via an  $Lyt-1^+$  helper pathway. Studies by others (12) have shown that  $Lyt-1^+$  cells responsible for induction of  $Lyt-2,3^+$  suppressor cells are  $Qa-1^+$ , whereas  $Lyt-1^+$  helper inducers are  $Qa-1^-$ . Current studies are underway to investigate the possibility that C3H/HeJ mice lack an  $Lyt-1^+$ ,  $Qa-1^+$  inducer T cell population in GALT.

TABLE I  
*Abrogation of Unresponsiveness in Peyer's Patch and Spleen Cell Cultures from C3H/HeN Mice Given SRBC by GI for a 2-wk Period\**

Cell source	Treatment	Anti-SRBC		
		IgM	IgG	IgA
Peyer's patch	None	41 ± 7	28 ± 4	7 ± 2
	Anti-Lyt-2.1	682 ± 28	895 ± 64	2814 ± 51
Spleen	None	48 ± 12	37 ± 17	30 ± 12
	Anti-Lyt-2.1	396 ± 30	1343 ± 56	1466 ± 59

\* Single cell preparations of PP or spleens from C3H/HeN mice given SRBC by GI for 2 wk were either untreated or treated with anti-Lyt-2.1 and C, cultured ( $5 \times 10^6$  cells), and incubated with SRBC ( $2 \times 10^6$  to  $3 \times 10^6$ ).

‡ Values are the mean ± SEM of triplicate determinations per experiment and three separate experiments.

The demonstration of significant  $T_s$  cell activity in both the PP and the spleen of C3H/HeN mice given SRBC orally for prolonged periods suggests that this mechanism is a major mode of regulation for host immune responses. Furthermore, the removal of  $T_s$  cells from lymphoid tissue of these orally tolerized mice restored full immune responses, including IgG and IgA isotype responses. These results clearly suggest that oral administration of antigen also results in induction of both T and B cells required for antibody synthesis. The finding that high IgA anti-SRBC PFC occurred in PP cells treated with anti-Lyt-2.1 antibody further documents the propensity of B cells committed to IgA responses in GALT.

A direct correlation between LPS responsiveness and the induction of oral tolerance is suggested by this study. Three LPS-responsive strains, became tolerant to SRBC after prolonged oral exposure, in sharp contrast to the LPS-nonresponsive C3H/HeJ animal. The lack of induction of  $T_s$  cells in orally treated C3H/HeJ mice and the presence of a significant number of  $T_h$  cells (9) suggest an association between the inability to respond to LPS and the failure to induce  $T_s$  cells with orally administered antigen. This correlation raises the possibility that LPS from the Gram-negative intestinal microflora is involved in maturation of precursor  $T_s$  cells in GALT. Current studies that would directly prove a relationship between LPS exposure and oral tolerance suggest that gastric administration of LPS to germ-free mice renders them sensitive to oral tolerance induction (manuscript in preparation). Several previous studies had suggested that administration of LPS to mice before antigen results in unresponsiveness (13, 14) and that LPS may induce suppression via a direct effect on T lymphocytes (13, 14). The inability to orally induce  $T_s$  cells and the lack of oral tolerance in C3H/HeJ mice offer a unique model for the study of  $T_h$  cell pathways and for investigation of regulation of precursor IgA B cells in immune responses to orally administered, thymic-dependent antigens.

### Summary

Daily gastric intubation of lipopolysaccharide (LPS)-responsive C3H/HeN, BALB/c, and Swiss mice with SRBC for 2 wk resulted in oral tolerance, whereas similarly treated LPS-nonresponsive C3H/HeJ mice gave splenic anti-SRBC PFC responses, including the IgA isotype, after systemic challenge with antigen. Oral tolerance in LPS-responsive C3H/HeN mice was due to T suppressor ( $T_s$ ) cells because significant  $T_s$  cell activity was demonstrated in both Peyer's patches (PP) and spleens of these animals. On the other hand, T cells from PP and spleens of

identically treated C3H/HeJ mice exhibited mainly T helper cell activity. Prior treatment of PP or spleen cell preparations from tolerant C3H/HeN mice with anti-Lyt-2.1 resulted in good in vitro anti-SRBC PFC responses, especially IgA isotype responses in PP cell cultures. These results indicate that oral administration of a thymic-dependent antigen (SRBC) to LPS-responsive mice induced a T<sub>h</sub> cell population in PP, which, after migration to peripheral lymphoid tissue (e.g., spleen), suppressed responses to systemically administered antigen. LPS-nonresponsive mice lack this T<sub>h</sub> cell pathway and continually respond to oral administration of antigen.

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