We have recently reported that the nonspecific plant mitogens concanavalin A (Con A) and phytohemagglutinin can either enhance or suppress the in vitro primary humoral immune responses of mouse spleen cells to heterologous erythrocytes (1, 2). Whether enhancement or suppression of immune responses was observed depended upon the dose of mitogen employed and the time of treatment of cultures with mitogen.

When submitogenic doses of Con A were added to spleen cell cultures at initiation, enhancement of plaque-forming cell (PFC) responses was observed. If cultures were treated with mitogenic doses of Con A at initiation, PFC responses were profoundly suppressed; however, addition of mitogenic doses of Con A after 48 h incubation produced enhancement of PFC responses.

Furthermore, spleen cells obtained from mice injected intravenously 24 h previously with a low dose of Con A (6 µg) developed enhanced PFC responses when stimulated in vitro with sheep erythrocytes (SRBC). Enhancement of PFC responses was also observed when spleen cells from mice injected with a large dose of Con A (150 µg) were mixed in vitro with normal spleen cells in a ratio of 1:100. Cells mediating the enhancement of PFC responses in vitro after activation with Con A in vivo were thymus-derived and resistant to irradiation. The enhancement of PFC responses was observed only when small numbers of spleen cells from mice injected with the large dose of Con A were mixed with large numbers of normal spleen cells. However, when spleen cells from mice injected with the large dose of Con A were cultured alone or in a ratio of 1:1 with normal spleen cells, PFC responses were suppressed.

Related observations have recently been made by Dutton (3). When spleen cells activated in vitro by mitogenic doses of Con A were mixed with normal spleen cells,

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1 Abbreviations used in this paper: B cell, bone marrow-derived lymphocyte; Con A, concanavalin A; HBSS, Hanks' balanced salt solution; PFC, plaque-forming cell(s); SRBC, sheep erythrocytes; T cell, thymus-derived lymphocyte.
suppression of PFC responses resulted. Sjöberg et al. (4) have also recently reported suppression of PFC responses when large numbers of Con A-activated thymocytes were added to spleen cell cultures depleted of thymus-derived lymphocytes (T cells).

The present experiments were undertaken to further investigate the phenomenon of suppression of PFC responses by Con A-activated cells. The data clearly demonstrate that the previously described suppression of PFC responses is mediated by mitogen-activated lymphocytes. The suppressor lymphocyte population is thymus-derived, resistant to irradiation after activation, and generated in largest numbers in cultures of cells from peripheral lymphoid tissues.

Materials and Methods

Mice.—Male C57BL/6j mice, 2-4 mo old, (Jackson Laboratory, Bar Harbor, Maine) were maintained on acidified-chlorinated water and laboratory chow ad libitum.

Concanavalin A.—Twice recrystallized Con A (Nutritional Biochemicals Corp., Cleveland, Ohio) was stored at room temperature in saturated NaCl solution and sterilized by filtration through a Con A-saturated 0.45 μm Millipore membrane (Millipore Corp., Bedford, Mass.). Dilutions were prepared in Hanks’ balanced salt solution (HBSS), stored at 4°C, and used within 1 wk. Con A was iodinated by a modification of the method of Greenwood, Hunter, and Glover (5) previously described (2). Counts per minute (cpm) per nanogram of [125I]Con A were determined for solutions of known Con A content in a Packard gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Transfer of [125I]Con A with cultured spleen cells was quantified by reference to counts per minute per nanogram of Con A in these standard solutions.

Antigen.—SRBC (Grand Island Biological Co., Grand Island, N.Y.) were obtained in Alsever’s solution and washed three times with HBSS.

Cell Culture.—Preparation of spleen cell suspensions and techniques of cell culture have been previously described in detail (6). Briefly, single-cell suspensions of spleen, lymph node, and thymus were prepared by gentle teasing and sedimentation of tissue fragments and debris. Perithymic lymph nodes were marked by intraperitoneal injection of carbon 2 h before removal of thymus and were excluded from thymocyte cultures. Bone marrow was flushed from femoral and tibial cavities with HBSS through a 25 gauge needle. Cells were incubated in Eagle’s minimal essential medium supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.; lot E 21806) and containing penicillin, streptomycin, and nystatin. 1-ml cultures with 10^7 spleen cells were maintained at 37°C in 35-mm plastic Petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) on a continuously rocking platform in a humidified atmosphere of 10% CO₂, 7% O₂, and 83% N₂. Antigen-stimulated cultures contained 10⁶ SRBC; mitogen-stimulated cultures contained 750 ng of Con A. Cells from Con A-activated cultures and control cultures were washed twice with HBSS and resuspended in fresh culture medium at the desired cell density before mixing with antigen-stimulated cultures. Experimental groups contained three cultures that were pooled for PFC assay.

Hemolytic Plaque Assay.—IgM and IgG PFC responses in antigen-stimulated spleen cell cultures were assayed after 5 days’ incubation utilizing the slide modification of the Jerne localized hemolysis-in-gel technique previously described (6). PFC responses are expressed as PFC per culture. In those tables in which data from two to six experiments are presented, control responses are normalized to a PFC response of 1,000 IgM PFC per culture and experimental data are derived by multiplying observed responses by the normalization factor (2).

Enumeration of Cells and Viability Determination.—Nucleated cells were enumerated with
a model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). Cell viability was determined by trypan blue exclusion (2).

*Treatment of Spleen Cells with Anti-θ Serum and Complement.*—AKR anti-θ C3H/HeJ serum was prepared by the method of Raff (7) and stored at −20°C until use. Spleen cell suspensions were treated with anti-θ serum and noncytotoxic guinea pig serum as a source of complement as previously described (2) to specifically kill T cells.

*Cell Irradiation.*—Spleen cells were X-irradiated (2,000 R) with a General Electric Maximar 250 type III X-ray therapy unit (250 KVP, 15 mA) at 140 R/min through 0.5 mm Cu, 1.0 mm Al filters (General Electric Co., Schenectady, N. Y.). After irradiation, cells were washed with HBSS and resuspended in fresh medium at the desired cell density.

**RESULTS**

*Generation of Suppressor Cells with Concanavalin A.*—After incubation for 48 h with or without Con A, 750 ng/culture, spleen cells were harvested, washed twice with HBSS, and resuspended in fresh culture medium. Graded numbers of control or Con A-activated cells were then added to fresh SRBC-stimulated spleen cell cultures or to SRBC-stimulated spleen cell cultures established 48 h previously. PFC responses were determined in all cultures after 5 days of incubation with SRBC. Marked suppression of PFC responses was obtained by adding as few as 10^4 Con A-activated spleen cells to either fresh or 48-h antigen-stimulated cultures (Table I). Control spleen cells, not exposed to Con A, did not mediate suppression. Total viable cell recoveries in cultures to which control or Con A-activated spleen cells were added were comparable. Consequently, the suppressed PFC responses were not the result of nonspecific cytotoxicity of the Con A-activated cells on the normal spleen cells.

Suppression of PFC responses was consistently more profound when Con A-

| Suppressor cells* | IgM PFC culture† |
|-------------------|------------------|
|                   | Mixed at 0 h     | Mixed at 48 h |
| Treatment         | Cell dose × 10^6 |              |
|                   | Medium           | 1,000        | 1,000       |
| None              | 0.1              | 1,284        | 1,094       |
| None              | 0.5              | 826          | 1,196       |
| None              | 1.0              | 919          | 1,076       |
| Con A             | 0.1              | 164          | 388         |
| Con A             | 0.5              | 31           | 283         |
| Con A             | 1.0              | 16           | 150         |

* Spleen cells were incubated with or without Con A, 750 ng/culture, for 48 h, then washed twice and added to SRBC-stimulated cultures.

† Mean 5-day IgM PFC responses. Spleen cell cultures initially were established with 10^7 spleen cells and 10^7 SRBC. Graded numbers of control or Con A-activated cells or an equal volume of medium were added to these cultures at 0 h or after 48 h incubation with SRBC.
activated cells were mixed with fresh SRBC-stimulated spleen cells than when mixed with 48-h antigen-stimulated spleen cells. We have previously demonstrated, however, that 750 ng of Con A suppressed PFC responses when added to cultures at initiation, but that this dose of Con A enhanced PFC responses when added after 48 h incubation (1, 2). Subsequent experiments designed to characterize the suppressor cell population were therefore performed by adding Con A-activated cells to 48-h SRBC-stimulated cultures, thus permitting clear differentiation between the effect of Con A per se and the effect of Con A-activated cells.

**Transfer of Con A with Activated Spleen Cells.**—The amount of Con A transferred to SRBC-stimulated cultures with Con A-activated cells was measured directly by determination of cell-associated Con A after 48 h incubation of 10^7 spleen cells with 750 ng of [125I]Con A (Table II). Surface-associated and internalized Con A were differentiated by displacement of Con A from the lymphocyte membranes, either at culture initiation or 1 h before harvesting, with 60 mM methyl α-D-mannopyranoside, a specific competitive inhibitor of Con A binding (8). After 48 h incubation with Con A, cells were harvested, washed twice, counted, and assayed for radioactivity in a gamma scintillation spectrometer. Approximately 62 ng of [125I]Con A were associated with 10^6 spleen cells after 48 h incubation. The amount of cell-associated Con A at 48 h was reduced 93% by the addition of 60 mM methyl α-D-mannopyranoside to cultures at 0 h; 23% of cell-associated Con A could be displaced from cell membranes by methyl α-D-mannopyranoside added during the final hour of incubation.

**Effects of Suppressor Cells on Primary IgG and Secondary PFC Responses.**—Mice were immunized intraperitoneally with 10^8 SRBC; 12 days later spleen cells from immunized and normal mice were cultured with SRBC in the usual manner. After 48 h incubation, 10^6 Con A-activated or control spleen cells were added to the antigen-stimulated cultures. IgM and IgG PFC responses were assayed at 5 days (Table III). In vitro primary IgM and IgG PFC responses were suppressed by the Con A-activated cells. In vitro secondary IgM and IgG PFC responses were also both suppressed by Con A-activated cells although the degree of suppression of secondary PFC responses was less than that of primary PFC responses.
TABLE III
Suppression of In Vitro Primary IgG PFC Responses and Secondary IgM and IgG PFC Responses by Con A-Activated Spleen Cells

| 48 h SRBC-stimulated spleen cells | Suppressor cells* | PFC/culture† |
|----------------------------------|-------------------|--------------|
|                                  |                   | IgM | IgG |
| Primary response                 | Medium            | 5,125| 465 |
| Primary response                 | Control cells     | 5,750| 685 |
| Primary response                 | Con A-activated cells | 195 | 2   |
| Secondary response§              | Medium            | 2,450| 1,240|
| Secondary response               | Control cells     | 3,100| 1,210|
| Secondary response               | Con A-activated cells | 345 | 385 |

* $1 \times 10^6$ spleen cells incubated with or without Con A, 750 ng/culture for 48 h, or an equal volume of medium were added to antigen-stimulated cultures established 48 h previously with $10^7$ spleen cells and $10^7$ SRBC.

† PFC/culture on day 5. Data from a representative experiment.

§ Spleen cells obtained from mice injected intraperitoneally with $10^8$ SRBC 12 days before culture initiation.

Effect of Anti-θ Serum and Complement on Suppressor Cell Activity.—Derivation of the Con A-activated cells with suppressor activity was determined by reacting spleen cells with AKR anti-θ C3H/HeJ serum and complement to kill T lymphocytes. After treatment, cell suspensions were incubated in the usual manner with or without 750 ng of Con A for 48 h. Then, $10^6$ Con A-activated or control anti-θ-treated spleen cells were added to antigen-stimulated cultures initiated 48 h previously. Suppressor cell activity was not detected in cell populations previously depleted of T cells by anti-θ serum and complement (Table IV).

Effect of Irradiation on Suppressor Cell Function.—Sensitivity of suppressor cell function to irradiation was determined by exposing cultures of $10^7$ spleen cells stimulated with 750 ng of Con A to 2,000 R X-irradiation at three different times during the 48 h activation period (Table V). When cells were irradiated 1 h after addition of Con A, generation of suppressor cell activity was entirely abrogated. Conversely, suppressor cell activity of cells exposed to 2,000 R X-irradiation after the 48 h activation period was radioresistant and comparable to unirradiated Con A-activated cells. Partial radioresistance of suppressor activity was demonstrated in cells derived from cultures irradiated after 24 h incubation with Con A.

Tissue Sources of Con A-Activated Suppressor Cells.— Cultures of spleen, lymph node, thymus, and bone marrow were compared as sources of Con A-activated suppressor cell activity (Table VI). Cells from normal bone marrow inhibited PFC responses slightly although Con A-treated bone marrow cells did not. Thymocytes stimulated with Con A exhibited little if any suppressor activity. Suppressor cells were found in Con A-activated cultures of both spleen
TABLE IV
Effect of Anti-0 C3H Serum and Complement on Generation of Suppressor Cell Activity

| Suppressor cells* | Anti-0 serum and complement+ | IgM PFC/culture§ |
|-------------------|-------------------------------|------------------|
| Medium            | --                            | 1,000            |
| Control cells     | No                            | 958              |
| Con A-activated cells | No                      | 228              |
| Control cells     | Yes                           | 888              |
| Con A-activated cells | Yes                     | 1,197            |

* 1 X 10⁶ spleen cells incubated for 48 h with or without Con A, 750 ng/culture, or an equal volume of medium were added to antigen-stimulated cultures established 48 h previously with 10⁷ spleen cells and 10⁷ SRBC.

† Before incubation with or without Con A, spleen cells were treated with neat AKR anti-0 C3H serum (10 s cells/ml antiserum) for 30 min at 37°C. Cells were then washed and incubated for 30 min at 37°C with nontoxic guinea pig complement (1 ml of a 1:4 dilution/10⁵ cells) containing 0.1 mg/ml DNase.§ Day 5 PFC response.

TABLE V
Effect of Irradiation on Generation of Suppressor Cell Function

| Suppressor cells* | Irradiation+ | IgM PFC/culture§ |
|-------------------|--------------|------------------|
| Medium            | --           | 1,000            |
| Control cells     | None         | 1,031            |
| Control cells     | 1            | 960              |
| Control cells     | 24           | 1,057            |
| Control cells     | 48           | 956              |
| Con A-activated cells | None       | 174              |
| Con A-activated cells | 1           | 1,069            |
| Con A-activated cells | 24           | 561              |
| Con A-activated cells | 48           | 256              |

* 1 X 10⁶ spleen cells incubated for 48 h with or without Con A, 750 ng/culture, or an equal volume of medium were added to antigen-stimulated cultures established 48 h previously with 10⁷ spleen cells and 10⁷ SRBC.

† 2,000 R X-irradiation administered at times indicated during 48 h activation period of Con A-stimulated cells. Cells were washed and resuspended in fresh medium after irradiation, and cultured with or without Con A for the duration of the activation period. § Day 5 PFC response.

and lymph node cells. Interestingly, spleen cells were slightly more efficacious as suppressors on a per cell basis than lymph node cells. Viability of cells cultured from various lymphoid tissues was comparable and differences in cell survival did not account for the observed compartmentalization of suppressor cell activity.
TABLE VI

| Source        | Treatment | IgM PFC/culture |
|---------------|-----------|-----------------|
| Medium        |           | 1,000           |
| Spleen        | None      | 1,134           |
| Lymph node    | None      | 1,059           |
| Thymus        | None      | 923             |
| Bone marrow   | None      | 760             |
| Spleen        | Con A     | 208             |
| Lymph node    | Con A     | 338             |
| Thymus        | Con A     | 837             |
| Bone marrow   | Con A     | 1,046           |

*Lymphoid cells from the tissue indicated were incubated for 48 h with or without Con A, 750 ng/culture. 1 X 10^6 cells or an equal volume of medium were added to antigen-stimulated cultures established 48 h previously with 10^7 spleen cells and 10^7 SRBC.

† Day 5 PFC response.

DISCUSSION

The nonspecific plant mitogen Con A stimulated a population of T cells in mouse spleen that can either enhance or suppress in vitro primary IgM and IgG responses to heterologous erythrocytes (1, 2). The Con A-activated enhancing cells share important functional characteristics with antigen-activated ‘helper’ T cells (9). The generation of the enhancing activity of both Con A and antigen-activated cells is abrogated by treatment with anti-θ serum and complement. After activation by either mechanism, however, the enhancing function is resistant to X-irradiation.

The present studies have further characterized important parameters of the suppressive effects of Con A-activated cells on PFC responses to SRBC by normal spleen cells. We have shown that a population of T lymphocytes activated by Con A can suppress PFC responses by bone marrow-derived (B) cells in vitro. Generation of suppressor cell activity is sensitive to treatment with anti-θ serum and complement and to irradiation before activation with Con A; however, after activation, suppressor cell function is radioresistant. In this regard, suppressor cells are similar to antigen-specific helper T cells and Con A-activated enhancing cells. Con A-induced suppressor cells are active in suppressing both IgM and IgG PFC responses and actively suppress both primary and secondary immune responses in vitro. They are generated in greatest numbers in cultures of spleen and lymph node cells. Thymus is a relatively poor source of Con A-induced suppressor cells and we have been unable to generate them from bone marrow.

Sjöberg and colleagues (4) have also recently reported suppression of PFC
responses to SRBC by Con A-activated thymus cells. These investigators demonstrated that thymocytes activated by Con A for 24 h could reconstitute PFC responses in T cell-depleted spleen cell cultures stimulated with SRBC. Reconstitution of PFC responses was optimal when \(2 \times 10^6\) Con A-activated thymocytes were added to \(4 \times 10^6\) B cells. When the number of Con A-activated thymocytes was increased to \(4-8 \times 10^6\) cells, however, less than optimal reconstitution was observed. Thus, they concluded, small numbers of Con A-activated thymocytes enhanced PFC responses, whereas larger numbers were inhibitory. These data are concordant with our observations (2) that spleen cells from mice injected 24 h previously with 150 \(\mu\)g of Con A enhanced PFC responses when mixed with normal spleen cells in a ratio of 1:100. When the ratio of Con A-activated spleen cells to normal spleen cells was equal to or greater than 1:1, PFC responses were markedly suppressed.

Generation of suppressor cells with Con A has also been recently described by Dutton (3). He observed substantial inhibition of in vitro PFC responses to SRBC when 10% or more Con A-activated spleen cells were mixed with normal spleen cells at culture initiation. He also reported that suppressor cell activity was abrogated by irradiation of cells before Con A exposure. Those data are consistent with the data presented above although we have observed further that suppressor cell function becomes radioresistant after activation.

It is not, as yet, clear whether the Con A-activated cells that mediate suppression and enhancement of PFC responses are functionally two distinct types of cells. Alternatively, suppression and enhancement of PFC responses may represent opposing activities of the same thymus-derived regulator cell population, enhancing antibody synthesis under certain circumstances and suppressing it in others. Consistent with the latter hypothesis are the observations of Sjöberg et al. (4) and ourselves (2) that small numbers of Con A-activated cells enhance PFC responses whereas larger numbers are suppressive. This alternative is also supported by our demonstration that both suppressing and enhancing activities are sensitive to treatment with anti-\(\theta\) serum and complement and resistant to irradiation after activation.

The observation that Con A-activated cells can suppress PFC responses when added to SRBC-stimulated cultures after 48 h incubation is critically important for two reasons. First, we have previously shown that although a mitogenic dose of Con A suppressed PFC responses when added at culture initiation, this same dose of Con A enhanced PFC responses when added at 48 h (1, 2). Consequently, addition of Con A-activated suppressor cells to antigen-stimulated cultures at 48 h clearly differentiates between the opposing effects on PFC responses of Con A per se and of Con A-activated cells. Second, Pierce et al. (6, 10, 11) have demonstrated that after 48 h incubation the antigen and macrophage-dependent phases of in vitro immune responses are completed, and PFC responses in cultures of spleen cells cannot be inhibited by depletion of macrophages or by addition of antibody to SRBC or \(\mu\)-chain. Furthermore, by 48 h incubation the
exponential expansion of the specifically stimulated clone of antibody-synthesizing cells has begun (6). Consequently, although the mechanism remains to be elucidated, the experimental protocol utilized in these experiments indicates that Con A-activated suppressor cells can directly inhibit the differentiation of already "activated" B cells into PFC rather than interfering with antigen or macrophage-dependent events important in the activation of B cells.

The present experiments with Con A-induced suppressor cells support and clarify suggestions from several laboratories that T cells activated by antigen in vivo can suppress as well as enhance antibody production. Baum et al. (12) observed enhancement of primary antibody responses to keyhole limpet hemocyanin, but suppression of responses to SRBC after treatment of rats with doses of antilymphocyte globulin that produced lymphopenia. Similarly, Eidinger and Pross (13) observed that PFC responses to horse erythrocytes in irradiated mice reconstituted with spleen cells were inhibited by concomitant administration of large numbers of cortison-resistant thymocytes or thymus-derived cells. Okumura and Tada (14) reported enhancement of homocytotropic antibody responses to DNP-Ascarris in adult rats splenectomized or thymectomized 3–10 days before primary immunization. Investigators in several laboratories have reported that immune responses to thymus-independent antigens can be suppressed by T cells. Baker et al. (15) reported enhancement of direct PFC responses to type III pneumococcal polysaccharide after treatment of mice with antilymphocyte serum; Kerbel and Eidinger (16) demonstrated enhanced antibody responses to polyvinylpyrrolidone after adult thymectomy; and Möller and Michael (17) have shown that T cells suppress antibody responses by bone marrow to Escherichia coli endotoxin.

In summary, data from laboratories utilizing diverse experimental models support the concept that T lymphocytes can be specifically or nonspecifically activated to actively suppress antibody production by B cells. Responses to both thymus-dependent and thymus-independent antigens can be affected. Important questions regarding the specificity of T–B cell interaction in generation of antibody responses remain to be resolved. T cell activation by antigen and certain aspects of T–B cell interaction may be relatively specific, although perhaps less specific than the interaction of antigen with B cells (18). The efficacy of Con A-activated T cells in suppression of PFC responses by B cells, however, suggests that the T cell signal to B cells for suppression of antibody synthesis is relatively or entirely nonspecific. In this regard, the suppressor activity of activated T cells in a humoral immune response is similar to the activities of the soluble mediators secreted by T cells in delayed hypersensitivity reactions. Synthesis and secretion of these T cell products can be stimulated either specifically, by antigen, or nonspecifically, by T cell mitogens (19, 20). Once secreted, however, the effects of these mediators on the activities of other cells are probably antigen or mitogen-independent and nonspecifically expressed (20–22). Studies currently in progress should ascertain whether the T cell suppressor ac-
tivity in antibody responses represents the expression of another soluble product of activated T cells.

SUMMARY

A population of thymus-derived lymphocytes has been identified that, upon activation by the nonspecific plant mitogen concanavalin A, suppresses the development of plaque-forming cell responses in fresh or 48-h antigen-stimulated cultures of mouse spleen cells. Suppressor cells can inhibit both primary and secondary IgM and IgG responses in vitro. X-irradiation before activation of peripheral thymus-derived cells by concanavalin A abrogates generation of suppressor cells. After a 48 h activation period, however, the function of concanavalin A-activated suppressor cells is radioresistant. As yet uncertain is whether these suppressor cells are a population of cells distinct from thymus-derived "helper" cells. In certain important regards, the cells mediating these two opposing functions share similar characteristics; the effect observed may be determined by the circumstances of activation or the numbers of activated cells, and may consequently represent different functions of a single thymus-derived regulator cell population.

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