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Contrasting effects of H-2 and Mls immunization on the polyclonal mitogenicity of murine lymphocytes

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Summary. During the immune response to H-2 and Mls alloantigens, murine lymphocytes showed altered sensitivity to polyclonal mitogens. The reactivity to the T-cell mitogen PHA followed a similar pattern in both H-2 and Mls-immunized mice while the reactivity to the B-cell mitogen LPS was contrasting in the two groups. In the former group, the response exceeded control levels by the seventh day after immunization and then gradually dropped below control levels; the response of Mls-immunized lymphocytes dropped below control levels soon after immunization and remained so for the period of study. Nylon wool column-purified Mls-immunized B cells also showed a suppressed reactivity to LPS, while the T-enriched populations from Mls-immune mice when added to normal B cells lowered their LPS reactivity. Soluble factors derived from cultures of Mls-immune lymphocytes had a suppressive effect on normal B cells.

INTRODUCTION

The functional capacity of lymphocytes is usually assessed by their *in vitro* response to polyclonal mitogens such as phytohaemagglutinin (PHA) for T lymphocytes and lipopolysaccharide (LPS) for B lymphocytes (Callard & Basten 1977; Callard, Möller & Gronowicz, 1977). The ability to mount a polyclonal response to LPS has been shown to be indicative of the ability to produce specific antibody to hapten–LPS conjugates; in experiments with backcross mice between genetic high and low responder strains to LPS, high polyclonal responses were linked with high antibody titres (Coutinho, Möller & Gronowicz, 1975; Watson & Riblet 1974). Fernandez & Möller (1977) also demonstrated that the lymphocyte-activating signal given by thymus-independent antigens is via their polyclonal B-cell activating properties. Whereas these experiments show a correlation between a humoral response to LPS conjugates and LPS mitogenicity, it is not known whether humoral or cell-mediated responses to other antigens have any effect on polyclonal responsiveness to mitogens.

This was examined using the H-2 and Mls alloantigenic systems in mice which give rise to contrasting responses during immunization; antibodies are produced against H-2 but not Mls determinants (Festenstein, Abbasi, Sachs & Oliver, 1972; Sachs, Huber, Pena-Martinez & Festenstein, 1973). Mice were immunized against H-2- or Mls-incompatible lymphocytes and during the immunization period the polyclonal responses of their lymphocytes to LPS and PHA were examined. This paper shows that the polyclonal response to LPS of Mls-immunized lymphocytes is suppressed and this is mediated by suppressor T cells and suppressor factor.

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MATERIALS AND METHODS

Mice

Mice of the following inbred strains and F_1 hybrids raised in our own laboratories were used: CBA/H (H-2^k, Mls^b), BRVR (H-2^k, Mls^a), BALB/c (H-2^d, Mls^b), DBA/2 (H-2^d, Mls^a) and CBA/H × DBA/2).

Immunizations

Mice were immunized by intraperitoneal injection of 10^7 lymphocytes from the appropriate strains.

Mitogenicity of PHA and LPS

Groups of CBA/H mice were immunized with (a) syngeneic cells (b) BRVR (Mls incompatible) and (c) BALB/c (H-2 incompatible) lymphocytes. At intervals after immunization lymph nodes were removed and cell suspensions were prepared in RPMI (Gibco) containing 10% foetal calf serum and antibiotics. Cells from each immunized group were adjusted to 1, 2 and 4×10^6/ml and 200 μl were dispensed in flat-bottomed Microtest II plates (Cooke). PHA (Wellcome Research Laboratories, Kent) or LPS (*E. coli* 055: B5, Difco Laboratories, Detroit, Michigan) was added to wells in triplicate to give a final concentration of 1 μg/ml and 10 μg/ml respectively. The plates were incubated for 72 h at 36° in a 5% CO_2 atmosphere. During the last 16 h the cultures were pulsed with a mixture of cold and ^{14}C-labelled thymidine. The cells were harvested on fibre filter strips using a semi-automatic harvesting machine and the radioisotope uptake was determined using a liquid scintillation spectrometer. Optimum counts were obtained with cell suspensions of 2×10^6/ml; these were used to express the results.

T-cell and B-cell enrichment of cell populations

Nylon wool columns were prepared in sterile 5 ml syringe barrels. Eagle's MEM (Wellcome) containing 20% foetal calf serum was passed through the columns which were incubated for 15 min at 37° before use. Cell suspensions containing up to 50×10^6 cells were overlayed on the columns and after a further 45 min incubation the non-adherent cells were eluted in 15 ml of MEM. The adherent cells were obtained by shaking the nylon wool in 15 ml of MEM in a suitable container. Microcytotoxicity of the separated populations using anti-θ and anti-Ig sera revealed a purity of separation of above 90%.

Preparation of MLS-induced suppressor factor

Lymph node cells from CBA/H mice injected 12 days previously with 10^7 spleen cells from H-2- identical Mls-incompatible BRVR mice were cultured for 5 days with (CBA/H × BRVR)F_1 or (CBA/H × DBA/2)F_1 lymphocytes. In either case the responder lymphocytes were re-exposed to the Mls^a allele carried by BRVR or DBA/2. The supernatants were harvested on the fifth day, centrifuged, filtered through sterile 0·2 μm Millipore filters (Millipore Corp., Bedford, Mass.) and stored at −20°. Both these supernatants had similar suppressive effects and were designated 'suppressor supernatant'.

To prepare control supernatants, lymph node cells from CBA/H mice pre-immunized with 10^7 syngeneic cells were cultured with (CBA/H × BRVR)F_1 lymphocytes. Culture supernatants were harvested after 5 days and treated as described above.

Testing of suppressor factor

The suppressor factor was tested using the cell-mediated lympholysis (CML) assay. This was performed as previously described (Matossian-Rogers & Festenstein, 1976). Sensitization cultures of CBA/H responder cells and (CBA/H × DBA/2)F_1 stimulator cells were set up in culture medium containing 25% or 50% suppressor supernatant. Control cultures were set up in similar mixtures of culture medium and control supernatant. Effector cells were harvested after 5 days and tested against DBA/2 PHA blasts.

RESULTS

Mitogen stimulation of lymphocytes from H-2 and Mls-immunized mice

The PHA and LPS responses of lymph node lymphocytes from the H-2- and Mls-immunized mice were examined various times after immunization. The results are expressed relative to the mitogen stimulation of syngeneically immunized lymphocytes (Fig. 1). The PHA response of the H-2- and Mls-immunized lymphocytes followed a similar pattern even though the latter were more responsive than the former. The radioisotope uptake of both populations was almost twice that of the syngeneically immunized animals by the third day of immunization. The response dropped below control levels by the fifth day and subsequently followed an upward trend.

The LPS response of the H-2- and Mls-immunized lymphocytes was dichotomous after an initial suppression. In the former case, the response exceeded control levels by the seventh day and then gradually dropped

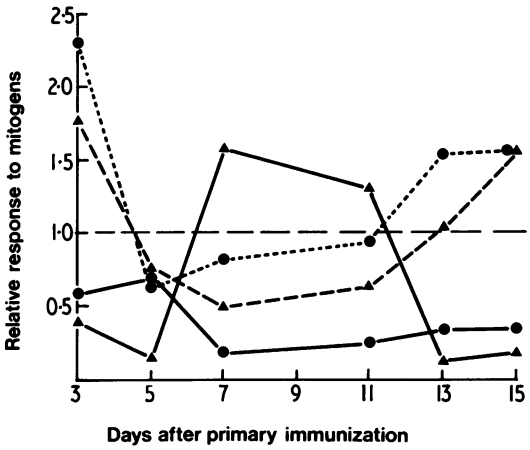

Figure 1. Time-course study of the mitogenic response of lymphocytes from H-2-immunized mice to PHA (▲ – – ▲) and LPS (▲ — ▲) and M-locus-immunized mice to PHA (● – – ●) and LPS (● — ●) expressed relative to the response of mice immunized with syngeneic cells (— —).

below control levels by the thirteenth day. The response of the Mls-immunized lymphocytes remained suppressed during the period of study.

Mitogen responses of T- and B-cell enriched populations from Mls and H-2 immunized mice

To eliminate the possibility that the suppressed LPS responsiveness of Mls-immunized lymphocytes could be due to activation in the lymph nodes of relatively different numbers of T and B cells compared to control and H-2-immunized animals, the lymphocytes from 7–11 day immunized mice were separated on nylon wool columns and the T- and B-cell enriched populations tested separately. Representative results are shown in Table 1. The radioisotope uptake after LPS stimulation of the Mls-immunized B cells was drastically suppressed compared to the H-2-immunized B cells. The difference in T-cell responses was not as pronounced but T cells from Mls-immunized mice were significantly more responsive than those from H-2-immunized mice.

Effect of T and B cells from Mls-immunized mice on the mitogen response of normal lymphocytes

T- or B-cell enriched cell populations from Mls-immunized mice (Mls-T or Mls-B) were mixed in varying proportions with similarly enriched normal cell populations (NB or NT) to examine whether the suppressed response to LPS could be transmitted by the suppressed cells to normal cells. Table 2 shows the effect of Mls-T cells on B and T cells from non-immunized control mice. As the quantity of normal B cells was increased from 100 to 175 μl of a standard suspension, the isotope uptake also increased from 3341 to 4140 c.p.m. The addition of Mls-T cells, however, to similar quantities of NB cells significantly reduced the responsiveness to LPS when the proportion of added Mls-T cells to NB cells was 1:3 or less. When Mls-T and NB were mixed in a proportion of 1:1, a slight improvement in responsiveness to LPS was noted. Mls-B cells also appeared to have a suppressive effect on normal B cells, but to a lesser degree than Mls-T cells (Table 3). The possibility that this may be due to contaminating T cells in the Mls-B population is not ruled out. Neither Mls-T nor Mls-B cells had any effect on the responsiveness to PHA of normal T cells (Tables 2 and 3).

Effect of factors released by lymphocytes from Mls-immunized mice on CBA cells in culture

Normal CBA/H and (CBA/H × DBA/2)F_1 cells were suspended in culture medium containing 25% and 50%

Table 1. Response to mitogens of nylon wool-enriched T- and B-cell preparations from M-locus and H-2-immunized mice

LPS 10 μg/ml (c.p.m. ± SD)			PHA 1 μg/ml (c.p.m. ± SD)		
Mls-B	H-2 B	Normal B	Mls-T	H-2 T	Normal T
370 ± 89	4211 ± 392	2807 ± 240	5967 ± 765	2587 ± 301	2838 ± 268

Mls-B, B cells from Mls-immunized mice. H-2 B, B cells from H-2-immunized mice. Mls-T, T cells from Mls-immunized mice. H-2 T, T cells from H-2-immunized mice.

Table 2. Effect of T cells from Mls-immunized mice on the response to mitogens of B and T cells from normal mice

	Polyclonal response of cell mixtures to:			
	LPS c.p.m. ± SD		PHA c.p.m. ± SD	
Cell mixture μl (a) + μl (b)	(a) (b) NB + Mls-T	(a) NB alone	(a) (b) NT + Mls-T	(200 μl) NT alone
100 + 100	3704 ± 208	3341 ± 136	2986 ± 305	2630 ± 346
150 + 50	3153 ± 119	3846 ± 278	3216 ± 288	
175 + 25	1739 ± 88	4140 ± 235	2829 ± 265	

NB, Normal B cells, Mls-T, see footnotes to Table 1, NT, normal T cells.

Table 3. Effect of B cells from Mls-immunized mice on the response to mitogens of B and T cells from normal mice

	Polyclonal response of cell mixtures to:				
	LPS (c.p.m. ± SD)			PHA (c.p.m. ± SD)	
Cell mixture μl (a) + μl (b)	(a) (b) NB + Mls-B	(a) NB	(b) Mls-B	(a) (b) NT + Mls-B	(a) (b) NT + NB
100 + 100	4226 ± 276	3341 ± 136	360 ± 22	4309 ± 332	3676 ± 255
150 + 50	3900 ± 310	3846 ± 278	340 ± 18	4864 ± 408	4549 ± 512
175 + 25	3799 ± 323	4140 ± 235	276 ± 20	5619 ± 459	6093 ± 530

See footnotes to Tables 1 and 2.

Table 4. Effect of soluble factors on the development of CBA/H cytotoxic effectors against DBA/2 targets and the mitogenic response of CBA/H lymphocytes to LPS

Supernatants added to cultures		Specific ^{51}Cr release (%) from DBA/2 targets at effector to target ratios: 40:1	20:1	10:1	Response to LPS (c.p.m. ± SD)	Percentage reduction
None	—	35·8	28·7	16·5	6086 ± 208	—
Suppressor	25%	25·8	17·4	6·2	3783 ± 138	38
Suppressor	50%	12·6	8·4	3·8	2452 ± 105	60
Control	25%	38·4	25·6	17·2	5811 ± 328	5
Control	50%	40·7	27·6	19·8	5658 ± 277	7

Differences in specific ^{51}Cr release between assays in normal culture medium and those in medium containing 25% or 50% suppressor supernatant were significant within the range $P < 0{\cdot}25$ to $P < 0{\cdot}001$ estimated by analysis of variance tests. Assays were carried out with triplicates at each ratio and standard deviations from the means were invariably less than 5%. Spontaneous release of radioisotope was always less than 10% of the total.

suppressor or control fluid. Effector cells harvested from these cultures were tested for cytotoxicity against DBA/2 PHA blasts. The results in Table 4 show that effector cells from cultures containing suppressor fluid were significantly less cytotoxic than those obtained from standard culture medium or from culture medium mixed with control supernatant. CBA/H cells suspended in mixtures of culture medium and suppressor fluid were significantly less responsive to LPS than if suspended in mixtures of medium and control fluid.

DISCUSSION

Lymph node cells from mice immunized against Mls-incompatible lymphocytes were significantly less responsive to LPS than normal lymphocytes and remained so for at least 15 days after primary immunization. In contrast, H-2-immunized lymphocytes showed an improved responsiveness to LPS between the sixth and eleventh day of primary immunization. The mechanism of B-lymphocyte triggering is not yet clearly understood but it appears from these data that the specific stimulus for antibody production, which the H-2 alloantigen provides, is accompanied by an improved response to the polyclonal B-cell mitogen, LPS. This may be due to helper effects of accessory cells or factors released by such cells which by a common mechanism facilitate the specific and polyclonal triggering of B lymphocytes. In fact, Norcross & Smith (1977) demonstrated that activated T cells augmented B-cell proliferation stimulated by LPS.

The function of T cells in modulating the polyclonal response of B cells to LPS is controversial because of the selective effects of LPS on T and B cells and the T-independent nature of LPS immunogenicity (Andersson, Möller & Sjöberg, 1972; Gery, Krüger & Spiesel;, 1972; Andersson & Blomgren 1971). Yoshinaga, Yoshinaga & Waksman (1972) and more recently Norcross & Smith (1977) demonstrated that T cells augmented B-cell proliferation stimulated by LPS. The adjuvant effects of LPS *in vivo* on antibody production have also been shown to involve T lymphocytes (Newburger, Hamaoka & Katz, 1974; Ness, Smith, Talcott & Grunet, 1976, Shinohara & Kern 1976).

The present experiments demonstrate that T cells do play a modulatory role on the polyclonal response of B cells, and while the effect of H-2-immunization is enhancing, during some part of the primary immune response the effect of Mls-immunized T cells on normal B cells is predominantly suppressive (Table 3). When large numbers of Mls-T cells were mixed with normal B cells, a slight amplification of the response to LPS was noted, possibly due to the presence of some helper T cells. With lower concentrations, the helper function was diluted out and the suppressor effect was dominant. This finding may explain why antibodies were not formed to Mls determinants (Festenstein *et al.*, 1972; Sachs *et al.*, 1973); during immunization against Mls determinants, sufficient helper activity may not be generated to collaborate with B cells in producing antibody. In fact it has previously been demonstrated that suppressor cells are generated in mice in response to injection of Mls-incompatible lymphocytes and that these suppressor cells mixed in culture with normal lymphocytes can suppress the development of cytotoxic cells against H-2 alloantigens (Matossian-Rogers & Festenstein, 1977).

In this report, it was also shown that Mls-induced suppressor cells could release a suppressor factor in culture which, besides reducing the cytotoxic potential of normal T cells *in vitro*, also mediated the poor responsiveness of B cells to LPS (Table 4).

At first glance, the present data may appear conflicting with previous reports that Mls incompatibility amplifies B-cell function. For example, the antibody response to sheep red blood cells and DNP was shown to be enhanced by the simultaneous administration of Mls-incompatible lymphoid cells and antigen (Rollinghoff & Wagner 1975; C. A. Janeway Jr, personal communication). The initial effect of Mls-incompatible lymphocytes is enhancement of T-cell responses (Matossian-Rogers & Festenstein 1976) and possibly B-cell responses, and once B cells are switched on to antibody production the delayed addition of suppressor cells has no effect (Debré, Kapp & Benacerraf, 1975). Since there is a lapse of several days for Mls-induced suppressor cell generation (Matossian-Rogers *et al.*, 1976), it is possible that the suppressive effects of Mls on B-cell responses were missed in previous reports.

The Mls-induced suppressor cell is probably a T cell, since strong suppression of LPS-induced mitogenicity was noted when the eluted population of cells from a nylon wool column was mixed with normal B cells (Table 2). The lesser degree of suppression noted with the B-cell enriched fraction (Table 3) may have been due to contaminating suppressor T cells. These experiments also suggest that suppressor T cells can act directly on B cells (Mosier, Mathieson & Campbell, 1977) and not via T helper cells.

In conclusion, B-cell tolerance to Mls determinants can be seen to be mediated via a non-specific signal delivered directly to the B cells by suppressor T cells or soluble factor (Tables 2 and 4). Probably in analogous fashion, non-specific activation of B cells via polyclonal receptors is enhanced, albeit transiently, by helper effects generated during the immune response to H-2 alloantigens.

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