GENERATION OF T-HELPER CELLS IN VITRO
I. Cellular and Antigen Requirements*

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The cellular immune reactions to hapten-protein conjugates (in the generation of hapten-specific primary and secondary humoral responses) display a significant dependence upon carrier recognition (reviewed in reference 1). Experimental models, particularly the adoptive transfer of immunocompetent cells into irradiated hosts (2-5), have established that the cooperative interactions involved require a thymus-derived (T) lymphocyte (6) with specificity for the carrier protein (7-9) to induce an optimal response by hapten-primed, antibody-forming precursors (B cells). In vivo methods for generating carrier-reactive T-helper cells include preimmunization with free carrier before immunization with hapten-carrier conjugate (10, 11), adoptive transfer of carrier primed cells (6-9), and the use of thymus-derived cells obtained from spleens of irradiated mice injected with thymocytes and carrier (12-14). These cells, or products derived from them, have been analyzed in vivo (12-15) and in vitro (16-18).

The critical role of the macrophage in a variety of T-cell responses (19-25) suggests that an in vitro system in which macrophage-thymocyte synergy is used might be devised for generating carrier-reactive T cells. Cultured, adherent cells have been shown to substantially preserve thymocyte viability and function in vitro (26), and have been used to selectively enrich antigen-reactive T cells from mice primed in vivo (27). Until recently it has not been possible to generate carrier-reactive T-helper cells in vitro. In 1973, Kontianen and Feldmann (28) reported the induction of carrier-specific helper T cells in vitro by using spleen and lymph node cells as a source of unprimed T cells. Subsequently, Erb and Feldmann (29-31) have obtained evidence that interaction between macrophages and T cells is essential to the generation of carrier-specific T-helper cells. A detailed examination of the cellular requirements, antigen specificity, or mode of action of T-helper cells generated in vitro has not been reported.

The following experiments were initiated to determine whether helper T cells could be generated in vitro from purified cell populations. The expectation is that such a system would be a valuable model for examining the mechanism by which T-helper cells or their products enhance B-cell anti-hapten responses. We report the culture conditions and the cellular and antigen requirements for generating cells active in enhancing a hapten-specific response in vitro. The results provide evidence that: (a) the active cell is thymus derived, but requires macrophages for generation of helper cell function; (b) the culture system

* The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U S Department of Health, Education, and Welfare.
represents a true induction rather than enrichment of helper cells; (c) enhancement of a carrier-specific as well as nonspecific response is generated; and (d) the enhancement of a carrier-specific response requires an additional influence of macrophages to demonstrate enhanced anti-hapten B-cell responses, whereas the enhancement of the nonspecific response is not macrophage dependent.

Materials and Methods

Animals. (B10D2 × BALB/C) F1, hybrid and CBA/J mice were used. The F1 hybrids were raised in our animal facilities. The parental strains and CBA/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Both sexes were used, however, within any given experiment, mice of the same sex were used.

Antigens. 2,4-dinitrophenyl (DNP) groups were coupled to keyhole limpet hemocyanin (KLH) and fowl gamma globulin (FyG) by the method of Little and Eisen (32). Conjugation ratios were 123 mol DNP per 800,000 g KLH and 33 mol DNP per 160,000 g FyG. DNP-N-2-aminoethyl carbamylmethylated Ficoll (DNP-Ficoll) containing 39 mol DNP per 400,000 g Ficoll was prepared by the method of Inman (33).

Media and Culture Conditions. Eagle's minimal essential media (MEM) made with Earle's salt base was prepared from concentrated stock solutions (Center for Disease Control Tissue Culture Unit) diluted to 300–308 mosmol/liter at pH 7.2–7.4. Supplemented MEM contained nonessential amino acids, 1 mM Na pyruvate, 2 mM l-glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin, and 5 × 10⁻³ M 2-mercaptoethanol (2-ME). MEM containing 10 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES MEM), and 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N.Y.) was used for cell preparations and culture. Cells were cultured in 1.0-ml vol in Marbrook-Diener chambers (34) within a 1.5 cm inner chamber. The reservoir, 30–35 ml, contained supplemented MEM and 5% FCS. Cells were cultured at 37°C in 10% CO₂ in humidified air.

Cortisone-Resistant Thymocytes. Thymuses from 4- to 6-wk-old mice, injected intraperitoneally (i.p.) 2 days previously with 2.5 mg cortisone acetate, were aseptically removed taking care to avoid parathymic lymph nodes. Thymocytes were teased from the capsules through a no. 60-mesh stainless steel screen and washed twice at 300g with HEPES MEM. Thymocytes were then passed through a nylon wool column (35).

Macrophages. Peritoneal exudate cells (PEC) from mice injected i.p. with 1 cm³ of 10% protease peptone broth (Difco Laboratories, Detroit, Mich.) 4 days previously were obtained by peritoneal lavage with cold HEPES MEM containing 10 U/ml heparin. PEC were washed, plated on 15- by 100-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) at 2-3 × 10⁷ cells per dish in 20 ml HEPES MEM 10% FCS. Plates were incubated 90 mm at 37°C on a rocker platform (Bellco Glass, Vineland, N. J.). Nonadherent cells were vigorously washed off, and in some experiments, the remaining cells treated with anti-Thy-1.2 sera and complement (see below). Adherent cells were harvested with a rubber policeman, and dead cells were removed by rapid filtration over cotton wool in a low ionic strength buffer at 4°C (36).

Alternatively, 8–15 × 10⁶ PEC in 1.5 ml were drawn into a 3.0-ml plastic syringe fitted with a no. 20-gauge needle. The syringe contained 1.5 ml glass beads (CPG-10, 20/80 mesh, Electro-Nucleonics Inc., Fairfield, N. J.) which had been previously equilibrated with HEPES MEM 10% FCS. The syringe was incubated for 1 h at 37°C on a rocker platform with frequent manual rotation. Nonadherent cells were removed by dropping elution with 15–20 ml of prewarmed media by using gentle pressure on the plunger. Cells adhering to the glass beads were calculated by subtracting the number eluted from the syringe from the number introduced. Glass beads were removed by cutting the end of the syringe with a hot scalpel and introducing measured amounts of glass beads directly into culture vessels by advancing the plunger.

1 Abbreviations used in this paper: ATC, in vitro activated thymus-derived cell; CRL, lymphocyte with receptor for complement; DNP, dinitrophenyl; FCS, fetal calf serum, FyG, fowl gamma globulin; SIg, cell surface immunoglobulin; KLH, keyhole limpet hemocyanin; MEM, minimum essential media; NMS, normal mouse serum; PEC, peritoneal exudate cells; PFC, plaque-forming cells.
**Hapten-Primed Spleen Cells** Spleens from mice injected i.p with 25 μg DNP-Ficoll 8-4 mo previously were removed aseptically. Cells were gently teased from the cut capsule, expressed through a no. 60-mesh stainless steel screen, and washed twice in HEPES MEM; clumps and debris were allowed to settle at unit gravity, and the supernate containing a single cell suspension was adjusted to the desired concentration for culture.

**Sequential Culture Technique.** Unless stated otherwise, the standard procedure used is as follows. 1.5 × 10⁷ nylon wool-purified, cortisone-resistant thymocytes and 5 × 10⁶ glass-adherent PFC were cultured with carrier antigen (0.1 μg KLH or 0.5 μg FyG) in 1.0 ml HEPES MEM 10% FCS in Marbrook-Diener culture chambers. After 4 days, the cells were harvested, and the debris and glass beads were allowed to settle through a layer of FCS at unit gravity. The supernate was pelleted at 300 g for 7 min and reseeded in fresh media. A quantity of the cell suspension containing 5-10 × 10⁶ viable cells was then transferred to cultures containing spleen cells from hapten-primed mice together with hapten-protein conjugates (1 μg DNP-KLH or 5 μg DNP-FyG). The recipient cultures contained 15 × 10⁶ cells. After 4 days, triplicate cultures were harvested and assayed for DNP-specific plaque-forming cells (DNP-PFC). All experiments reported here were done at least three times.

**PFC Assay** Direct PFC were assayed by the Cunningham Szegner modification of the Jerne hemolytic-plaque technique (37) with 2,4,6-trinitrophenyl-conjugated sheep red blood cells (SRBC) (38). Specific DNP-PFC were calculated by subtracting the number of PFC formed in the presence of 5 × 10⁻⁴ M e-DNP-L-lysine (Sigma Chemical Co., St. Louis, Mo.) from the number of PFC formed in the absence of free hapten. Indirect (IgG) PFC were enumerated inhibiting direct PFC with a goat anti-mouse γ-chain serum (>90% inhibition) and developing IgG PFC with a rabbit anti-mouse γ-chain serum (39).

**Depletion of T Lymphocytes with Anti-Thy-1.2 Serum** Anti-Thy-1.2 serum (anti-θ) with a plateau microcytotoxicity titer of 1:128 (1 μl serum/3 × 10⁶ cells) was prepared according to the method of Reif and Allen (40). A 1:3 final dilution of anti-Thy-1.2 serum in HEPES MEM was reacted with cell suspensions of no more than 2 × 10⁶ cells per ml for 30 min at room temperature. The cells were pelleted and reseeded in a 1:10 dilution of guinea pig complement containing 10 μg/ml deoxyribonuclease (DNAse) (Worthington Biochemical Company, Freehold, N. J.) for 1 h at 37°C. The complement had previously been absorbed at 4°C with CBA or (B10D2 × BALB/c) F₁ spleen cells (two spleens/ml). When spleen cell preparations were treated with anti-Thy-1.2 serum, dead cells were removed by the technique of Von Boehmer and Shortman (36). Control cells were treated identically to the anti-Thy-1.2-treated cells, except that normal AKR mouse serum was used.

**Depletion of Macrophages.** Macrophages were removed from cell preparations by Sephadex G-10 column incubation according to the method of Ly and Mishell (41) with the following modifications. HEPES MEM 20% FCS was used instead of Hanks' balanced salt solution, and a 1-h incubation was performed at 37°C.

**Mitomycin C Treatment of Cells.** Thymocytes and macrophages at 5 × 10⁶ cells per ml were treated with 50 μg/ml mitomycin C (Laboratory and Educational Supplies, Los Angeles, Calif.) for 45 min at 37°C. The cells were washed twice with a 5-min 37°C incubation between washes. This dose diminished the DNA synthetic response of cells to mitogenic stimulation to less than 10% of control values, yet allowed preservation of cell viability in culture.

**Identification of Cells** Mononuclear phagocytes (macrophages) were identified by the ingestion of latex beads (1.1 μm, Dow Chemical, Midland, Mich.) during a 30-min incubation at 37°C in the presence of 20% FCS (42). Latex ingestion was performed on all cell preparations before being dispensed for other cell marker procedures. Surface immunoglobulin-bearing cells were determined with a fluorescein-conjugated, thymocyte-absorbed rabbit anti-mouse immunoglobulin reagent (a gift from C. B. Reimer, Center for Disease Control). Cells (1 × 10⁶) in a 0.5-ml vol were incubated with a 1:100 final dilution of antisera in 0.01 M phosphate-buffered saline (PBS), 0.1% Na azide, and 5% FCS for 30 minutes at 4°C. Cells were washed twice at 4°C over FCS, and positive cells were enumerated with an incident-illumination Leitz fluorescent microscope. T cells were determined by microcytotoxicity (43) or indirect immunofluorescence with anti-Thy-1.2 serum. The latter procedure was performed essentially as described by Schwartz et al (44). Cells were first incubated with either undiluted anti-Thy-1.2 serum or normal AKR mouse serum (NMS). The cells were washed and incubated with fluorescein-conjugated, rabbit anti-mouse immunoglobulin reagent. The percent of T lymphocytes was determined as the difference between the percentage of
fluorescent cells in the anti-Thy-1.2 serum and the NMS-incubated preparation. Complement receptor-bearing lymphocytes (CRL) were enumerated with sheep erythrocytes coated with a subagglutinating dose of rabbit 19s anti-SRBC (Cordis Laboratories, Miami, Fla.) and mouse complement (a 1:5 dilution of AKR mouse serum previously absorbed at 4°C against SRBC) (45) Antibody-coated SRBC without complement served as control. Only cells surrounded by three or more SRBC (macrophages excluded) were considered CRL. Lymphocytes bearing receptors for the Fc portion of immunoglobulin were enumerated indirectly according to the method described by Dickler and Kunkel (46) in which aggregated human IgG and a fluorescein-conjugated, goat anti-human γ-chain reagent were used (supplied by C. B. Relmer, Center for Diseases Control). The latter reagent did not bind mouse cells directly. At least 300 cells were examined for each determination. When a particular marker comprised a small percentage of the cells being examined, effort was made to examine 1,000 or more cells.

Mitogen Stimulation. All experiments were performed with several doses of mitogens: phytohemagglutinin, diluted according to manufacturer's instructions, 0.25-1.0 μl/ml (Difco Laboratories); concanavalin A, 0.1-1.0 μg/ml (Con A, Pharmacia, Piscataway, N. J.); and Salmonella enteritidis lipopolysaccharide, 1-10 μg/ml (Difco Laboratories). Results reported reflect optimums from dose-response curves. 1-ml cultures of 10⁶ cells in serum-free, supplemented MEM containing 5 × 10⁻⁵ M 2-ME were incubated for 3 days. 18 h before harvest, 1 μCi [³H]thymidine (6 7 Ci/mM, New England Nuclear, Boston, Mass.) was added. Cultures were harvested manually. Cells were pelleted, and the pellets were precipitated with cold 5% trichloroacetic acid, washed twice in methanol, and solubilized at 50°C in 0.4 ml NCS reagent (Amersham/Searle Corp., Arlington Heights, Ill.); radioactivity was determined by liquid scintillation counting.

Results

Cell Preparations and the Optimal Conditions for the In Vitro Induction of Helper Cell Function. Preliminary experiments were performed to determine the optimal thymocyte cell number, proportion of macrophages, antigen dose, and duration of the helper cell induction culture. It was found that 1.5 × 10⁷ thymocytes and 5 × 10⁵ macrophages cultured for 4 days with either 0.1 μg KLH or 0.5 μg FyG are the optimal conditions for generating helper cells. Cells from this culture enhance the anti-DNP PFC response of hapten-primed, spleen cell cultures to hapten-carrier conjugates. The enhancement of indirect (IgG) DNP-PFC responses varied in parallel with direct (IgM) DNP-PFC responses (Fig. 1). The presence of macrophages in the thymocyte cultures improves cell culture recovery and viability (25-45% viable cell recovery within the protocols outlined in Fig. 1), but does not obviate the addition of varying amounts of dead cells to the recipient cultures. This might affect recovery of spleen cells from the cultures and, therefore, the results expressed as DNP-PFC per culture. In the experiments reported here, individual spleen cell-culture recoveries were within 20% of the mean recovery for all cultures within any given experiment.

The characteristics of the cell preparations before and after 4 days of coculturing is shown in Table I. B-cell contamination is minimal as assessed by membrane markers (SIg, Fc, CRL) and by the absence of a response to the B-cell mitogen lipopolysaccharide (Table II). No PFC are generated in this culture (n = 9).

Nylon wool purification of corison-resistant thymocytes yields 50-70% recovery, removes contaminating B cells, and results in an effluent population with improved viability (90-98% vs. 60-80% viability for the starting pool).

PEC obtained from mice injected with proteose peptone broth are 60-75% phagocytic as judged by uptake of polystyrene beads. Purification by adherence substantially increases the proportion of phagocytic cells. Macrophages are
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FIG. 1. Optimal conditions for the in vitro generation of helper cells. Thymocyte-macrophage cultures primed to KLH and assayed for helper cell activity as described in the text, except that in (A) the number of macrophages in the carrier-induction culture was varied, (B) the duration of culture before transfer to the assay culture was varied, and (C) the dose of carrier (KLH) was varied. Direct (•—•) and indirect (○—○) DNP-PFC ± SD of triplicate cultures; horizontal lines represent background direct and indirect DNP-PFC responses of spleen cell cultures not receiving helper cells.

### TABLE I
Composition of Thymocyte-Macrophage Cultures

| Marker* | Thymocytes | Macrophages § | Thymocyte-macrophage culture | Normal spleen |
|---------|------------|---------------|------------------------------|--------------|
| Latex | 0.04 ± 0.07 (n = 14) | 96.9 ± 1.2 (n = 36) | 22 ± 0.4 (n = 16) | 34 ± 1.8 (n = 17) |
| O* (fluorescence) | 96.8 ± 4.7 (n = 9) | 1.4 ± 0.3 (n = 7) | 90.4 ± 4.2 (n = 8) | 40.5 ± 5.3 (n = 17) |
| O* (cytotoxicity) | 99.9 ± 0.0 (n = 14) | ND | 96.6 ± 1.6 (n = 5) | 44.4 ± 2.2 (n = 10) |
| Sig | 0.02 ± 0.08 (n = 13) | 0.5 ± 0.2 (n = 9) | 0.09 ± 0.02 (n = 12) | 45.0 ± 5.4 (n = 17) |
| CRL* | 0.03 ± 0.03 (n = 11) | 0.3 ± 0.1 (n = 9) | 0.01 ± 0.01 (n = 10) | 37.3 ± 7.0 (n = 14) |
| Fc* | 0.02 ± 0.09 (n = 8) | 0.3 ± 0.3 (n = 7) | 0.04 ± 0.10 (n = 10) | 53.6 ± 8.3 (n = 14) |

* Markers latex*, percent cells phagocytosing latex beads; O*, percent cells with O (thy-1 2) isoeantigen, percent cells (bearing surface membrane immunoglobulin; CRL*, percent cells with receptors for complement (macrophages excluded); Fc*, percent cells with receptors for the Fc portion of immunoglobulin (macrophages excluded).
† Cortisone resistant, nylon wool purified thymocytes.
§ Adherent peritoneal exudate cells.
| Thymocyte-macrophage cultures | 1.5 × 10⁶ thymocytes and 5 × 10⁶ macrophages | Markers done after 4 days of co-culturing.
§ Percent positive cells ± SD; n = number of determinations.

recovered for culture by scraping with a rubber policeman, which, in our hands, results in as much as a 50% loss of viability. Unless removed by rapid filtration at 4°C over cotton wool in low ionic strength buffer (36), dead macrophages markedly depress thymocyte cell-culture recovery and the induction of helper cell function.

If glass beads that contain adherent macrophages are introduced directly into the thymocyte culture, the cell loss sustained by scraping and removing dead cells is eliminated. The disadvantage of this procedure is that the adherent population cannot be adequately characterized. Viability can be evaluated by tube incubation with trypan blue and examination of the beads under low-power microscopy. The number of cells added to the glass-bead column minus the number of nonadherent cells recovered served as an estimate of the number of macrophages bound to a given volume of beads. This method of enumeration, although it may represent a slight overestimation, gives a macrophage dose-response curve similar to that obtained with plastic dish-adherent macrophages (Fig. 1 A). Moreover, when membrane marker studies are done on the eluted, nonadherent cell fraction, there is virtually complete recovery of the nonphagocytic component of the PEC population (n = 4).
Table II
Response of Thymocyte-Macrophage Cultures to Mitogens*

| Mitogen  | Thymocytes§ | Macrophages¶ | Thymocyte-macro- | Normal spleen |
|----------|-------------|--------------|------------------|---------------|
| Media    | 0.8 ± 0.2   | 0.5 ± 0.1    | 1.6 ± 0.2        | 0.8 ± 0.1     |
| PHA      | 37.3 ± 3.1  | 6.6 ± 0.1    | 91.1 ± 6.3       | 75.8 ± 5.4    |
| Con A    | 10.6 ± 1.3  | 7.0 ± 0.2    | 149.6 ± 11.0     | 100.4 ± 7.1   |
| LPS      | 0.9 ± 0.2   | 0.5 ± 0.1    | 2.9 ± 0.3        | 51.0 ± 1.2    |

* Incorporation of tritiated thymidine expressed as cpm × 10⁻³ ± SD of triplicate cultures
† Values given for mitogens are optimums from dose-response curves (see text): PHA 0.5 μl/ml, Con A 1.0 μg/ml, LPS 10 μg/ml.
§ Cortisone resistant, nylon wool purified thymocytes (10⁶ cells/culture).
¶ Adherent peritoneal exudate cells (10⁶ cells/culture).
¶ Thymocyte culture containing 3% macrophages (10⁶ cells/culture).

FIG. 2. Dose response of in vitro generated helper cells. Varying numbers of cells (ATC_kLH) from thymocyte-macrophage cultures primed to KLH were transferred to hapten-primed spleen cell cultures with either 1 μg DNP-KLH (●—●), 5 μg DNP-FyG (△—△), or no hapten-carrier conjugate (□—□). After 4 days the direct DNP-PFC ± SD of triplicate cultures was determined.

The major nonphagocytic component of the PEC population is T cells. Pretreatment of the adherent cell population with anti-Thy-1.2 serum and complement increases the percentage of macrophages in the population. It does not affect the generation of helper cell function, making it unlikely that the peritoneal exudate T cell contributes significantly to the generation of helper cell function in this system.

**Cellular Requirements for the In Vitro Induction of Helper Cells.** Early in these studies, it became apparent that, under certain conditions, the thymocyte-macrophage culture system nonspecifically enhanced anti-DNP-PFC responses. This is illustrated in Fig. 2. The thymocyte-macrophage culture cells stimulated splenic anti-DNP-PFC responses when added to cultures without antigen or to those with hapten coupled to a noncross-reacting carrier (DNP-FyG), i.e., a carrier other than the one used to prime the helper cell culture. This is evident particularly when greater numbers of helper cells are transferred. An identical
phenomenon is observed when the thymocyte macrophage culture is primed with a different carrier (FyG).

This finding prompted us to more carefully examine the cellular and antigen requirements for optimal generation of specific helper cell activity (Table III). Thymocytes cultured without macrophages are ineffective as helper cells in this system. Macrophages cultured alone with or without carrier and transferred in numbers comparable to that found in the thymocyte-macrophage culture are only marginally enhancing. Cells from thymocyte-macrophage cultures primed with carrier display optimal stimulation of splenic anti-DNP-PFC responses only when transferred with hapten conjugated to the same carrier. Transfer with carrier (not conjugated to hapten) or without antigen is not as effective. Conversely, thymocyte-macrophage cultures generated in the absence of carrier do not stimulate an optimal anti-hapten response. It is readily apparent, however, that in this system, the dependence on the appropriate carrier hapten-carrier combination for optimal stimulation is a relative phenomenon. A signifi-
significant stimulation of anti-hapten responses is provided by cells from thymocyte-macrophage cultures regardless of the antigen supplied.

**Carrier Specificity of Helper Cells Generated In Vitro.** With knowledge of the nonspecific enhancement by thymocyte-macrophage cultures, we were able to design experiments to determine whether these cultures develop significant carrier specificity. Thymocyte-macrophage cultures were generated in the presence of KLH, FyG, or without antigen. Cultures were then tested for helper cell function by transfer to hapten-primed, spleen cell cultures with either the homologous DNP-carrier conjugate, DNP conjugated to a noncross-reacting carrier, or without antigen. The results are shown in Table IV. Under these conditions, despite considerable nonspecific enhancement of background anti-DNP-PFC responses, optimal enhancement is seen only in a homologous-carrier, hapten-carrier combination. In this sense the culture can be considered to develop carrier-specific helper cells (designated ATCKLH or ATCFyg in the tables).

**Nature of the Active Cell.** Table V indicates that the cell generated in thymocyte-macrophage culture which is active in enhancing carrier-specific and nonspecific anti-DNP-PFC responses is a T cell. Treatment of the culture with anti-Thy-1.2 serum and complement before transfer abolishes both the carrier-specific and nonspecific stimulation. That the abolition of enhancement by anti-Thy-1.2 serum treatment was not caused by trivial effects such as the addition of dead cells to the second culture or the generation of immune complexes was confirmed by mixing cells treated with anti-Thy-1.2 serum with cells treated...
Table V

| First culture* | Second (recipient) spleen cell culture$ |
|----------------|--------------------------------------|
|                | Anti-DNP-PFC response (direct DNP-PFC ± SD per culture) |
|                | Antigen added to second culture |
|                | None | DNP-KLH | DNP-FyG |
| $5 \times 10^7$ ATClH (anti-Thy-1 2 + C') | 86 ± 36 | 93 ± 27 | 86 ± 43 |
| $10 \times 10^7$ ATClH (anti-Thy-1 2 + C') | 82 ± 21 | 106 ± 33 | 96 ± 30 |
| $5 \times 10^7$ ATClH (NMS + C') | 134 ± 41 | 690 ± 139 | 192 ± 70 |
| $10 \times 10^7$ ATClH (NMS + C') | 155 ± 27 | 797 ± 42 | 200 ± 89 |
| $5 \times 10^8$ ATClH (anti-Thy-1 2 + C') plus $5 \times 10^7$ ATClH (NMS + C') | 89 ± 84 | 704 ± 46 | 179 ± 100 |
| No cells transferred | 79 ± 21 | 93 ± 41 | 69 ± 21 |
| $5 \times 10^8$ ATClH (mφ depleted) | 182 ± 51 | 725 ± 26 | 127 ± 29 |
| $10 \times 10^8$ ATClH (mφ depleted) | 165 ± 17 | 884 ± 119 | 273 ± 27 |
| $5 \times 10^8$ ATClH (no treatment) | 192 ± 21 | 811 ± 70 | 237 ± 37 |
| $10 \times 10^8$ ATClH (no treatment) | 189 ± 53 | 824 ± 55 | 268 ± 17 |
| $5 \times 10^8$ ATClH (mφ depleted) plus $5 \times 10^8$ ATClH (no treatment) | 141 ± 31 | 790 ± 57 | 258 ± 41 |
| No cells transferred | 69 ± 13 | 14 ± 16 | 38 ± 16 |

* Thymocyte macrophage culture cells primed with KLH (ATClH) were treated with anti-thy-1 2 sera or NMS and complement before transfer to spleen cell cultures. In the second set of experiments ATClH were depleted of macrophages by Sephadex G-10 column incubation before transfer.

With NMS. When macrophages are removed from the thymocyte-macrophage culture before transfer, no significant effect is seen (Table V). The active cell is therefore a T-cell.

Requirement for In Vitro T-Cell Proliferation in the Generation of Functional T-Helper Cells. In vivo models of T-helper cell generation require a critical 2- to 3-day period of T-cell proliferation in the presence of antigen. Once generated, the T-helper cell is functionally resistant to X-irradiation or anti-mitotic drugs (9, 14, 47–49). It was therefore of interest to examine whether cellular proliferation was required in the thymocyte-macrophage culture system before helper cell activity was manifest.

Thymocyte-macrophage cultures were treated with mitomycin C, either at the initiation of culture or after 4 days, just before transfer, and then tested for helper cell activity. Efficacy of mitomycin C treatment was assessed by the reduction in PHA and Con A responses to less than 10% of control values. Control cultures with mitomycin C-treated cells and either untreated ATC or DNP-Ficoll (which stimulates B cells directly) were used to monitor the potential responsiveness of the recipient spleen cell cultures, i.e., to insure that any inhibition found was not due to mitomycin C leaching from the cells. The results are shown in Table VI. Mitomycin C treatment of thymocyte-macrophage preparations at the onset of culture abolished the generation of helper cell function. If the culture is treated after 4 days, just before transfer, helper cell activity can be demonstrated. This is true for both the carrier-specific and nonspecific enhancing effects.

In separate experiments, thymocytes and macrophages were treated separately with mitomycin C, combined with untreated macrophages or thymocytes, respectively, and cultured for 4 days with carrier protein. The abolition of helper...
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TABLE VI

Mitomycin C Treatment of Macrophage-Thymocyte Cultures

| First culture* | Second (recipient) spleen cell culture† | Anti-DNP response (direct DNP-PFC per culture ± SD) | Cultured in the presence of |
|----------------|----------------------------------------|---------------------------------------------------|-----------------------------|
|                | Cells transferred                       | DNP-KLH                                          | DNP-FyG                     | DNP-Ficol, x 1@ ATC_KLH |
|                |                                        | 93 ± 27                                          | 79 ± 52                     | 93 ± 47                   | 2,143 ± 53 | 1,126 ± 51 |
| 5 × 10⁵ ATCₖloth mitomycin treated day 0 | 183 ± 43 | 855 ± 64 | 367 ± 67 | 2,178 ± 247 | 1,229 ± 98 |
| 5 × 10⁵ ATCₖloth mitomycin treated day 4 | 179 ± 97 | 1,075 ± 88 | 340 ± 63 | 2,782 ± 462 | 1,343 ± 99 |
| 5 × 10⁵ ATCₖloth treated – | 103 ± 10 | 41 ± 40 | 82 ± 74 | 2,616 ± 152 | 1,075 ± 88 |

* Thymocyte-macrophage cultures primed with KLH (ATCₖloth) were treated with mitomycin C at the initiation (day 0) or after 4 days of culture just before transfer to spleen cell cultures.
† 1.5 × 10⁷ spleen cells per culture were co-cultured with cells transferred from the first culture as indicated and either no additional antigen, 1 µg DNP-KLH, 5 µg DNP-FyG, 50 ng DNP-Ficol, or 5 × 10⁶ untreated ATC*KLH with 1 µg DNP-KLH. After 4 days, the direct DNP-PFC ± SD of triplicate cultures was measured.

Cell activity was seen only in cultures containing mitomycin C-treated thymocytes. Treatment of macrophages alone did not diminish the generation of helper cell function. Thymocyte proliferation in vitro before the manifestation of helper cell function is a critical inductive event in this system. These results correlate with in vivo requirements for the generation of helper cells from unprimed cells (9, 14).

Cooperative Interactions Between the In Vitro Generated T-Helper Cell and Hapten-Primed Spleen Cells. In vitro generated helper cell function is ultimately expressed as a stimulation of B-cell maturation and proliferation to DNP-specific PFC within the spleen cell cultures. This stimulation could require additional splenic T-cell cooperation (a T-T-B-cell interaction), be mediated through cooperative effects of splenic macrophages (a T-mΦ-B-cell interaction), or act directly on B cells (a T-B-cell interaction). To test this, recipient spleen cell cultures were treated with anti-Thy-1.2 serum and complement or depleted of macrophages. Effectiveness of depletion was assessed by membrane marker studies and, functionally, by the primary in vitro response to sheep erythrocytes, a response known to require T cells (50) and macrophages (51). Preservation of B-cell precursors in these populations was assessed, functionally, by the DNP-PFC response to the T-independent antigen, DNP-Ficol (52).

Results of T-cell depletion experiments are shown in Table VII. Splenic T cells are not absolutely required for the carrier-specific or nonspecific enhancement of the DNP-PFC response by T-helper cells generated in vitro.

Macrophage depletion of the responding spleen cell culture, sufficient to abrogate the primary in vitro response to SRBC, markedly blunts the carrier-specific enhancement, but has little or no effect on nonspecific enhancement. It is noteworthy that macrophages had to be removed from both the spleen cell population and the transferred thymocyte-macrophage culture in order for these differences to be observed (Table VIII). Apparently, the small number of macrophages transferred with the cultured thymocytes is sufficient to restore the carrier-specific stimulation of macrophage-depleted spleen cultures.
## Generation of T-Helper Cells in Vitro. I.

### Table VII

**Effect of In Vitro Generated Helper Cells on T-Depleted Spleen**

| First culture* | Second (recipient) spleen cell cultures§ | Anti-DNP response (direct DNP-PFC ± SD per culture) |
|----------------|----------------------------------------|----------------------------------------------------|
|                | Cells transferred                       | Anti-Thy 1 2 + C' treated spleen  | NMS + C' treated spleen |
|                | Antigen§                               |                                    |                       |
| 5 × 10⁸ ATC<sub>KLH</sub> | -                                      | 124 ± 10                              | 82 ± 10                |
| 5 × 10⁸ ATC<sub>KLH</sub> | DNP-KLH                                | 1,030 ± 93                            | 769 ± 49               |
| 5 × 10⁸ ATC<sub>FyG</sub> | DNP-FyG                                | 168 ± 16                              | 127 ± 21               |
| 5 × 10⁸ ATC<sub>FyG</sub> | -                                      | 113 ± 20                              | 106 ± 16               |
| 5 × 10⁸ ATC<sub>FyG</sub> | DNP-KLH                                | 179 ± 39                              | 151 ± 6                |
| 5 × 10⁸ ATC<sub>FyG</sub> | DNP-FyG                                | 780 ± 113                             | 673 ± 70               |
| 5 × 10⁸ ATC<sub>control</sub> | -                                      | 93 ± 41                               | 76 ± 16                |
| 5 × 10⁸ ATC<sub>control</sub> | DNP-KLH                                | 192 ± 31                              | 158 ± 12               |
| 5 × 10⁸ ATC<sub>control</sub> | DNP-FyG                                | 144 ± 37                              | 141 ± 6                |
| -              | -                                      | 52 ± 10                               | 38 ± 21                |
| -              | DNP-KLH                                | 58 ± 22                               | 45 ± 6                 |
| -              | DNP-FyG                                | 58 ± 42                               | 35 ± 12                |
| -              | DNP-Ficoll                             | 1,508 ± 41                            | 958 ± 68               |
| -              | SRBC                                   | 107 ± 44                              | 2,487 ± 44             |

* Thymocyte-macrophage culture cells primed with KLH (ATC<sub>KLH</sub>), FyG (ATC<sub>FyG</sub>), or without carrier (ATC<sub>control</sub>)

† Spleen cells treated with either anti-Thy 1 2 sera and complement or control AKR NMS and complement, dead cells removed, and cultured at 1 × 10⁷ cells per culture.

§ Antigens DNP-KLH, 1.0 μg; DNP-FyG, 5 μg; DNP-Ficoll, 50 ng, or 3 × 10⁸ SRBC.

I Direct (IgM) DNP-specific PFC per culture ± SD of triplicate cultures

¶ Primary in vitro response to unconjugated SRBC.

### Discussion

The present work was designed to establish the conditions for a reliable in vitro system of generating T-helper cells by using well-defined populations of cells. Cortisone-resistant thymocytes were selected because they represent a more mature population of thymus cells enriched for helper cell precursors (53). Nylon wool filtration of thymocytes and combination of these cells with adherent, peritoneal exudate macrophages effectively establish a viable thymocyte culture which can be primed with carrier protein to develop significant carrier-specific T-helper cell function (Tables III-V).

B-cell contamination in this culture system is negligible (Tables I and II). Minor nonphagocytic or nonthymocytic cellular components in this culture system are peritoneal exudate T cells. These cells are reportedly a good source of antigen-reactive T cells (44). Several lines of evidence mitigate against a major role for this cell in the system described. Anti-Thy-1.2 serum and complement treatment of the adherent cell population before combination with thymocytes does not affect the generation of helper cells. Pretreatment of the adherent cell population with mitomycin C does not affect the generation of helper cells, whereas pretreatment of the thymocytes or the combined culture (Table VI) abolished helper cell generation.

With the cell populations described in this communication, our results confirm the basic observations of Erb and Feldmann (29-31) that macrophages are
TABLE VIII
Effect of In Vitro Generated Helper Cells on Macrophage-Depleted Spleen

| First culture* | Second (recipient) spleen cell culture‡ | Anti-DNP response (direct DNP-PFC per culture ± SD) |
|----------------|-----------------------------------------|---------------------------------------------------|
|                |                                         | Antigen                                           |
|                |                                         | m° depleted spleen cells                          |
|                |                                         | Normal spleen cells                               |
| $5 \times 10^5$ m° depleted ATC_{KLH} | –                                       | 127 ± 26                                          |
| $5 \times 10^5$ m° depleted ATC_{KLH} | DNP-KLH                                 | 330 ± 45                                          |
| $5 \times 10^5$ m° depleted ATC_{KLH} | DNP-FYG                                  | 161 ± 57                                          |
| $10 \times 10^5$ m° depleted ATC_{KLH} | –                                       | 124 ± 64                                          |
| $10 \times 10^5$ m° depleted ATC_{KLH} | DNP-KLH                                 | 337 ± 26                                          |
| $10 \times 10^5$ m° depleted ATC_{KLH} | DNP-FYG                                  | 103 ± 63                                          |
| $5 \times 10^5$ ATC_{KLH} | –                                       | 151 ± 31                                          |
| $5 \times 10^5$ ATC_{KLH} | DNP-KLH                                 | 797 ± 84                                          |
| $5 \times 10^5$ ATC_{KLH} | DNP-FYG                                  | 254 ± 39                                          |
| $10 \times 10^5$ ATC_{KLH} | –                                       | 244 ± 16                                          |
| $10 \times 10^5$ ATC_{KLH} | DNP-KLH                                 | 491 ± 65                                          |
| $10 \times 10^5$ ATC_{KLH} | DNP-FYG                                  | 258 ± 66                                          |
| –              | DNP-KLH                                 | 69 ± 12                                           |
| –              | DNP-FYG                                  | 14 ± 16                                           |
| –              | DNP-FYG                                  | 38 ± 16                                           |
| –              | DNP-Ficoll                               | 1,067 ± 109                                       |
| –              | SRBC§                                    | 148 ± 30                                          |

* Thymocyte-macrophage culture cells primed to KLH are transferred directly to spleen cell cultures or macrophage depleted by Sephadex G-10 column incubation before transfer.

‡ Spleen cell cultures either macrophage depleted by Sephadex G-10 column incubation or cultured without depletion at 1.0 × 10^7 cells per culture in the presence of either 1 μg DNP-KLH, 5 μg DNP-FYG, 50 ng DNP-Ficoll, or 3 × 10^7 SRBC. After 4 days the direct DNP-PFC response ± SD of triplicate cultures was measured.

§ Primary in vitro PFC response to SRBC.

essential for inducing an active T-helper cell in vitro (Tables III and V). We extend these results to show that thymocyte proliferation is required for the induction of functional T-helper cells (Table VI), and that these cells display an antigen-specific as well as nonspecific capacity to stimulate B-cell responses to antigen (Tables III and IV).

The macrophage has been shown to be essential to a variety of T-cell responses, including antigen presentation (19–21), T-cell proliferation in the presence of antigen (22–23), mitogen responsiveness (24, Table II), and the mixed lymphocyte reaction (25). Macrophages improve thymocyte cell culture recovery and viability (26).

The antigen requirements (Table III) and specificity (Table IV) of this culture system suggest a more critical role for the macrophage than merely facilitating thymocyte integrity in vitro. The experiments with mitomycin C (Table VI) indicate that T-cell proliferation is required in this system before the expression of helper cell function. Based on our data, the predominant role of the macrophage in this system is in facilitating the proliferative expansion of thymocytes into functional carrier-reactive helper cells. While it is probable from available data (19, 21, 22, 30) that antigen acts in the induction of specific T-cell prolifera-
tion, we have not presented formal evidence that this is the mechanism in our system. It is possible that the thymocyte proliferation in this system is undirected and that antigen-bound macrophages serve to selectively enrich proliferating cells which are antigen specific. The requirement for T-cell proliferation distinguishes this mechanism of generating helper cells from another in vitro system described by Ben-Sasson et al. (27), where antigen-pulsed macrophages have been used to enrich antigen-specific T cells from mice presensitized in vivo. Their system enriches by selection rather than proliferation.

Analysis of the antigen requirements reveals that carrier-specific and nonspecific T-helper cell function is generated in vitro. Optimal stimulation of splenic DNP-PFC responses to a hapten-carrier conjugate is provided by thymocyte-macrophage cultures primed to the carrier. Stimulation is substantially less if the helper cell culture is not primed with carrier or is primed with a noncross-reacting carrier (Tables III and IV). In the sense that optimal enhancement is seen only in the appropriate combination of carrier in the helper cell culture and carrier-hapten conjugate in the assay culture, this system generates specific, carrier-reactive T-helper cells.

Enhancement of the anti-DNP-PFC response is also seen under conditions that cannot be considered antigen specific. Both carrier-primed and unprimed cells from helper cell cultures stimulate a small but reproducible enhancement of background splenic DNP-PFC responses in the absence of hapten-carrier conjugate (Tables III and IV). This nonspecific effect is particularly striking if greater numbers of activated T cells are used (Fig. 2). The nonspecific response is further enhanced in the second culture in the presence of hapten coupled to a noncross-reacting carrier (Fig. 2, Table IV). The marginal but consistent augmentation of the nonspecific response in the presence of an irrelevant hapten-carrier conjugate requires that macrophages be present in the assay culture (Table VIII), and its mechanism is further clarified in the companion paper.

Nonantigen-specific enhancing effects of activated T cells have been described in other systems. The activation of T cells to histocompatibility differences results in a cell active in enhancing B-cell responses to T-dependent antigens (1, 53-55). When looked for, nonspecific enhancing effects have even been demonstrated with in vivo generated, carrier-specific T-helper cells (18, 56). In their system, Erb and Feldmann do not describe an enhancing effect of cultured T cells that is independent of antigen (29-31). There are several possible reasons for this discrepancy. They used unprimed spleen cells as recipients of activated T cells, whereas we used hapten-primed spleen cells. In our system, the major differences between primed and unprimed spleen cells in culture are in the magnitude of the IgM-PFC response and the fact that IgG-PFC are not detected unless hapten-primed cells are used. It is noteworthy in this regard that the allogeneic effect, a T-cell derived nonspecific stimulation of B-cell responses to antigen, is generally not demonstrable in unprimed cells (1, 57). The low levels of background DNP-PFC in unprimed cells, the inherent variability between cultures, polyclonal B-cell activation from other sources in vitro such as FCS (55), and the method of enumerating DNP-specific PFC (hapten inhibition vs. subtraction technique), make precise demonstration of a nonspecific stimulatory effect on unprimed cells more difficult.
Macrophage depletion of recipient spleen cell cultures revealed a difference in the mechanism of the specific and nonspecific stimulation of B-cell responses by cultured T-helper cells. It is difficult to totally deplete a cell population of macrophages. In our hands, the most effective method without proportionate changes in T and B cells is Sephadex G-10 column incubation. Macrophage depletion of both the spleen cell cultures and the transferred thymocyte culture markedly blunts the carrier-specific effect of in vitro generated T-helper cells (Table VIII), findings consistent with those observed with in vivo generated T-helper cells (16). Although macrophages are not the active cell (Tables III and IV), the small number transferred with the primed thymocyte-culture cells is apparently sufficient to restore the T-\(\mu\)-B-cell interaction in macrophage-depleted spleen cell cultures. The nonspecific enhancement of background DNP-PFC due to cultured thymocytes does not require additional macrophage interaction and appears to act directly on B cells.

Summary

A sequential mouse cell culture system is described for the induction and assay of T-helper cells. Unprimed, cortisone-resistant, nylon wool-purified thymocytes cultured with adherent peritoneal exudate cells can be primed in vitro with soluble carrier protein to generate carrier-reactive helper cells. These cultured cells enhance the anti-hapten plaque-forming response of hapten-primed spleen cell cultures to hapten carrier conjugates. The culture conditions, cellular manipulations, and antigen requirements for the optimal induction of helper cells with these purified cell populations is presented. The active helper cell generated in this culture system is a thymus-derived cell which requires macrophages for its induction and must be proliferate in vitro before the manifestation of helper-cell function. Helper cells generated in vitro stimulate both carrier-specific and nonspecific enhancement of splenic anti-hapten responses. The carrier-specific and nonspecific enhancement can be distinguished by the requirement for antigen in the helper cell and spleen cell cultures, the dose of helper cells added to the spleen cell cultures, and by the requirement for additional splenic adherent accessory cell interactions.

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