

REGULATION OF LYMPHOCYTE RESPONSES IN VITRO

I. REGULATORY EFFECT OF MACROPHAGES AND THYMUS-DEPENDENT (T) CELLS ON THE RESPONSE OF THYMUS-INDEPENDENT (B) LYMPHOCYTES TO ENDOTOXIN*

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It has recently been shown that activated thymus-dependent (T) lymphocytes, in addition to their well-recognized ability to cooperate with thymus-independent (B) lymphocytes, can exert "suppressor" effects on antibody formation in vivo (1-4) and in vitro (5, 6).¹ This effect is exerted both on thymus-dependent and thymus-independent antibody responses, and appears to be mediated by soluble factors. We present here evidence for a similar suppressor effect on the nonspecific mitotic response of B cells to lipopolysaccharide endotoxin (LPS).

Materials and Methods

Animals.—Inbred DA rats were thymectomized at 8 wk of age, irradiated 2 wk later with 900 R (Siemens Stabilipan, 250 kv; Siemens Corp., Iselin, N. J.), and reconstituted with 3×10^8 syngeneic bone marrow cells. Their spleens and lymph nodes (cervical, axillary, inguinal, and popliteal) harvested 7 wk after irradiation provided "T-deprived" cells, which were compared with cells of nontreated animals.

Cell Suspensions and Culture Media.—Teased lymph node cells (LNC) were gently squeezed with sterile slides in ice-cold medium, passed through nylon mesh, and washed twice by centrifugation. Spleen cells, prepared in the same way, were treated with tris(hydroxymethyl)amino-methane-ammonium chloride buffer, pH 7.2, to remove red cells and washed three times. Both suspensions were routinely diluted to 4×10^6 /ml viable cells (estimated by trypan blue dye exclusion). Ham's F10 medium (Microbiological Associates, Inc., Bethesda, Md.) containing 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) (FCS), 100 units/ml penicillin, and 100 μ g/ml streptomycin was used throughout. LPS (lipo-

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¹ Sjöberg, O. Effect of allogeneic cell interaction on the primary immune response in vitro: Cell types involved in suppression and stimulation of antibody synthesis. Submitted to *Clin. Exp. Immunol.*

polysaccharide B, *Escherichia coli* 0111:B4; Difco Labs., Inc., Detroit, Mich.) was dissolved in phosphate-buffered saline at various concentrations and 0.1 ml of the solution added to cultures.

Preparation of Macrophages.—Peritoneal exudate cells (PEC) were collected 4 days after intraperitoneal injection of 8 ml of Brewer's thioglycolate medium (Difco Labs., Inc.). 10^7 nucleated PEC were incubated for 30 min in 60×15 mm Falcon plastic dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) in 1.5 ml of 10% heat-inactivated DA rat serum, washed vigorously, and cultured for 48 hr in 4 ml of fresh medium. After further vigorous washing, the remaining adherent cells were resuspended with a rubber policeman in F10 medium with 10% heat-inactivated FCS. The final yield was usually $2-4 \times 10^6$ cells/animal. Of these "purified macrophages," more than 99% took up neutral red, and more than 93% phagocytized formalinized sheep red cells (7).

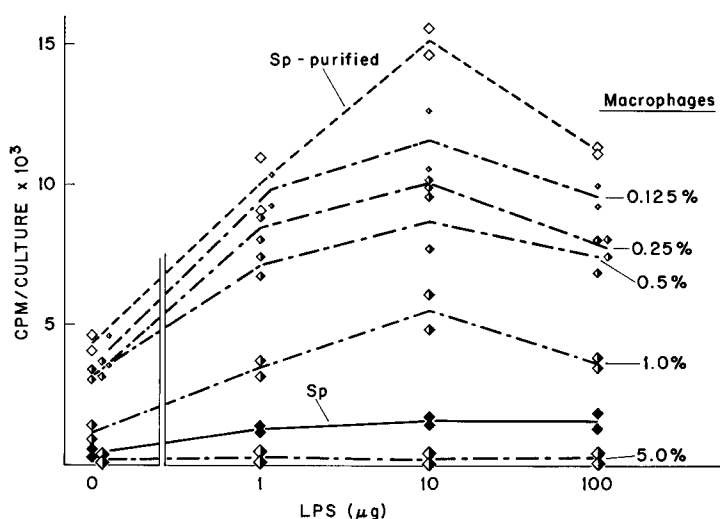


FIG. 1. DNA synthesis in cultures of normal spleen cells (◆—), nonadherent spleen cells (◇---), and nonadherent cells supplemented with varying numbers of purified macrophages (half-filled diamond, — —) when these were stimulated with different amounts of LPS. Sp = spleen.

Removal of Adherent Cells from Spleen and Lymph Node Suspensions.—Washed glass beads (type 100-5005; Minnesota Mining and Manufacturing Co., Reflective Products Division, St. Paul, Minn.) were packed in 10-ml syringes up to 5 ml and in 35-ml syringes up to 20 ml and equilibrated with medium. 1 ml of LNC (2×10^8 cells) and 4 ml of spleen cells ($10-12 \times 10^8$ cells) were incubated on 5- or 20-ml columns, respectively, for 30 min at 37°C and then eluted with 30 or 80 ml of warm medium. The yield of nonadherent purified cells was usually 20–30% and included an average of 0.3% (in all cases less than 1%) neutral red-staining cells.

Culture.—1 ml of LNC or spleen cells (4×10^6 /ml) was mixed with 0.1 ml of macrophage suspension and 0.1 ml of LPS solution or comparable volumes of control solution. These were cultured for 48 hr, pulsed with thymidine-³H an additional 24 hr, harvested, processed, and counted as in previous studies (8). Counts in duplicate cultures regularly differed from the mean value by less than 15%.

RESULTS

Normal rat spleen cells were stimulated to a low level of DNA synthesis by LPS at all doses between 1.0 and 100 μg (Fig. 1). LNC cultured with LPS showed a similar dose-response curve, but both background and peak values were higher (e.g. 4407 and 7721 cpm at 0 and 10 μg LPS, respectively). After removal of adherent cells, the background with spleen cells increased somewhat and the reaction to LPS at all doses tested was strikingly enhanced (Fig. 1). LNC showed a similar enhancement, for example 12,698 cpm (after purification) compared with 7721 (before purification) with 10 μg of LPS. Addition of purified

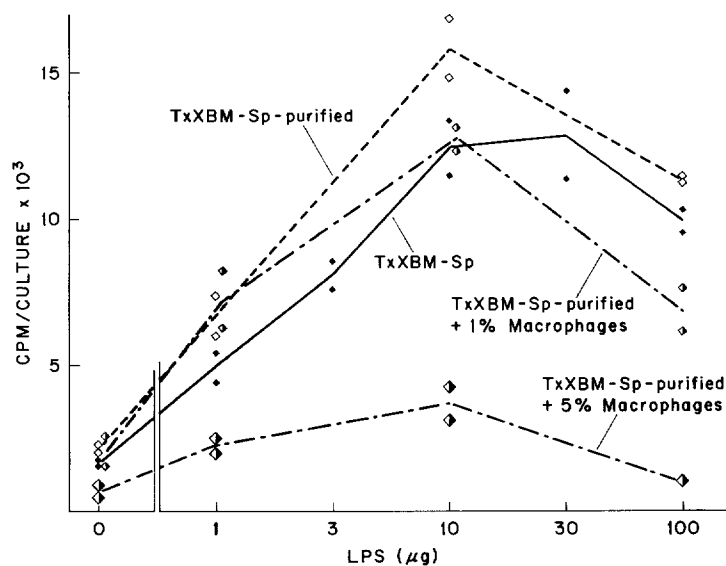


FIG. 2. DNA synthesis in cultures of TxXBM spleen cells with LPS. Symbols as in Fig. 1.

macrophages to the nonadherent spleen cells at a concentration of 0.125–1% (actual cell numbers were $5\text{--}40 \times 10^3/\text{culture}$) resulted in a substantial decrease of DNA synthesis, and 2.5 or 5% macrophages ($1\text{--}2 \times 10^3/\text{culture}$) virtually wiped out the response. The inhibitory activity of macrophages on DNA synthesis of purified spleen cells was roughly proportional to the number of macrophages on a logarithmic scale.

With adult thymectomized, lethally irradiated, and bone marrow-restored (TxXBM) rat spleen cells cultured 4 wk after irradiation, LPS gave only slight DNA synthesis (e.g. 915 cpm at maximal stimulation). By 7 wk after thymectomy, irradiation, and bone marrow injection, however, the response of TxXBM spleen to LPS was much higher than that of normal spleen (Fig. 2), and removal of adherent cells enhanced it only slightly. When 1% purified

macrophages (corresponding roughly to the proportion found in whole spleen cell suspensions) were added in this system, the response returned to the level seen with unpurified TxXBM spleen. 5% macrophages, as before, virtually wiped out the response.

Since the suppressive effect of purified macrophages on the nonadherent spleen cell response to LPS varied considerably from one experiment to another, a single batch of macrophages was used in an experiment comparing the reactions of purified normal and purified TxXBM spleen cells (Table I). The findings shown in Figs. 1 and 2 were fully confirmed. The difference in DNA synthesis between purified normal spleen cells and purified normal spleen cells plus 1% macrophages was 9463 cpm while that figure with TxXBM cells was 3140. Similarly the difference between the responses of purified and original

TABLE I
Effect of Macrophages on the Response of Spleen Cells from Normal and Thymus-Deprived Rats to LPS

Cells cultures	LPS	Normal spleen	TxXBM spleen
	μg	<i>cpm/culture*</i>	
Original spleen cells	0	1,388	1,834
	10	3,431	12,395
Purified spleen cells	0	5,973	2,097
	10	16,793	15,881
Purified spleen cells + 1% macrophages	0	1,618	1,688
	10	7,330	12,741

* Average of duplicate values

normal spleen cells was 13,362 cpm, while that with TxXBM cells was 3486. This implies the existence in the normal spleen population of a suppressive mechanism which is both macrophage dependent and thymus dependent.

In several experiments, DNA synthesis elicited by LPS in LNC from TxXBM rats varied in parallel with the responses of spleen cells, but the differences were much less marked. In a few experiments, lymph node cells behaved like spleen cells and, in one instance, the differences were more clear-cut with LNC than with spleen.

DISCUSSION

Macrophages in sufficient concentration (5%) directly inhibit DNA synthesis by spleen cells or LNC responding to LPS. Conversely the response is enhanced by purification, with removal of adherent cells. An additional inhibition of the response at low concentrations of macrophages is seen with normal spleen cells but not with T-deprived spleen cells. Such inhibition may result from the action

of suppressor T cells (4, 6).¹ That it is observed only in the presence of macrophages agrees with evidence that many T cell responses are macrophage dependent (8-11). Some difficulty in disentangling the respective roles of the two cells in suppressing *in vitro* antibody responses was noted by Sjöberg.¹ He concluded, however, that suppressor effect of the T cell might be mediated by macrophage activation (see references 12 and 13). This seems unlikely since our experiments gave consistent results in the presence of a range of LPS concentrations capable of stimulating macrophages markedly. It is difficult to imagine that T cells could activate these macrophages further nonspecifically.

Considerable variation in the magnitude of the effects described here was observed between experiments. This may have been due to intercurrent infection with varying degrees of macrophage activation, to stress with generalized T cell depletion, or to variations in the rate of B cell regeneration in TxXBM rats. It did not affect the character of the results obtained.

The suppressor effect was much more striking with spleen cells than with LNC. This agrees with an increasing body of evidence which suggests that suppressor T cells localize predominantly but not exclusively in the spleen.²

SUMMARY

DNA synthesis of normal rat spleen cells in response to endotoxin increases markedly if adherent cells are first removed from the cell suspension. Addition of small numbers of purified macrophages to the cultures restores the response to a low level. T-deprived cells show these effects in very much lesser degree. Large numbers of macrophages completely suppress the response of both normal and T-deprived spleen. We conclude that two mechanisms of suppression are at work: a direct effect of macrophages and a macrophage-dependent "suppressor T cell" effect.

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² Gershon, R. K., K. Kondo, and E. M. Lance. 1972. Immunoregulatory role of spleen-seeking thymocytes. Submitted to *J. Exp. Med.*

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