REGULATION OF B-CELL PROLIFERATIVE RESPONSES TO LIPOPOLYSACCHARIDE BY A SUBCLASS OF THYMUS T CELLS*

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Endotoxin (lipopolysaccharide, LPS) is selectively mitogenic for a subpopulation of B lymphocytes, but is not directly mitogenic on peripheral or thymus T lymphocytes (1-6). On the other hand, the adjuvant effects of LPS in vivo in enhancing antibody formation may involve T lymphocytes (7-11). LPS effects on in vitro antibody production and B-cell mitogenesis (12) have also been associated with T-cell action (13-15). Forbes et al. (16) and Ozato et al. (17) reported that LPS stimulated the immunocompetent minor subpopulation of thymus cells in synergy with small amounts of the T-mitogen concanavalin A (Con A) or alloantigen. Similar effects have been reported using human peripheral T cells (18). Others (14, 19) have failed to find synergy of LPS responses in mixtures of spleen or lymph node and thymus cells. This paper shows that the major immunoincompetent subpopulation of mouse thymus contains cells which act in synergy with peripheral lymphoid B cells to regulate proliferative responses to LPS.

Materials and Methods

Animals. Female C57BL/10, C57BL/10 A, C3Heb/Fej, CBA/H-T6J, or CBA/Caj C3H/Hej 6-20 wk of age were obtained from The Jackson Laboratory, Bar Harbor, Maine. In experiments not reported here, it was found that the LPS high responder (C3Heb/Fej) and low responder strains (C3H/Hej) are not mutually stimulatory in MLC at a level detectable in the culture system employed.

Preparation of Cells. Thymus, spleen, or lymph node cells were obtained by pressing small tissue fragments through 60-mesh stainless steel screens and then serially drawing the suspension in RPMI-1640 (Grand Island Biological Company, Grand Island, N. Y.) through 19-, 23-, and 25-gauge needles.

Bovine Serum Albumin (BSA) Gradient Separation. BSA gradients were prepared according to methods described elsewhere (20) using BSA concentrations as follows: A-layer above 23%, B-layer above 27%, C-layer above 29%, and D-layer above 35%. Cells to be separated were layered on the upper layer, centrifuged at 13,000 rpm in a Beckman SW50.1 rotor for 30 min, washed with RPMI, and resuspended in complete culture medium (see below).

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Abbreviations used in this paper: BSA, bovine serum albumin; BSS, balanced salt solution; Con A, concanavalin A; [3H]TdR, tritiated thymidine; LAF, lymphocyte-activating factor; LN, lymph node; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; PHA, phytohemagglutinin.
Preparation of Cell Subpopulations Selected by Antiserum Treatment. 2 × 10^8 cells were suspended either in 10.0 or 2.6 ml RPMI; 10 ml or 0.2 ml antisera; and 10 ml (1:5) or 0.2 ml undiluted guinea pig complement (C') (Grand Island Biological Company), respectively, then the mixture was incubated at 37°C for 45 min. The residual cells were washed three times, tested for viability by trypan blue exclusion, and then suspended in complete medium. AKR anti-C3H anti-Thy-1.2 was obtained from Litton Bionetics, Inc., Kensington, Md., anti-Ig from Dr. B. M. Gebhardt (Tumor Biology Unit), goat anti-mouse immunoglobulin from Dr. Rebecca Blackstock (Tumor Biology Unit), and the goat anti-IgM and anti-IgG antisera from Meloy Laboratories, Inc., Springfield, Va.

Separation by Plastic Adherence. Spleen cells suspended in RPMI without serum were incubated for 2 h in 5% CO_2 at 37°C on 100 x 15-mm Falcon plastic Petri dishes (Falcon Plastics, Oxnard, Calif.). Nonadherent cells were gently poured off, the plate washed three times with RPMI, and the residual adherent cells removed with a rubber policeman.

Separation by Nylon Wool Adherence. Spleen cells were separated on nylon wool columns by a modification of the technique of Julius et al. (21). 1.5-2.5 × 10^8 cells were first passed through glass wool at room temperature, then resuspended in 2 ml of media (RPMI containing 10% fetal calf serum [Gibco]), and added to a washed prewarmed nylon wool column. An additional 1 ml of media was used to wash the cells into the column. The filled column was ressealed loosely and incubated for 45-60 min at 37°C in 5% CO_2. Cells not adherent to nylon were harvested by dropwise elution with 20 ml warm medium. Another 15 ml media was passed through the column, and the loosely adherent cells ("retarded") were removed by a modified form of the Handwerger and Schwartz technique (22). 20 ml warm media was added to the column, and then the nylon wool was squeezed with the syringe plunger. This was repeated three times with 5-ml vol of medium, and then twice again, after turning the nylon wool over. The samples of each fraction were pooled, washed, and the cells resuspended in complete medium.

Microplate Culture System. The method employed has been described in detail elsewhere (23). 5 × 10^3 thymus cells were cultured in tissue culture microplates (Falcon Plastics) or Microtest II plates (Linbro Chemical Co., Inc., New Haven, Conn.) in combination with varying numbers of peripheral lymphoid cells taken from lymph node or spleen in a vol of 0.2 ml. The mitogens were solubilized in RPMI and added in 10-μl vol. *Salmonella typhimurium* type-7 endotoxin (LPS) was obtained from Dr. J. R. Shands, Department of Immunology and Medical Microbiology, University of Florida College of Medicine; *Salmonella minnesota* R-595 from Dr. W. H. Adler, Gerontology Research Centre, Baltimore City Hospital, Baltimore, Md.; phytohemagglutinin (PHA-P), Con A, and *S. typhimurium* LPS were obtained from Difco Laboratories, Detroit, Mich. The cultures were incubated for 72 or 48 h where indicated at 37°C in 5% CO_2. [3H]thymidine ([3H]TdR) incorporation was assayed as described previously (23). Culture results are expressed in terms of mean cpm/culture ± SE for quadruplicate cultures.

Medium - RPMI. 1640 medium, to which 100 U of penicillin, 100 μg streptomycin/ml, and Fungizone (Gibco) were added plus 5% heated (56°C for 30 min) human serum, was used for final cultures in all experiments. Mitomycin C (Nutritional Biochemicals, Cleveland, Ohio) was used to block thymidine incorporation in some experiments at a concentration of 50 μg/0.1 ml per 15 × 10^6 cells/0.5 ml, incubated at 37°C for 30 min. The cells were washed three times with RPMI-1640 and resuspended in culture medium. Irradiation: 1,000 R was delivered to cell suspensions by a 137Cs source.

Determination of T6 Chromosome Marker. Cultures prepared as described above were treated with colcemid (Gibco) 0.4 μg/ml 4 h before harvest, then washed in Hanks' balanced salt solution (BSS) (Gibco), and resuspended for 15 min in hypotonic BSS (24). Cells were then centrifuged and resuspended in a cold methanol:acetic acid mixture (1:3) for 15 min. The fixed preparation was again centrifuged and resuspended in two drops of the fixative, placed on a clean microscope slide, and flamed briefly burning off the methanol. The slides were stained with Giemsa stain, and metaphases were identified and counted. Only those mitotic figures allowing full visualization of all chromosomes were evaluated. Approximately one-half of the metaphases in such preparations were unsatisfactory for evaluation.

Results

**LPS-Induced Proliferation of Thymus Cells in the Presence of Peripheral Lymphoid Cells.** The basic observation explored in the series of experiments to
be described is that cultures of mouse thymus cells, which proliferate minimally or not at all in response to LPS (4), appear to do so strongly when numbers of peripheral lymphoid cells too low to respond detectably are added. Table I shows four experiments typical of the range of proliferative responses observed when thymus cells and spleen cells were combined in a 25:1 ratio in the presence of LPS. The cultures containing thymus lymphocytes alone proliferate minimally; when splenic lymphocytes are added in numbers that respond minimally to LPS alone, a highly significant amplification of proliferation appears.

This apparent synergistic effect is dependent upon the ratio between the cells of the mixture and the amount of LPS added. Dose-response kinetics, explored through experiments such as that illustrated in Fig. 1, resemble those of B-cell mitogens rather than T-cell mitogens (25). That is, a linear relationship was found between dose and degree of \(^{3}H\)Tdr incorporation at low LPS levels reaching a plateau at high ranges, without a significant inhibitory effect upon further addition of LPS.

When lymph node (LN) cells were used as a peripheral cell source, similar amplified proliferation occurred in LPS-stimulated cultures (Table I). The dose-response kinetics (Fig. 1) were similar to those seen using spleen cells. On the other hand, synergistic effects of LN cells at various ratios had kinetics somewhat different from that of spleen cells (Figs. 2, 3). At high peripheral cell/thymus ratios, proliferation of spleen, but not LN cells alone, masked any

### Table I

**Comparison of Proliferation Induced in C57BL/10 Thymus, Peripheral, or Thymus-Peripheral Cell Mixtures by LPS**

| Experiment number | Cells in culture | Thymus/peripheral cell ratio | \(^{3}H\)Tdr incorporation (mean cpm ± SE culture) |
|-------------------|------------------|-----------------------------|--------------------------------------------------|
|                   |                  |                             | Unstimulated culture | LPS added |
| 1                 | Thymus           |                             | 81 ± 10               | 301 ± 51  |
|                   | Spleen           |                             | 211 ± 105             | 371 ± 188 |
|                   | Thymus + spleen  | 1:0.04                      | 214 ± 77              | 2,738 ± 203 |
| 2                 | Thymus           |                             | 126 ± 21              | 565 ± 17  |
|                   | Spleen           |                             | 142 ± 25              | 5,866 ± 31 |
|                   | Thymus + spleen  | 1:0.04                      | 273 ± 56              | 21,417 ± 2,381 |
| 3                 | Thymus           |                             | 124 ± 57              | 127 ± 7   |
|                   | Spleen           |                             | 92 ± 23               | 956 ± 48  |
|                   | Thymus + spleen  | 1:0.04                      | 205 ± 32              | 4,011 ± 290 |
| 4                 | Thymus           |                             | 145 ± 26              | 208 ± 30  |
|                   | Spleen           |                             | 285 ± 32              | 591 ± 111 |
|                   | Thymus + spleen  | 1:0.04                      | 362 ± 47              | 4,964 ± 230 |
| 5                 | Thymus           |                             | 140 ± 8               | 393 ± 18  |
|                   | LN cells         |                             | 92 ± 11               | 209 ± 44  |
|                   | Thymus + LN cells| 1:0.1                       | 172 ± 9              | 3,960 ± 742 |

*5 × 10⁶ C57BL/10 thymus cells and 2 × 10⁴ spleen or 1 × 10⁶ LN cells were incubated separately or together and with or without 4 μg LPS per culture for 72 h. \(^{3}H\)Tdr was added during the last 24 h of culture. Data represent mean cpm ± SE of four replicate cultures.*
synergism that may have occurred. Table II summarizes this group of experiments in terms of an amplification index. The synergy occurring with spleen cells was greatest at ratios between 1:0.1 and 1:0.04.

When the thymus cell concentrations were varied, but the peripheral cell concentration was kept constant (Fig. 4), proliferation in the resultant mixture was approximately proportional to the number of thymus cells added. This suggests that a cell component of the thymus population was the limiting element in the combined responses. When peripheral cell concentrations were varied and thymus remained constant under the same culture conditions (Table II), different kinetics emerged; high cell concentrations usually inhibited or reduced thymus/peripheral cell synergy.

**Synergy Requires that Both Thymus and Peripheral Cells Proliferate.** To determine the contribution of each cell type to the augmented reaction, the thymus or the spleen cell component was either treated with mitomycin C (26) or irradiated (25) before culture (Table III). When proliferation was inhibited in either cell component the synergistic two-cell response to LPS was essentially eliminated. The spleen cell contribution appeared more sensitive to block than that of the thymus; when blocked spleen cells were combined with thymus, proliferation was reduced to the level of thymus cells alone. When blocked thymus cells were combined with spleen cells, the combination retained the response expected of that number of spleen cells alone. It was concluded that the synergistic effect observed involves proliferation of both cellular elements.

**Subpopulation Characteristics of Cells in the Proliferating Thymus Component.** Immunocompetent thymus cells constitute a minor subpopulation identified by responsiveness to T mitogens and alloantigens, graft-versus-host reactivity, low net density, low Thy-1, and high H-2 antigen distribution. The major subpopulation has high net density and high membrane Thy-1 antigen, but has none of those attributes that indicate immunocompetence (26, 27). To determine
which thymus subpopulation was involved in synergistic proliferation, the cells were selected either on the basis of density on BSA gradients or selection with anti-Thy-1.2 antisera and complement. Table IV illustrates the findings. Selective elimination of cells highly sensitive to anti-Thy-1.2 and C yielded a subpopulation in which Con A responsiveness was apparently concentrated, but in which the capacity to support synergistic proliferation remained equivalent to whole unfractionated thymus. This suggested that the functions involving synergy are independent of Con A responsiveness.

Cells active in promoting synergy were found at all density levels in BSA gradients (Fig. 5), although any direct LPS activity observed in thymus cells alone was limited to cells of lowest net density (26, 27). Low density thymus fractions cultured with peripheral cells and LPS gave synergy not significantly greater than that given by high density fractions. Selective elimination of high Thy-1.2 cells from the high-density fractions with dilute anti-Thy-1.2 plus C yielded a residual subpopulation of 20-30% of cells which supported synergy as well as the untreated fraction. Higher concentrations of anti-Thy-1.2 eliminated all cells in this fraction.

The thymus cell subpopulation participating in the synergistic effect can therefore be characterized as one having relatively low Thy-1.2 representation, variable net density, and of a different subclass from those cells that respond strongly to Con A.

Characteristics of the Proliferating Peripheral Lymphoid Cell Population. The peripheral cell involved in the synergy effect was examined for properties of adherence to glass or nylon and for sensitivity to anti-Ig, anti-IgG,
Fig. 3. Effect of varying spleen cell concentration on thymus/spleen mixture LPS responses. $5 \times 10^5$ C57BL/10 thymus cell were co-cultured with spleen cells in levels as follows: $5 \times 10^5$, $1 \times 10^5$, $5 \times 10^4$, and $2 \times 10^4$ corresponding to the ratios 1:1, 1:0.2, 1:0.1, and 1:0.04, respectively. Data represent mean cpm ± SE for four replicate cultures. Spleen alone (○); thymus + spleen (◇); thymus alone (□).

Table II

Calculated Synergy of LPS-Induced Proliferation in Cultures of Mixtures of Thymus and Peripheral Lymphoid Cells Effects of Varying Cell Ratios*

| Experiment number and cells used | Amplification ratio, by cell ratio used (thymus: peripheral lymphocyte) |
|----------------------------------|-------------------------------------------------------------------------|
|                                  | 1:1           | 1:0.5         | 1:0.2         | 1:0.1          | 1:0.05        | 1:0.04        |
| 1. Spleen                        | 0.79          | 2.6           | 4.0           | 4.6            |
| 2. Spleen                        | 1.0           | 2.0           | 3.0           | 4.0            |
| 3. Spleen                        | 1.1           | 1.1           | 1.9           | 4.7            |
| 4. LN                            | 4.0           | 4.7           | 9.7           | 8.8            |
| 5. LN                            | 1.6           | 6.5           | 5.5           | 4.0            |

* $5 \times 10^5$ thymus cells were cultured with varying numbers of spleen or LN cells and either $5 \mu g$ (exp. 1, 2) or $1 \mu g$ (exp. 3-5) LPS. Synergy ratios were calculated: $A \cdot R = (cpm \text{ thymus-peripheral cell mixture})/(cpm \text{ thymus alone}) + (cpm \text{ peripheral cell alone})$.

Anti-IgM, or anti-Thy-1.2 plus C'. Table V shows that both adherent and nonadherent spleen cells had essentially equal activity in combination with thymus cells, while the nonadherent subpopulation had slightly lower levels of response to LPS alone. Nylon-column separation (Table VI) concentrated the proliferating peripheral cell in the retarded fraction. The cell population retarded by nylon had been previously filtered through glass wool, thus essentially eliminating a role of macrophages that are reported to potentiate T-cell functions in other contexts (28-31). The retarded subpopulation appeared directly responsive to LPS to a degree equal to that of the unseparated cells and contained cells giving synergistic activity when combined with thymus cells.

Selection of cell populations by treatment with anti-Ig, anti-IgG, or anti-IgM plus C' reduced LPS-induced proliferation and eliminated most B-cell functions.
Fig. 4. Effect of varying thymus cell concentration on synergy. 5 × 10⁴ spleen cells were cultured with 5 μg LPS alone and with thymus cells in the indicated concentrations. [³H]TdR incorporation was measured over the last 24 h of a 72-h culture period and represented as mean cpm ± SE for quadruplicate. Thymus + LPS (●); thymus + spleen + LPS (○).

(32). As is illustrated in Table VII, this treatment both reduced drastically the LPS response level of spleen cells and essentially eliminated synergistic proliferation with thymus cells. Anti-Thy-1.2 and complement used in similar experiments either had no effect or in some cases enhanced synergistic proliferation (Table VIII).

Comparison of the Contribution of LPS Responder or Nonresponder Status to the Synergy Effect. Spleen cells from C3H/HeJ mice are low responders to LPS, in contrast to most C3H strains (33-35). Combinations of thymus and spleen from the low-responder strain (C3H/HeJ) with cells from a closely related and non-MLR reactive high-responder strain (C3Heb/FeJ) were tested to determine how responder status contributed to synergistic proliferation in the system. It is evident from Table IX that the synergy effect occurs only when the peripheral component is of responder origin. The thymus cell component did not require responder status. These results are interpreted to mean that the peripheral cell must be LPS responsive to be synergistic with T cells.

Karyotypic Analysis Using Mixtures of T6 and Non-T6 Thymus and Spleen Cells. The relative contribution of each cell component to synergistic proliferation was resolved using CBA/H-T6J (T6) and CBA/Caj (Ca) histocompatible thymus and spleen combinations (Table X). Either T6 thymus or spleen cells were cultured together with Ca thymus or spleen cells, and after 72 h, the presence of the T6 markers was determined in each satisfactory metaphase. Replicative cultures were assayed for [³H]TdR incorporation.

The thymus cell contribution, although detectable in every culture, proved to be minimal. Only 2-10% of the total dividing cells in cultures showing augmented proliferation in response to LPS were of thymus origin. 90-98% of proliferating cells in the synergistic cultures were of peripheral cell origin.

Discussion

The data presented demonstrate that synergistic proliferation occurs when a low Thy-1, non-Con A responsive thymus T-cell subpopulation is cultured with small numbers of peripheral B cells in the presence of LPS. Synergy requires
### Table III

**Effect of Mitomycin Treatment or Irradiation of Thymus or Peripheral Cell Component on Synergy of LPS Response**

| Cells in culture and treatment | Thymus: spleen ratio | $[^3]$H$^*$TdR incorporation (mean cpm $\pm$ SE/culture) |
|-------------------------------|---------------------|-------------------------------------------------------|
|                               |                     | Unstimulated                                         | LPS                                           |
| 1. Thymus alone (5 x $10^5$)   |                     | 345 $\pm$ 111                                        | 684 $\pm$ 92                                 |
| Thymus [M] alone (5 x $10^5$)  |                     | 190 $\pm$ 31                                          | 271 $\pm$ 26                                 |
| Spleen alone (5 x $10^5$)      |                     | 175 $\pm$ 16                                          | 184 $\pm$ 25                                 |
| Spleen (4 x $10^5$)            |                     | 3,285 $\pm$ 270                                       | 20,968 $\pm$ 830                             |
| Spleen alone (2 x $10^5$)      |                     | 409 $\pm$ 155                                         | 257 $\pm$ 173                                |
| Spleen [M] (2 x $10^5$)        |                     | 141 $\pm$ 23                                          | 383 $\pm$ 48                                 |
| Thymus + spleen (2 x $10^5$)   | 1:0.04              | 493 $\pm$ 155                                         | 5,895 $\pm$ 157                              |
| Thymus [M] + spleen (2 x $10^5$)| 1:0.04              | 156 $\pm$ 29                                          | 254 $\pm$ 48                                 |
| Thymus + spleen [M] (2 x $10^5$)| 1:0.04              | 271 $\pm$ 52                                          | 665 $\pm$ 119                                |
|                               |                     |                                                       |                                              |
| 2. Thymus alone (5 x $10^6$)   |                     | 100 $\pm$ 17                                          | 160 $\pm$ 20                                 |
| Spleen (4 x $10^6$)            |                     | 4,559 $\pm$ 439                                       | 14,460 $\pm$ 1,045                          |
| Spleen [M] (4 x $10^6$)        |                     | 55 $\pm$ 6                                            | 67 $\pm$ 4                                  |
| Spleen alone (2 x $10^6$)      |                     | 69 $\pm$ 6                                            | 219 $\pm$ 94                                 |
| Thymus + spleen (2 x $10^6$)   | 1:0.04              | 125 $\pm$ 22                                          | 5,314 $\pm$ 488                              |
| Thymus + spleen [M] (2 x $10^6$)| 1:0.04              | 125 $\pm$ 23                                          | 235 $\pm$ 11                                 |
|                               |                     |                                                       |                                              |
| 3. Thymus alone (5 x $10^6$)   |                     | 320 $\pm$ 150                                         | 257 $\pm$ 21                                 |
| Thymus [X] alone (5 x $10^6$)  |                     | 46 $\pm$ 4                                            | 69 $\pm$ 17                                  |
| Spleen alone (5 x $10^6$)      |                     | 16,804 $\pm$ 1,485                                    | 54,796 $\pm$ 685                             |
| Spleen [X] alone (5 x $10^6$)  |                     | 844 $\pm$ 96                                          | 1,076 $\pm$ 86                               |
| Thymus + spleen (5 x $10^6$)   | 1:1                 | 15,115 $\pm$ 717                                      | 56,796 $\pm$ 5,747                           |
| Thymus [X] + spleen (5 x $10^6$)| 1:1                 | 15,444 $\pm$ 1,015                                    | 59,218 $\pm$ 4,894                           |
| Thymus + spleen [X] (5 x $10^6$)| 1:1                 | 905 $\pm$ 127                                         | 1,560 $\pm$ 33                               |
| Spleen (1 x $10^6$)            |                     | 1,646 $\pm$ 89                                        | 15,704 $\pm$ 592                             |
| Spleen [X] (1 x $10^6$)        |                     | 133 $\pm$ 8                                           | 251 $\pm$ 37                                 |
| Thymus + spleen (1 x $10^6$)   | 1:0.2               | 3,384 $\pm$ 221                                       | 32,921 $\pm$ 685                             |
| Thymus [X] + spleen (1 x $10^6$)| 1:0.2               | 2,756 $\pm$ 118                                       | 17,427 $\pm$ 584                             |
| Thymus + spleen [X] (1 x $10^6$)| 1:0.2               |                                                       | 546 $\pm$ 22                                 |
| Spleen (5 x $10^4$)            |                     | 618 $\pm$ 49                                          | 6,091 $\pm$ 94                               |
| Spleen [X] (5 x $10^4$)        |                     | 152 $\pm$ 80                                          | 83 $\pm$ 8                                   |
| Thymus + spleen (5 x $10^4$)   | 1:0.1               | 1,196 $\pm$ 98                                        | 19,051 $\pm$ 245                             |
| Thymus [X] + spleen (5 x $10^4$)| 1:0.1               | 1,295 $\pm$ 132                                       | 5,987 $\pm$ 353                              |
| Thymus + spleen [X] (5 x $10^4$)| 1:0.1               | 145 $\pm$ 14                                          | 419 $\pm$ 49                                 |
| Spleen (2 x $10^4$)            |                     | 183 $\pm$ 24                                          | 1,057 $\pm$ 23                               |
| Spleen [X] (2 x $10^4$)        |                     | 68 $\pm$ 11                                           | 66 $\pm$ 13                                  |
| Thymus + spleen (2 x $10^4$)   | 1:0.04              | 398 $\pm$ 59                                          | 5,234 $\pm$ 288                              |
| Thymus [X] + spleen (2 x $10^4$)| 1:0.04              | 303 $\pm$ 28                                          | 860 $\pm$ 117                                |
| Thymus + spleen [X] (2 x $10^4$)| 1:0.04              | 103 $\pm$ 10                                          | 251 $\pm$ 14                                 |

*C57BL/10 thymus and spleen were mitomycin [M]-treated or irradiated [X] with 1,000 R then used in combinations above adding 4 $\mu$g LPS to stimulate cultures. $[^3]$H$^*$TdR was added during the last 24 h and the data presented as the mean values for quadruplicate cultures $\pm$ SE.

The proliferation of both the thymus and peripheral lymphoid cell component. However, the major proliferative activity is due to greatly augmented cell division of the peripheral B-cell elements; the T-cell contribution is apparently to provide a regulatory effect.
### Table IV

**Effect of Anti-Thy-1.2 Plus Complement Treatment of Thymus Cells on Proliferative Response to LPS of Thymus/Spleen Cell Mixtures**

| Experimental groups | Thymus: spleen ratio | (H)TdR incorporation (mean cpm ± SE/culture) |
|---------------------|----------------------|---------------------------------------------|
|                     |                      | Unstimulated | LPS | Con A |
| 1. Whole thymus (C') (5 x 10⁶) | 57 ± 2 | 78 ± 7 | 2,715 ± 143 |
| Anti-Thy-1.2 + C'-treated thymus (5 x 10⁶) | 83 ± 9 | 120 ± 5 | 15,005 ± 1,223 |
| Spleen (5 x 10⁶) alone | 149 ± 24 | 2,407 ± 126 | 3,530 ± 104 |
| Thymus (C') + spleen (5 x 10⁶) | 316 ± 8 | 16,129 ± 774 | |
| Anti-Thy-1.2 plus C'-treated thymus + spleen (2 x 10⁶) | 355 ± 20 | 14,179 ± 379 | |
| 2. Low density thymus (C') (5 x 10⁶) | 42 ± 2 | 29 ± 1 | 479 ± 103 |
| Low density thymus (anti-Thy-1.2 plus C' treated) (5 x 10⁶) | 64 ± 1 | 48 ± 2 | 2,564 ± 69 |
| Spleen (5 x 10⁶) | 75 ± 24 | 314 ± 36 | 1,224 ± 88 |
| Low density thymus (C') (5 x 10⁶) + spleen (5 x 10⁶) | 85 ± 4 | 1,916 ± 39 | |
| Low density thymus (anti-Thy-1.2 plus C' treated) (5 x 10⁶) + spleen (5 x 10⁶) | 88 ± 3 | 3,214 ± 134 | |
| 3. High density thymus (C') (5 x 10⁶) | 58 ± 2 | 67 ± 7 | 308 ± 52 |
| High density thymus (anti-Thy-1.2 plus C') (5 x 10⁶) | 78 ± 8 | 102 ± 4 | 2,343 ± 149 |
| Spleen (5 x 10⁶) | 61 ± 6 | 3,069 ± 109 | |
| High density thymus (C') (5 x 10⁶) + spleen (5 x 10⁶) | 193 ± 30 | 13,510 ± 1,012 | |
| High density thymus (anti-Thy-1.2 plus C') (5 x 10⁶) + spleen (5 x 10⁶) | 206 ± 28 | 13,787 ± 926 | |

* Nylon column passed C57BL/10 whole thymus cells (exp. 1), low density (fraction "A + B") (exp. 2), or high density (fraction "D") (exp. 3) thymus cells were treated with anti-Thy-1.2 antiserum, 1:100 plus 1:15 guinea pig complement or complement alone at 37°C for 45 min and washed. 5 x 10⁶ cells of the control or anti-Thy-1.2-resistant subpopulations were cultured with spleen cells and 1 μg LPS or 1 μg Con A as indicated. (H)TdR incorporation was assayed during the last 24 h of a 48-h culture. Mean cpm ± SE are for four replicate cultures. 1:100 anti-Thy-1.2 plus complement killed 70–75% of high density cells, 30–35% of low density cells, and 50% of whole thymus cells. 1:50 anti-Thy-1.2 killed 96% of high density thymus cells and 75% of whole thymus.

The thymus cell component which supplies this amplifier effect has characteristics that do not identify it clearly with those thymus cell subpopulations previously described (20, 25, 27, 36). Moreover, it appears not to require macrophages to function. The responsible cell was distributed equally in high- and low-density thymus cells. The latter contain those immunocompetent T-cell subpopulations that are both T-mitogen and alloantigen responsive, and have T-helper cell functions; the former does not exhibit these functions. The amplifier cells are in the high-density fraction which is killed by higher concentrations of anti-Thy-1.2 and C'. Those anti-Thy-1.2 concentrations which appear to concentrate immunocompetent cell functions, retain amplifier function but not at a level greater than the whole thymus or untreated high density cells. The amplifier cell is therefore characterized as a thymus T cell of varying density, having Thy-1.2 representation, but apparently not an immunocompetent T cell.

The peripheral cell has properties that identify it as a B lymphocyte, including nonadherence to glass or plastic, adherence to nylon, and sensitivity to lysis.
REGULATION OF LPS B-CELL RESPONSE

**Fig. 5.** Effect of separation of thymus cells on discontinuous BSA density gradients on LPS synergy with spleen cells. 5 x 10⁵ C57BL/10 thymus cells obtained from BSA gradients were incubated with 5 x 10⁴ spleen cells for 72 h after adding indicated mitogens. [³H]TdR incorporation was assayed during the last 24 h; the data represent mean cpm of four cultures ± SE. Alloantigen responses were to 0.25 x 10⁶ irradiated CBA spleen cells (responder to target ratio 2:1). Unstimulated (○), LPS alone (■); spleen + LPS (■); alloantigen (■).

**Table V**
Adherence Properties of Spleen Cells in Synergistic Thymus/Spleen LPS Response*

| Cells                     | [³H]TdR incorporation (mean cpm + SE culture) |
|---------------------------|---------------------------------------------|
|                           | Unstimulated  | LPS (4 μg)      |
| Thymus (5 x 10⁵)         | 359 ± 63     | 936 ± 79        |
| Spleen (whole) (4 x 10⁵) | 2,343 ± 288  | 24,933 ± 997    |
| Spleen nonadherent (4 x 10⁵) | 1,664 ± 79 | 18,634 ± 1,403  |
| Spleen adherent (4 x 10⁵) | 1,499 ± 69   | 25,843 ± 1,304  |
| Spleen (whole) (2 x 10⁴) | 285 ± 45     | 406 ± 43        |
| Spleen nonadherent (2 x 10⁴) | 277 ± 48  | 199 ± 34        |
| Spleen adherent (2 x 10⁴) | 127 ± 11     | 183 ± 11        |
| Thymus + (whole) spleen (2 x 10⁴) | 483 ± 155 | 5,896 ± 157     |
| Thymus + nonadherent spleen (2 x 10⁴) | 194 ± 52 | 4,783 ± 367     |
| Thymus + adherent spleen (2 x 10⁴) | 277 ± 43 | 6,279 ± 254     |

* C57BL/10 spleen cells were plated in Petri dishes and incubated for 2 h at 37°C in 5% CO₂. Nonadherent cells were poured off, the plate washed three times, and then the nonadherent cells removed with a rubber policeman. These subpopulations were cultured 72 h as indicated assaying for [³H]TdR incorporation during the last 24 h. Data represent mean value ± SE for four replicate cultures.

by anti-Ig, anti-IgG, or anti-IgM antiserum plus C'. Optimal synergy is seen when the peripheral cells are used in relatively low numbers, which alone react minimally to LPS. Numbers of peripheral cells which showed good responses to LPS alone did not demonstrate detectable synergy when combined with thymus cells under these conditions.
TABLE VI
Effect of Nylon Column Passage of Spleen Cells Upon LPS Synergy in Thymus/Spleen Cell Mixtures*

| Cells | Thymus: spleen ratio | Unstimulated \[^{3}H\]TdR incorporation (mean cpm ± SE/culture) | LPS \[^{3}H\]TdR incorporation (mean cpm ± SE/culture) | PHA \[^{3}H\]TdR incorporation (mean cpm ± SE/culture) |
|-------|---------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Thymus alone (5 × 10⁴) | 109 ± 11 | 239 ± 27 | 296 ± 9 |
| Spleen alone (5 × 10⁴) | 8,623 ± 755 | 83,853 ± 2,720 | 62,383 ± 3,168 |
| Passed spleen alone (5 × 10⁴) | 3,875 ± 296 | 18,883 ± 473 | 92,906 ± 3,878 |
| Retarded spleen alone (5 × 10⁴) | 7,219 ± 427 | 84,836 ± 1,251 | 63,580 ± 2,350 |
| Spleen alone (5 × 10⁵) | 276 ± 9 | 2,007 ± 176 | |
| Passed spleen alone (5 × 10⁵) | 237 ± 36 | 333 ± 33 | |
| Retarded spleen alone (5 × 10⁵) | 213 ± 20 | 3,150 ± 75 | |
| Thymus + spleen (5 × 10⁴) 1:0.1 | 610 ± 43 | 14,035 ± 288 | |
| Thymus + passed spleen (5 × 10⁴) 1:0.1 | 490 ± 86 | 1,871 ± 128 | |
| Thymus + retarded spleen (5 × 10⁴) 1:0.1 | 377 ± 71 | 22,482 ± 1,931 | |
| Spleen alone (2 × 10⁶) | 171 ± 10 | 517 ± 40 | |
| Passed spleen alone (2 × 10⁶) | 140 ± 10 | 241 ± 10 | |
| Retarded spleen alone (2 × 10⁶) | 136 ± 20 | 648 ± 61 | |
| Thymus + spleen (2 × 10⁶) 1:0.04 | 270 ± 0 | 4,875 ± 407 | |
| Thymus + nonadherent spleen (2 × 10⁶) 1:0.04 | 485 ± 188 | 856 ± 154 | |
| Thymus + adherent spleen (2 × 10⁶) 1:0.04 | 348 ± 24 | 6,245 ± 1,485 | |

* C57BL/10 spleen cells were first filtered through glass wool and then incubated for 60 min at 37°C in a nylon wool column. The column was eluted, the passed cells collected, washed, and used as above. The cells remaining (“retarded”) were removed by compressing the nylon collecting the loosely adherent cells. These cells were cultured as indicated with 5 × 10⁴ thymus cells. Counts represent \[^{3}H\]TdR incorporation for four replicate cultures ± SE.

TABLE VII
Effect of Anti-Immunoglobulin Plus Complement on Capacity of Spleen Cells to Support LPS Synergy when Cultured with Thymus Cells*

| Experiment and procedures | Thymus: spleen ratio | \[^{3}H\]TdR incorporation (mean cpm ± SE/culture) |
|---------------------------|---------------------|-------------------------------------------------|
| 1. Thymus alone (5 × 10⁴) | 284 ± 95 | 347 ± 39 | |
| Spleen alone (4 × 10⁵) | 1,533 ± 355 | 25,639 ± 1,503 | |
| Anti-Ig plus C'-treated spleen alone (4 × 10⁴) | 346 ± 65 | 2,130 ± 194 | |
| Spleen alone (4 × 10⁴) | 292 ± 55 | 822 ± 30 | |
| Anti-Ig plus C'-treated spleen alone (4 × 10⁴) | 293 ± 105 | 553 ± 47 | |
| Thymus + spleen (4 × 10⁴) 1:0.08 | 198 ± 53 | 6,294 ± 604 | |
| Thymus + anti-Ig plus C'-treated spleen (4 × 10⁴) 1:0.08 | 249 ± 72 | 1,185 ± 134 | |
| 2. Thymus alone (5 × 10⁵) | 91 ± 11 | 301 ± 41 | |
| Complement-treated spleen alone (4 × 10⁴) | 7,904 ± 947 | 48,324 ± 1,215 | 65,711 ± 3,550 |
| Complement-treated spleen alone (2 × 10⁴) | 597 ± 187 | 523 ± 192 | |
| Anti-IgG plus C'-treated spleen alone (4 × 10⁴) | 803 ± 56 | 11,038 ± 1,227 | 80,819 ± 1,286 |
| Anti-IgG plus C'-treated spleen alone (2 × 10⁴) | 36 ± 3 | 166 ± 56 | |
| Anti-IgM plus C'-treated spleen alone (4 × 10⁴) | 2,048 ± 322 | 21,140 ± 1,085 | 70,572 ± 4,378 |
| Anti-IgM plus C'-treated spleen alone (2 × 10⁴) | 138 ± 62 | 243 ± 121 | |
| Thymus + C'-treated spleen (2 × 10⁴) 1:0.04 | 270 ± 153 | 3,154 ± 254 | |
| Thymus + Anti-IgG plus C'-treated spleen (2 × 10⁴) 1:0.04 | 201 ± 73 | 670 ± 108 | |
| Thymus + Anti-IgM plus C'-treated spleen (2 × 10⁴) 1:0.04 | 294 ± 62 | 608 ± 57 | |

* C57BL/10 spleen cells were treated with 1:15 dilutions anti-Ig, anti-IgG, or anti-IgM, plus 1:15 guinea pig complement, incubated at 37°C for 45 min, and then washed. The residual subpopulation was cultured alone or in combination with thymus cells, with or without 4 µg LPS or 0.5 µg Con A. \[^{3}H\]TdR incorporation assayed in four replicate cultures.
**REGULATION OF LPS B-CELL RESPONSE**

### TABLE VIII

**Effect of Anti-Thy-1.2 and Complement Treatment of Spleen on Thymus-Spleen LPS Synergy**

| Experiment and procedure | Thymus: spleen ratio | \[^{3}H\]Tdr incorporation (mean cpm ± SE/culture) |
|--------------------------|----------------------|-----------------------------------------------|
|                          |                      | Unstimulated | LPS | PHA |
| 1. Thymus (5 x 10^5)     |                      | 46 ± 7      | 326 ± 24 | 32,335 ± 741 |
| Complement-treated spleen (5 x 10^5) | 1,092 ± 122 | 63,339 ± 1,755 | 47,187 ± 1,177 | 1,890 ± 157 |
| Anti-Thy-1.2 plus C'-treated spleen (5 x 10^5) | 520 ± 60 | 47,187 ± 1,177 | 47,187 ± 1,177 | 1,890 ± 157 |
| Complement-treated spleen (2 x 10^5) | 41 ± 13 | 1,274 ± 110 | 1,274 ± 110 | 1,274 ± 110 |
| Anti-Thy-1.2 plus C'-treated spleen (2 x 10^5) | 45 ± 12 | 1,532 ± 202 | 1,532 ± 202 | 1,532 ± 202 |
| Thymus + C'-treated spleen (2 x 10^5) | 1:0.04 | 188 ± 50 | 5,013 ± 286 | 5,013 ± 286 |
| Thymus + Anti-Thy-1.2 plus C'-treated spleen (2 x 10^5) | 1:0.04 | 122 ± 17 | 5,311 ± 205 | 5,311 ± 205 |
| 2. Thymus (5 x 10^5)     |                      | 174 ± 19    | 280 ± 15 | 32,335 ± 741 |
| Complement-treated spleen (5 x 10^5) | 863 ± 58 | 53,700 ± 720 | 28,658 ± 1,156 | 28,658 ± 1,156 |
| Anti-Thy-1.2 plus C'-treated spleen (5 x 10^5) | 2,148 ± 98 | 73,697 ± 526 | 3,073 ± 190 | 3,073 ± 190 |
| Complement-treated spleen (5 x 10^5) | 77 ± 4 | 2,778 ± 116 | 2,778 ± 116 | 2,778 ± 116 |
| Anti-Thy-1.2 plus C'-treated spleen (5 x 10^5) | 101 ± 7 | 7,751 ± 391 | 7,751 ± 391 | 7,751 ± 391 |
| Thymus + C'-treated spleen (5 x 10^5) | 1:0.1 | 248 ± 33 | 10,034 ± 376 | 10,034 ± 376 |
| Thymus + Anti-Thy-1.2 plus C'-treated spleen (5 x 10^5) | 1:0.1 | 314 ± 62 | 19,135 ± 499 | 19,135 ± 499 |

* C57BL/10 spleen cells were treated with 1:45 dilution of anti-Thy-1.2, plus 1:15 guinea pig complement, incubated at 37°C for 45 min, and washed. The residual populations were cultured alone or in combination with thymus cells together with 5 μg LPS or 0.05 μl PHA added as indicated. Data represent mean cpm ± SE for four cultures incubated for 72 h assaying \[^{3}H\]Tdr incorporation during the last 24 h.

### TABLE IX

**Contribution of LPS-Responder Strain (C3HeB/FeJ) or Nonresponder (C3H/HeJ) Spleen or Thymus Cells to LPS Synergy**

| Origin of cells cultured | Thymus: spleen ratio | \[^{3}H\]Tdr incorporation (mean ± SE/culture) |
|--------------------------|----------------------|-----------------------------------------------|
|                          |                      | Unstimulated | LPS | added |
| C3H/HeJ thymus alone     |                      | 328 ± 42    | 121 ± 13 | 121 ± 13 |
| C3HeB/FeJ thymus alone   |                      | 353 ± 18    | 125 ± 5 | 125 ± 5 |
| C3H/HeJ spleen alone     |                      | 125 ± 16    | 120 ± 18 | 120 ± 18 |
| C3Heb/FeJ spleen alone   |                      | 135 ± 16    | 1,064 ± 100 | 1,064 ± 100 |
| C3H/HeJ thymus + C3H/HeJ spleen | 1:0.04 | 360 ± 33 | 538 ± 33 | 538 ± 33 |
| C3HeB/FeJ thymus + C3HeB/FeJ spleen | 1:0.04 | 478 ± 48 | 4,746 ± 433 | 4,746 ± 433 |
| C3H/HeJ thymus + C3HeB/FeJ spleen | 1:0.04 | 610 ± 102 | 5,807 ± 596 | 5,807 ± 596 |
| C3HeB/FeJ thymus + C3HeB/FeJ spleen | 1:0.04 | 459 ± 23 | 542 ± 46 | 542 ± 46 |

* C3HeB/FeJ or C3H/HeJ thymus cells (5 x 10^5) were cultured with 2 x 10^4 spleen cells of the same or other strain together with or without 1.0 μg LPS. Data are mean values ± SE for four replicate cultures.

The extent of proliferation by each component was assessed by using the T6 chromosome markers; most cell division was contributed by the B-lymphocyte component with only 2–10% of dividing cells in the mixture being of thymus origin. The B-cell-like dose-response kinetics of the synergistic effect are consistent with this finding: The minimal level of thymus proliferation observed appears to be essential for amplified B-cell responses.

The data available permit construction of a working hypothesis involving a
| Experiment | Cell source and strain of origin | LPS (5 μg) | \[^3H\]Tdr incorporation (mean cpm ± SE/culture) | Number T6 metaphases/number of non-T6\* | Percent metaphases of thymus origin |
|------------|---------------------------------|------------|-----------------------------------------------|-----------------------------------------|------------------------------------|
| 1          | Thymus (T6)                     | –          | 105 ± 21                                       |                                        |                                    |
|            |                                 | +          | 123 ± 23                                       |                                        |                                    |
|            | Spleen (Ca)                     | –          | 95 ± 36                                        |                                        |                                    |
|            |                                 | +          | 364 ± 65                                       |                                        |                                    |
|            | Thymus (T6) + spleen (Ca)       | –          | 190 ± 21                                       |                                        |                                    |
|            |                                 | +          | 4,012 ± 333                                    | 1/66                                   | 1.5                                |
|            | Thymus (Ca)                     | –          | 83 ± 8                                         |                                        |                                    |
|            |                                 | +          | 94 ± 6                                         |                                        |                                    |
|            | Spleen (T6)                     | –          | 67 ± 3                                         |                                        |                                    |
|            |                                 | +          | 447 ± 28                                       |                                        |                                    |
|            | Thymus (Ca) + spleen (T6)       | –          | 311 ± 55                                       | 219/23                                 | 9.5                                |
|            |                                 | +          | 7,261 ± 410                                    |                                        |                                    |
| 2          | Thymus (T6)                     | –          | 57 ± 11                                        | 0/0\‡                                  | 0                                  |
|            |                                 | +          | 144 ± 21                                       |                                        |                                    |
|            | Spleen (Ca)                     | –          | 61 ± 11                                        |                                        |                                    |
|            |                                 | +          | 4,801 ± 360                                    |                                        |                                    |
|            | Thymus (T6) + spleen (Ca)       | –          | 141 ± 27                                       |                                        |                                    |
|            |                                 | +          | 19,596 ± 1,817                                 |                                        |                                    |

* CBA/CaJ (Ca) or CBA/H-T6J (T6) thymus (5 x 10⁴) or spleen (5 x 10⁴) cells were cultured 48 h (exp. 1) or 72 h (exp. 2); \[^3H\]Tdr was added during the last 24 h. To replicate cultures, 4 μg/ml Colcemid was added during the last 4 h of culture; metaphase plates were examined for the presence or absence of the T6 chromosome markers.

\‡ Numbers given are for those metaphases technically satisfactory for evaluation. The relatively higher value for thymus (underlined) may reflect difficulty in identifying T6 markers even in technically satisfactory preparations.

§ No metaphases observed.

sequence of cellular interactions. Briefly, the first step is postulated to involve direct LPS stimulation of the small subpopulation of peripheral B cells added to the mixture. As a result of this stimulation a subclass of thymus T cells is activated. The activated T cells in some way exert a stimulatory or regulatory effect on the ongoing LPS-induced B-cell proliferation which greatly increases the level of \[^3H\]Tdr incorporation finally measured.

The initial step appears to be a direct B-mitogen effect because (a) the thymus component does not respond to LPS directly, and (b) the B-cell-containing population is known to respond to LPS without any helper cell requirement (37, 38).
The next step is postulated to involve activation of the thymus cell component because (a) synergy requires these T cells and (b) supernates from LPS-stimulated peripheral B cells cause proliferation of high-density thymus cells with T-cell-like kinetics (M. A. Norcross and R. T. Smith, unpublished data). It is unlikely that this factor is macrophage derived, as is apparently true of the lymphocyte stimulating factor (LAF) described by others (28-30). Both the peripheral population involved and the thymus cells can be completely depleted of macrophages without interfering with synergy. A very small proportion of B cells (less than 5% of the cultured cell mixture) is required to activate the thymus cell component. Conceivably, apparent macrophage effects seen in other systems could be explained through the contribution of small numbers of B cells in the adherent cell fractions employed as a macrophage source.

In the last proposed step the activated T-cell component augments or regulates ongoing B-cell proliferation stimulated by LPS. This regulatory effect might occur (a) by recruiting previously unresponsive B cells; (b) by nonspecifically facilitating proliferation of cells already dividing; or (c) by releasing B cells from suppression. In any case, the B cells in the augmented response could be responding to a T-cell product alone, to LPS, or to both.

If the artificial system we have described is interpreted correctly, perhaps an analogous peripheral T-cell component regulates B-cell responses to LPS in peripheral lymphoid tissues. Tumor-bearing mice and Bacille Calmette Guerin-treated mice show greatly augmented LPS responses in regional lymph nodes, far in excess of any increase in T- or B-cell numbers in those nodes (23). This could represent an in vivo reflection of the in vitro phenomenon described. However, this does not appear congruent with the observation (5) that peripheral lymphoid cells pretreated with anti-γ and C' either show comparable or enhanced LPS proliferation in the residual population. The known subclasses of peripheral T cells are quite sensitive to anti-Thy-1 and C' treatment; a diminished response therefore would be expected unless a suppressor T cell were also eliminated or a helper cell were concentrated. Pre-T cells are present in spleen and bone marrow of the normal mouse, but not in peripheral blood or lymph nodes. Both of these cell populations are poor responders to LPS (39). Pre-T cells are found in spleens of both nude (40) or thymectomized (41) mice which show excellent LPS responses. Pre-T cells, if exposed to thymosin, develop characteristic thymus antigens (41, 42). It seems possible that the pre-T cell might provide an equivalent of the amplifier thymus cell subpopulation in augmenting LPS-induced B-cell proliferation. Attempts to identify a T-regulatory cell among peripheral lymphoid cells are in progress.

Summary

When thymus cells which are unresponsive to LPS are combined with numbers of peripheral lymphoid cells giving minimal responses to LPS, synergistic incorporation of [3H]thymidine occurs. Synergy requires that both components proliferate, but most of the augmented response is the result of peripheral cell proliferation. The thymus cell is a T cell of variable density, low in thy-1.2 antigen, not concanavalin A responsive, present in the major thymus subpopulation, and may be from lipopolysaccharide (LPS)-unresponsive strains. The
peripheral cell is sensitive to anti-IgG or IgM plus complement (C'), resistant to anti-Thy-1.2 and C', exhibits adherence properties of B lymphocytes, and must be from LPS-responsive strains. Synergistic responses depend on critical thymus/peripheral cell ratios, inhibition occurring at high peripheral cell numbers. The data provide evidence that B-cell proliferative responses to LPS may be regulated by a subclass of thymus T cells.

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