IN VITRO STUDIES OF THE RABBIT IMMUNE SYSTEM
V. Suppressor T Cells Activated by Concanavalin A Block the
Proliferation, not the Induction of Antierythrocyte Plaque-Forming
Cells*

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The rabbit immune system may be particularly useful for the study of cellular
control of antibody formation. A comparison of the responsiveness of rabbit and
mouse spleen cell cultures in vitro suggests that rabbit spleen populations may
contain greater numbers of cells with suppressive activity. This possibility was
suggested by the fact that dissociated spleen cells from normal unprimed mice of
several strains usually respond to erythrocyte antigens in vitro, whereas similar
cultures of rabbit spleen cells do not always respond and when responses do
occur they are usually much lower than those produced by mouse spleen cell
cultures (1–3). Further support of increased suppressor cell activity in the rabbit
comes from earlier studies in which we observed that fewer plaque-forming cells
(PFC) per 10^6 cultured cells were found when higher densities of normal rabbit
spleen cells were cultured as compared to cultures containing lower densities of
cells. These results were not explicable by differences in the viable cell recover-
ies at different cell densities. One interpretation of these results is that in-
creased numbers of suppressor cells in the cultures with high cell numbers
might be inhibiting the response. Subsequently, it was shown that addition of
the mitogen concanavalin A (Con A) profoundly inhibited the PFC response of
normal spleen cells cultured with sheep erythrocytes (SRBC). A suppressive
effect was also produced by rabbit peripheral blood lymphocytes (PBL) which
were stimulated with Con A and added to spleen cultures after Con A removal
(4). More recently we have developed methods of separating rabbit lymphocytes
into isolated populations exhibiting T or B functions (5). By applying these
methods to the study of the suppressive phenomena, we now report that Con A-
induced suppression in the rabbit is mediated by activated T cells. The target of

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Abbreviations used in this paper: ATS 82C, goat antirabbit thymocyte serum; BSS, balanced
salt solution; Con A, concanavalin A; FBS, fetal bovine serum; MAM, methyl-α-D-mannopyra-no-
side; 2-Me, 2-mercaptoethanol; NGS, normal goat serum; PBL, peripheral blood lymphocytes;
PFC, plaque-forming cells; PHA-P, phytohemagglutinin-P; SIRS, soluble immune response sup-
pressors.
the "suppressor cell" appears to be proliferating B cells because a suppressive effect can be obtained at a time when T help is no longer needed. Further, the evidence suggests that these suppressor cells are different from those which perform other T-cell functions.

Materials and Methods

**Animals and Antigens.** Young adult female rabbits obtained from a local supplier were used as normal cell donors or immunized with SRBC as previously described (1). 6-mo-old BDF1 mice (DBA/2 x C57 BL/6) were generously provided by P. Trefz and R. W. Dutton, Department of Biology, University of California at San Diego, La Jolla, Calif.

**Media and Sera.** Minimal essential medium (Eagle's) (MEM), RPMI 1640 (Microbiological Associates, Bethesda, Md. and Grand Island Biological Co., Grand Island, N. Y.), balanced salt solution (BSS), and fetal bovine serum (FBS) from International Scientific Industries Inc., Cary, Ill. (lot 721 B 301) or from Flow Laboratories, Inc., Rockville, Md. (lot 4055865) were employed as previously described (1).

**Mitogens and Antithymocyte Serum.** Con A was prepared by the affinity chromatography method of Agrawal and Goldstein (6) from Jack Bean Meal (General Biochemicals, Chagrin Falls, Ohio). The purified Con A produced a single band on polycrylamide gel electrophoresis, had an extinction coefficient within 5% of published values and was as active in stimulating mitogenesis as commercially available Con A (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.; generously provided by R. W. Dutton). The Con A used in culture was sterilized by filtration and stored in frozen aliquots of 0.50 ml. Frozen aliquots were thawed and diluted in medium or BSS immediately before use.

Phytohemagglutinin-P (PHA-P) was obtained from Difco Laboratories, Detroit, Mich., and reconstituted with medium or BSS to the recommended vol (5 ml/vial), stored at 4°C, and used within 10 days. This stock solution which contained 15 mg PHA-P/ml was additionally diluted and added as described in the text.

Goat antirabbit thymocyte serum (ATS 82C) was the antiserum described and characterized previously. Briefly, goat 82 was immunized in multiple sites with 10^8 thymocytes emulsified in complete Freund's adjuvant and bled at intervals. The 36d bleed was adsorbed with rabbit erythrocytes, sterilized by filtration, and stored frozen in 1.0 ml aliquots. The normal goat serum (NGS) used in these experiments was preimmune serum from the same goat which had been processed in the same manner as ATS 82C.

**PFC Cultures and Plaque Assays.** Spleen cells from normal rabbits or from rabbits immunized 6-12 days previously with ~10^8 SRBC intravenously were incubated at a density of ~5 x 10^6 cells/ml/dish for 1-6 days as described (1). Two to four replicate dishes per condition were pooled and assayed on various days of culture by the microscope slide modification (3) of the Jerne-Nordin technique (7). Responses are expressed as PFC per culture or per 10^6 spleen cells originally put into culture. Viable cell recoveries were determined by hemocytometer counts with trypan blue and are expressed as a percentage of the viable cells originally cultured (1). Some experiments had 5 x 10^{-5} M 2-mercaptoethanol (2-Me) included in the medium. The presence of 2-Me has made no

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2 Redelman, D., C. B. Scott, H. W. Sheppard, Jr., and S. Sell. 1976. In vitro studies of the rabbit immune system. II. Functional characterization of rabbit T and B populations separated by adherence to nylon wool or lysis with anti-thymocyte serum and complement. Cell. Immunol. In press.

3 Sheppard, Jr., H. W., D. Redelman, and S. Sell. 1976. In vitro studies of the rabbit immune system. IV. Differential mitogen responses of isolated T and B cells. Cell. Immunol. In press.

4 The ATS 82C used in these studies has the following properties: (a) With C, it kills 100% of thymocytes, 40-60% of spleen, and virtually all nylon-passed spleen cells; (b) PFC arising from immunization in vivo or in vitro are not lysed; (c) treatment of primed spleen cells before antigen addition abrogates the in vitro anti-SRBC response which can be reconstituted with nylon-passed spleen cells from the same animal; and (d) ATS plus C survivors are not stimulated to transform by Con A or PHA but do respond to stimulation by anti-immunoglobulin.
difference in the experiments in which Con A was directly added to culture. The effect, if any, of 2-Me on the suppressive activity of prestimulated cells or their supernates has not been examined.

Preparation of Prestimulated Lymphocytes and Supernates. PBL were obtained from defibrinated blood sedimented through gelatin as previously described (8). The resulting cell suspensions were highly enriched for leukocytes which were 90–95% small lymphocytes by light microscopy. PBL were incubated in tubes or dishes alone or with stimulatory amounts of Con A for 24–48 h. The optimal dose of Con A varied according to the source and proportion of serum used to supplement the medium. Stimulation of blastogenesis was determined by morphology and/or the incorporation of [14C]thymidine or [3H]-labeled iododeoxuridine ([3H]UdR). Spleen cells were stimulated by culturing ~5 × 10^6 cells/ml/dish alone or with 10 μg Con A for 24 h. After incubation, the cells were washed, resuspended, counted, and the stated numbers added to spleen cell cultures.

Several procedures were employed to insure that Con A was not transferred with prestimulated cells in sufficient quantity to mediate suppression. Con A was labeled with [125I] to determine the amount adhering to cells after washing in medium or BSS, or BSS containing 5 mg/ml methyl-α-D-mannopyranoside (MAM-BSS). Comparable amounts of Con A were then added to cultures of normal spleen cells and found to have no detectable suppressive effect. In other experiments, Con A was added to control cultures just before harvesting and washing. Control cells treated with Con A in this manner were essentially indistinguishable from fresh cells or cells which were never exposed to Con A. There was no evidence from any of these procedures that the suppressive effects of stimulated cells were mediated by transferred Con A.

Supernates from Con A-stimulated cells were prepared by incubating PBL collected 2 days before sacrifice alone or with Con A for 24 h, which was sufficient to stimulate a large proportion of the cells irreversibly (9). After washing with MAM-BSS the PBL were incubated an additional 24 h and the supernate from the second incubation was then added to spleen cultures from the same rabbit. The 48-h control or stimulated PBL were added to other cultures to compare the effectiveness of the cells and their supernates.

Mitogen-Stimulated Proliferative Cultures. The stimulation of mouse and rabbit spleen cells by Con A was determined in microcultures. Briefly, spleen cells were suspended in RPMI 1640 supplemented with 5% FBS and 0.20 ml containing ~5 × 10^6 cells was added to each well in Falcon Microtest II plates (BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md.). 10 μl of BSS or medium containing various amounts of Con A was added to six replicate wells for each condition to be tested. Cells were incubated in humidified gas-tight boxes flushed with 4% CO₂-air and supplemented daily with 10 μl of a mixture of RPMI:FBS:100 times vitamins, 15:7.5:1, respectively. In the experiments reported below, cultures were pulsed at 48 h with 0.10 μCi [3H]UdR (New England Nuclear, Boston, Mass.; catalogue no. NEX-072) in 10 μl and harvested 24 h later using a multiple sample harvester (Otto Hiller Co., Madison, Wis.). The data shown are the geometric means ± SE determined from the log₂ transformed counts per minute incorporated in six replicate cultures. Other pulse times and/or cell densities have been examined without significant differences being observed in the optimal stimulatory dose of mitogen required.

Results

Inhibition of the Anti-SRBC PFC Response by Con A. Optimal Con A suppression of the PFC response in mouse spleen cell cultures has been found to occur with Con A doses equivalent to the optimal mitogenic dose (10, 11). Con A is a potent stimulant for rabbit cells but the dose response is slightly different from that for the mouse cells. Fig. 1 depicts dose response profiles of rabbit and mouse spleen cells incubated under the same conditions in microculture. The peak proliferative response of rabbit cells occurred at a higher dose and extended over a wider range of Con A concentrations. The PFC response of normal rabbit spleen cells was then examined with varying doses of Con A and mitogen concentrations of 1–10 μg/ml were found to inhibit the PFC response completely. Complete inhibition was observed when Con A was added at 0 or 24 h.

We next examined the effects of Con A on the in vitro PFC response of spleen
cells from SRBC-primed rabbits. The results of experiments performed with spleen cells from seven primed rabbits are depicted in Table I. Con A doses of 0.3-10 μg/ml inhibited the PFC response from 45 to 96%. The observed inhibition was not caused by gross toxic effects as the viable cell recoveries from cultures with sufficient Con A (3-5 μg/ml) to inhibit the PFC response by 83-94% were greater than in cultures without Con A. The optimal mitogenic and PFC inhibitory doses were similar as can be seen by comparing the data in Fig. 1 and Table I. Most of our studies of Con A-induced inhibition have been performed with Con A doses of 5 or 10 μg/ml.

Routinely, only direct (IgM) anti-SRBC PFC are enumerated in our system as we have found that spleen cells from both normal rabbits and those primed with a single immunization of ~10^6 SRBC produce a marginal, indirect (IgG) PFC response in vitro. It was possible that Con A addition caused an accelerated "switchover" from IgM to IgG PFC with a concommitant apparent loss of responsiveness. This was examined by determining the effect of Con A on both direct and indirect PFC in spleen cell cultures from normal and primed rabbits. The addition of 5 or 10 μg of Con A to normal cell cultures at initiation completely abrogated both direct and indirect PFC on day 6. Primed cell cultures received 10 μg Con A at 48 h which caused a gradual decline in direct PFC through day 6. On day 6, both direct and indirect PFC were reduced ~90%, relative to untreated control cultures which had received no Con A. Thus, the data did not support an IgM to IgG shift caused by Con A.
The Effects of Con A on the In Vitro Anti-SRBC PFC Response*

| Con A‡ | % PFC§ (replicate) | Viable cell|| (replicate) recovery |
|-------|--------------------|------------------|
| µg/culture | % | % |
| 0 | 100 (28) | 100 (42) |
| 0.30 | 54.26 ± 5.44 (4) | 95.8 (6) |
| 1.00 | 18.82 ± 1.84 (4) | 153.2 (6) |
| 3.00 | 16.70 ± 2.68 (7) | 133.7 (12) |
| 5.00 | 6.13 ± 1.94 (6) | 117.3 (14) |
| 10.0 | 3.81 ± 1.02 (12) | 78.9 (15) |

* Spleen cells from seven rabbits primed 6–8 days previously with ~10⁶ SRBC intravenously were cultured alone or with ~10⁶ SRBC for 4 or 5 days and assayed as described in the text. 0.1 ml BSS containing the indicated amount of Con A was added at the initiation of culture. Spleen cells from individual rabbits were all cultured without Con A and one or more doses of Con A.

§ The number of antigen-specific (stimulated less background) PFC was determined for cultures with and without Con A from each rabbit. Percent of response was calculated as follows: % response = [SRBC-specific PFC in cultures with Con A – mean SRBC-specific PFC in cultures without Con A] / mean SRBC-specific PFC in cultures without Con A] × 100%.

|| The number shown is the mean from the indicated number of replicate sets of cultures. The viable cell recoveries were determined for each rabbit from all cultures with the indicated Con A doses. These values were then normalized relative to the untreated cells from the same rabbit.

PHA, which is another potent mitogen for rabbit lymphocytes, has also been examined for its suppressive activity. Table II reports the PFC responses and viable cell recoveries from primed spleen cells with PHA or Con A added. The higher doses of PHA were toxic but 3.75 µg PHA-P (0.10 ml of 1:400) induced blast transformation, increased the viable cell recoveries, and profoundly reduced the PFC response.

Kinetics of Con A-Induced Suppression. Con A has been added to normal spleen cultures at 0 and 24 h and found to be totally suppressive over a range from 1 to 10 µg/ml/culture as stated above. More extensive studies of the response after Con A addition at various times have been carried out in cultures of spleen cells from SRBC-primed rabbits. The 4-day PFC responses of primed spleen cells after adding 5 µg Con A at the initiation of culture, or 1–3 days later, are summarized in Table III. Addition at 0 or 24 h inhibited ~96% of the PFC response but there was markedly less inhibition if Con A was added at later times. Although this suggests that Con A acted on some early phase of the response, we knew from earlier kinetic studies that the in vitro PFC response of primed cells proceeds very rapidly with a detectable response as early as 24 h (1). A more detailed examination was performed in the experiment depicted in Fig. 2. Primed spleen cells were cultured alone or with ~10⁶ SRBC added at the start of incubation or with 10 µg Con A added at 24-h intervals. The number of PFC...
### Table II

The Effect of Con A and PHA on the Anti-SRBC PFC Response in Cultures of Primed Spleen Cells*

| 5 × 10⁴ SRBC | Additions at 0 h† | Viable %§ recovered | PFC/10⁶ cultured cells ± SE |
|--------------|------------------|---------------------|----------------------------|
|              | -                | 483 ± 23.8          | 32.5                       |
|              | +                | 8,500 ± 447         | 30.0                       |
|              | +                | 7,840 ± 479         | 37.0                       |
|              | - 0.10 ml with 15.0 μg PHA-P | 285 ± 16.2 | 12.5                       |
|              | +                | 166 ± 24.0          | 10.5                       |
|              | +                | 55.0 ± 13.1         | 19.0                       |
|              | - 0.10 ml with 7.50 μg PHA-P | 66.0 ± 6.7 | 19.0                       |
|              | +                | 244 ± 12.1          | 17.5                       |
|              | +                | 210 ± 30.0          | 24.5                       |
|              | - 0.10 ml with 3.75 μg PHA-P | 416 ± 36.6 | 45.0                       |
|              | +                | 367 ± 35.9          | 35.0                       |
|              | +                | 417 ± 3.33          | 41.7                       |
|              | - 0.10 ml with 5 μg Con A | 73.6 ± 8.9 | 24.0                       |
|              | +                | 81.3 ± 22.8         | 27.0                       |
|              | +                | 71.7 ± 10.4         | 41.5                       |

* Spleen cells from a rabbit primed 6 days previously with 2.3 × 10⁶ SRBC intravenously were suspended to 5 × 10⁶ cells/ml and 1.0 ml added to duplicate dishes for each condition. Pairs of dishes were cultured alone or with SRBC as indicated.
† Difco PHA-P, control number 58400, was reconstituted with 5.0 ml BSS and further diluted, i.e. 1:100 with 15.0 μg/0.10 ml, 1:200 with 7.50 μg/0.10 ml, and 1:400 with 3.75 μg/0.10 ml. Con A was also diluted in BSS to contain 5.0 μg/0.10 ml.
§ Duplicate dishes were harvested and pooled for assay after 4 days incubation. Arithmetic means and standard errors were calculated for sets of duplicate dishes from the four to eight slides enumerated. Viable cell recoveries were determined by trypan blue exclusion.

present on each day after treatment indicate that Con A addition stopped the logarithmic increase of the PFC response and maintained it near a constant level throughout the succeeding period. We previously determined that the major portion of the PFC increase during the logarithmic phase is due to the T-independent proliferation of PFC or their immediate precursors but that some T-dependent "recruitment" may occur as late as 72 h. If one also assumes that the PFC generated by in vitro stimulation with SRBC have a lifetime comparable to those arising in vivo (the open circles, Fig. 2), then some recruitment may occur after Con A addition. Therefore, the target of Con A inhibition appears to be the late proliferative phase of the PFC response and not the early stage of induction or recruitment.

**The Cell Type Affected by Con A.** Other investigators have shown that Con A-stimulated suppression in mouse spleen cells is caused by T-cell activation (10, 12). Our findings that Con A could exert effects late in the rabbit system

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5 Redelman, D., D. J. Anderson, and S. Sell. 1976. In vitro studies of the rabbit immune system. III. T cell requirement for initiation but not maintenance of anti-SRBC plaque-forming cells. Cell. Immunol. In press.
TABLE III

Suppression of the In Vitro Anti-SRBC PFC Response by 5 μg Con A Added at Various Times*

| 5 μg Con A added at: | Mean PFC/10^6 cultured cells | Control resp. | Viable cells recovered |
|---------------------|------------------------------|---------------|-----------------------|
| h                   |                              |               |                       |
| None                | 9262                         | 100           | 40.4 ± 4.05           |
| 0                   | 362                          | 3.91          | 70.0 ± 3.06           |
| 24                  | 404                          | 4.36          | 48.8 ± 4.42           |
| 48                  | 1747                         | 18.9          | 46.7 ± 3.94           |
| 72                  | 4457                         | 48.1          | 30.7 ± 7.22           |

*Spleen cells from a rabbit primed 6 days before with 2.3 x 10^8 SRBC intravenously were cultured at a density of ~5 x 10^6 cells/ml/dish alone or with ~10^6 SRBC. 5μg Con A was added at the indicated times.

† Three to six pairs of replicate dishes were separately assayed after 4 days as described in the text. Mean numbers of PFC in antigen-stimulated cultures less background are shown. Mean background for all conditions was 275 PFC/10^6 cultured cells. Viable cells were determined by trypan blue and are expressed as a percentage of the original number.

made it necessary to establish whether Con A acted directly on the antigen-responding cells or through an intermediate cell. We have found that both Con A-stimulated spleen cells and PBL can suppress the PFC response. Table IV summarizes an experiment in which spleen cells were cultured alone or with Con A for 24 h, washed extensively with MAM-BSS to remove Con A, and added to other spleen cultures from the same animal. The addition of control spleen cells incubated without Con A slightly elevated the response but Con A prestimulated cells reduced the PFC response on day 5 by as much as 50%. The effects of prestimulated PBL are shown in Tables V and VI. The PFC response of 5 x 10^6 normal spleen cells was highly sensitive to suppression by as few as 10^5 Con A-prestimulated PBL as shown in Table V. In an experiment not shown, as few as 10^4 prestimulated PBL suppressed the response ~50%. In another experiment not reported here, PBL incubated with Con A at 39-40°C remained viable but were only poorly stimulated and these cells were not suppressive. Primed spleen cells were also sensitive to suppression by prestimulated PBL but higher numbers of PBL were required and the resulting reduction of the response was not so great (Table VI). The kinetics of the response after PBL addition is depicted in Fig. 3. In this experiment PBL were collected 1 day before and immediately before sacrifice of a primed rabbit. Spleen cells were cultured in the normal manner and PBL incubated 48 h alone or with Con A were added 24 or 48 h after the start of the spleen cell incubation. Prestimulated PBL rapidly exerted a suppressive effect, as did the direct Con A addition depicted in Fig. 2, but the suppression by Con A-prestimulated PBL was overcome with additional incubation and the number of PFC increased. The final reduction in the response on days 4 and 5 was similar to that reported in Tables IV and VI.
Suppression of the in vitro anti-SRBC PFC response by 10 μg Con A added at various times. Spleen cells from a rabbit primed 6 days before with 2.3 × 10⁶ SRBC intravenously were cultured at a density of 5 × 10⁶ cells/ml/dish alone or with −10⁶ SRBC. The indicated cultures received 10 μg Con A at the specified times after initiation. Two or more pairs of dishes for each condition were assayed daily as described in the text. The values plotted are the means of two or more pairs of cultures assayed separately.

The data reported above indicate that cells prestimulated with Con A could exert a suppressive effect. This was not due to Con A carried over with the prestimulated cells for the following reasons. First, the amount of Con A associated with prestimulated cells as determined by studies with ¹²⁵I-labeled Con A was insufficient to cause the observed effects. Second, cells washed with BSS alone or with BSS containing 5 mg/ml MAM were equally suppressive. If cell-associated Con A were responsible, then washing the cells with the Con A-binding saccharide should have reduced the suppression. Third, cells incubated with Con A for 24 h, washed, incubated an additional 24 h in fresh medium, and washed again before addition retained suppressive activity (Table VII). Thus, a slow release of Con A after cell addition would not explain the suppression. Fourth, normal cells incubated 24 h alone but with Con A added just before harvesting and washing were not effective as inhibitors. Finally, PBL incubated 24 h with Con A but under conditions which were not favorable to blast transformation were also not active as suppressors. Therefore, we concluded that Con A did not act directly on the cells involved in the PFC response but exerted its effects via an activated population with suppressor capacity.

Suppression by Supernates from Prestimulated PBL. The effectiveness of very small numbers of prestimulated PBL implied that these cells might be acting via a secreted soluble factor. Our attempts to remove Con A from cell supernates were not entirely satisfactory so prestimulated cells were reincubated in fresh medium and the second supernate was employed. This was based on previous observations that cells incubated for 24 h with Con A were irreversibly stimulated (9). Table VII shows that both the stimulated PBL and their
TABLE IV
Suppression of the Anti-SRBC PFC Response in Primed Spleen Cells by Con A
Prestimulated Spleen Cells

| Additions to 5 $\times$ 10$^6$ spleen cells† | SRBC* | Day 5§ |
|---------------------------------------------|-------|-------|
|                                             | PFC/10$^6$ cultured cells | Viable cells recovered |
| None                                        | $709 \pm 37.0$ | 18.00 |
| +                                           | $11,837 \pm 481$ | 39.50 |
| +                                           | $11,558 \pm 385$ | 36.50 |
| $1.4 \times 10^6$ normal spleen cells at 27 h| $1,853 \pm 163$ | 36.50 |
| +                                           | $12,680 \pm 436$ | 39.50 |
| +                                           | $15,836 \pm 640$ | 49.00 |
| $1.4 \times 10^6$ Con A-stimulated spleen cells at 27 h| $653 \pm 28.1$ | 33.00 |
| +                                           | $5,408 \pm 265$ | 41.50 |
| +                                           | $5,499 \pm 180$ | 29.50 |
| $4.6 \times 10^4$ Con A-stimulated spleen cells at 27 h| $2,260 \pm 230$ | 33.50 |
| +                                           | $5,156 \pm 208$ | 43.00 |
| +                                           | $6,648 \pm 312$ | 40.50 |
| $2.3 \times 10^4$ Con A-stimulated spleen cells at 27 h| $342 \pm 38.2$ | 19.00 |
| +                                           | $10,980 \pm 339$ | 20.50 |
| +                                           | $9,500 \pm 356$ | 33.00 |
| $9.2 \times 10^4$ Con A-stimulated spleen cells at 27 h| $437 \pm 67.5$ | 22.50 |
| +                                           | $1,411 \pm 80.5$ | 24.50 |
| +                                           | $2,120 \pm 165$ | 25.00 |

* Spleen cells from a rabbit primed 6 days previously with $\sim 10^6$ SRBC i.v. were incubated at a density of $5 \times 10^6$/ml/dish alone or with $\sim 5 \times 10^5$ SRBC added as indicated at the start of culture.
† Dishes containing $5 \times 10^6$ spleen cells from the same rabbit were incubated 24 h alone or with $10 \mu g$ Con A. After 24 h, $10 \mu g$ Con A was added to the unstimulated cells, the normal and stimulated cells were harvested separately, washed with BSS, with MAM-BSS, with BSS again, and finally resuspended in medium. The indicated numbers of cells were then added in 0.20 ml to spleen cells cultures.
§ Replicate dishes were pooled and assayed on day 5. Mean PFC/10$^6$ spleen cells originally cultured were determined as described in the text. Viable cell recoveries were calculated as described in the text based on the cells originally put into culture. The number of cells added at 27 h was not included in the PFC or cell recovery calculations.
ǁ $10 \mu g$ Con A in 0.10 ml BSS was added at 27 h.

Supernate suppressed the PFC response on day 4. The active component of the supernate was relatively stable as it was effective after freezing and thawing when tested in later experiments not shown. As yet, the factor(s) in the supernate have not been characterized.

The Suppressor Cell is a T Cell. The suppressor cell possessed presumptive
### Table V

*The Effect of PBL on the Anti-SRBC PFC Response in Cultures of Normal Spleen Cells*

| Antigen added at 0 h | Additions† | Viable % recovered | PFC/culture§ ± SE |
|----------------------|------------|--------------------|--------------------|
| No SRBC              | All conditions∥ | 26.0 ± 3.72        | 32.5 ± 3.71        |
| 2.5 × 10⁷ SRBC       | No PBI¶    | 32.4 ± 8.30        | 208 ± 6.42         |
| 2.5 × 10⁷ SRBC       | Unstimulated | 39.4               | 211 ± 17.2         |
| 2.5 × 10⁷ SRBC       | PBL        | 54.4               | 193 ± 10.6         |
| 2.5 × 10⁷ SRBC       | Con A stimulated | 40.9                | 28.0 ± 4.78        |
| 2.5 × 10⁷ SRBC       | PBL        | 38.0               | 88.0 ± 9.29        |

* Spleen cells from a normal rabbit were suspended at a concentration of 4.5 × 10⁶ cells/ml and 1.0 ml added to triplicate dishes for each condition.
† PBL were obtained 1 day preceding sacrifice and cultured with or without Con A for 29 h. Cultured PBL were washed in BSS with 5 mg/ml MAM and resuspended in BSS with 1 × 10⁶ or 1 × 10⁵ cells/0.10 ml.
§ Mean PFC/culture ± SE were calculated from the four to six slides enumerated for each set of cultures assayed on day 6.
∥ Triplicate sets of cultures with or without PBL with no SRBC added all produced similar numbers of PFC. The values shown are the arithmetic mean ± SE of these eight sets of cultures.
¶ Two sets of cultures were incubated with SRBC and 0.10 ml BSS without PBL.

### Table VI

*Suppression of the In Vitro α-SRBC Response in Primed Spleen Cells by Con A-Prestimulated PBL*

| 5 × 10⁶ Primed spleen cells/ml/dish | PFC/10⁶ cultured cells ± SE on day 6 |
|------------------------------------|--------------------------------------|
| No SRBC, all conditions            | 585 ± 45.0                           |
| 5 × 10⁶ SRBC added at 0 h plus no PBL | 7,218 ± 367                          |
| PBL cultured 24 h*                 | 8,381 ± 662                          |
| 6 × 10⁶                              | 6,442 ± 443                          |
| 1 × 10⁶                              | 6,764 ± 641                          |
| 1 × 10⁵                              | 3,544 ± 195                          |
| 1 × 10⁴                              | 3,723 ± 195                          |
| 1 × 10³                              | 6,332 ± 428                          |

* PBL washed with BSS and added in 0.10 ml BSS at the initiation of culture.

T-cell properties as both PHA and Con A are mitogens for rabbit T cells. The type of cell having suppressive activity was tested directly by determining the effect of specific ATS upon suppression. For the experiment shown in Fig. 4, ATS or NGS was added to primed spleen cultures 42 h after antigen, which we have demonstrated is too late to abrogate the PFC response, but which does deplete T cells. Con A was added 8 h later and the response measured after this
Fig. 3. Suppression of the in vitro anti-SRBC PFC response by Con A-stimulated PBL added at 24 or 48 h. Spleen cells from a rabbit primed 6 days previously with $2.3 \times 10^6$ SRBC intravenously were incubated alone or with $1 \times 10^5$ SRBC added at the initiation of culture. PBL were collected from the same rabbit both 24 h and immediately before excising the spleen and were cultured alone or with a stimulatory dose of Con A. After 48 h incubation the PBL were washed with MAM-BSS, resuspended in BSS, and $1 \times 10^6$ normal or stimulated PBL were added to spleen cell cultures. Unstimulated PBL exerted no significant effects on the PFC response. The closed circles represent the mean PFC response of cultures with no PBL, or $1 \times 10^5$ normal PBL added. The open circles give the means of all cultures without SRBC added (background response).

Late ATS addition had little effect on the ongoing PFC response but it markedly reduced the suppressive effect of Con A. Direct ATS addition without C does not eliminate all T cells immediately, so another experiment was performed in which cells were incubated for $48$ h with SRBC, treated with ATS plus C or NGS plus C, and recultured with or without Con A (Fig. 5). The cells surviving ATS plus C or NGS plus C treatment continued to produce increasing numbers of PFC at similar rates from days 3-5 as we have previously reported. However, the results given in Fig. 5 show that Con A markedly reduced the response in the NGS plus C controls but had virtually no effect on the ATS plus C-treated cells. The difference in the magnitudes of the PFC responses by NGS plus C and ATS plus C survivors in the absence of Con A probably represents abrogation of T-dependent recruitment between 48 and 72 h. The left-hand panel of Fig. 5 shows that 10 µg Con A added to NGS plus C-treated cells stopped the PFC increase rapidly as seen above in Fig. 2. Conversely, ATS plus C survivors were not affected by Con A addition (Fig. 5, right panel). Therefore, we conclude that Con A stimulates a T cell which then acts to suppress the PFC response.
Table VII

Suppression of the In Vitro Anti-SRBC Response of Primed Spleen Cells by Con A-Stimulated PBL and PBL Supernates

| Condition                                      | PFC/10^6 cultured cells on Day 4 |
|------------------------------------------------|----------------------------------|
| All conditions, no SRBC antigen added          | 300                              |
| Antigen (5 x 10^4 SRBC) added at 0 h plus     |                                  |
| 1 x 10^6 PBL cultured 48 h*                   |                                  |
| Unstimulated                                   | 5,382                            |
| Con A stimulated                               | 2,360                            |
| 0.50 ml PBL supernate from:*                   |                                  |
| Unstimulated                                   | 4,523                            |
| Con A stimulated                               | 2,522                            |

* PBL were cultured for 24 h alone or with Con A, washed with MAM-BSS, and resuspended in fresh medium for an additional 24 h. PBL were resuspended in BSS and 0.20 ml added at the initiation of spleen cultures. The 24-48 h PBL supernates were added to other cultures at the same time.

during a stage in which T-helper cells are no longer required for maintenance of the PFC response.

Discussion

The present studies identify and characterize cells which are capable of suppressing the in vitro humoral immune response of rabbit spleen cells. The mitogens Con A and PHA, which primarily stimulate T cells, inhibit the in vitro anti-SRBC PFC response of normal and primed rabbits, presumably via activation of suppressor T cells. Mitogen-induced inhibition is neither associated with cell death nor with direct action of the mitogen upon the responding cells; prestimulated cells added to ongoing cultures are also effective suppressors. Since a ATS specific for T cells abrogates mitogen-induced suppression, the suppressor population is identified as T cells. The ultimate target of the T-suppressor cell appears to be the proliferating stage of PFC as Con A can act at a time when T cells are no longer required to maintain the response in vitro. Soluble factors secreted after Con A activation are also inhibitory and may mediate the suppression alone or in combination with other cellular effects.

Although our previous studies demonstrated that the cellular interactions required for an in vitro antibody response by rabbit lymphoid cells are similar to those of the mouse, the present data reveal three interesting differences. First, rabbit spleen cells tolerate and are stimulated to transform by doses of Con A which are supraoptimal for mouse cells. Second, Con A addition to antigen-stimulated spleen cells can suppress the PFC response considerably later in the culture period. Third, a Con A-induced stimulatory effect on the antibody response is not demonstrable as it is for the tested mouse strains. Con A suppresses the mouse spleen PFC response if added early in culture but can stimulate the response if added later (10, 13) or at lower doses (11). However, Con A is effective in stopping the proliferation phase of the PFC response when added to rabbit spleen cell cultures as late as 72 h after antigen. A stimulatory
Fig. 4. Inhibition of Con A-mediated suppression by the addition of ATS. Spleen cells from a primed rabbit were cultured alone or with SRBC added at initiation. After 42 h, 10 μl of NGS or ATS was added as shown. 8 h later, 10 μg Con A was added to half of the cultures containing NGS or ATS. The plotted values represent the mean SRBC-specific (SRBC stimulated less background) PFC response for each condition. Ag Spec., antigen specific.

The results of the current study suggest that the ultimate target of the Con A-induced rabbit suppressor cell is the proliferating PFC or its immediate precursor B cell. The PFC response by mouse cells can also be initiated in the presence of suppressor cells but can be abrogated during the phase of rapid PFC increase by activated suppressor cells implying a similar direct B-cell target (16, 17). On the other hand, it has been reported that "soluble immune response suppressors" (SIRS) from prestimulated cells acts via macrophages (17). This factor is inactive in the presence of 2-Me even though suppression by direct Con A addition is unaffected by 2-Me (C. W. Pierce, personal communication). We do not yet know whether supernates from Con A-stimulated rabbit cells are rendered inactive by 2-Me or affect macrophages. However, the effects of direct Con A addition on the rabbit PFC response are unaltered by the presence of 2-Me. Therefore, it is likely that soluble factors are not solely responsible for the inhibition produced...
Abrogation of Con A-induced suppression by treatment with ATS plus C before Con A addition. Primed spleen cells were incubated alone or with SRBC for 48 h. Cells were then removed from culture and treated with 10 μl NGS or ATS and 50 μl guinea pig C per 10<sup>7</sup> cells originally present. After washing, the surviving cells were recultured in fresh medium and new dishes with or without 10 μg Con A. The left panel presents the PFC in cells surviving NGS plus C immediately after the treatment and on succeeding days. The right panel presents the same data for cells surviving ATS plus C. All values represent geometric means of two pairs of replicate dishes assayed separately for PFC.

by suppressor cells. A direct effect of suppressor T cells on target B cells must be considered as a possible mechanism.

Other studies strongly suggest that B cells are the target of suppressor cells as the responses to the T-independent antigens DNP-Ficoll and DNP-lipopolysaccharide can be suppressed by Con A addition (17). This type of suppressor effect has been put forth to explain antigenic competition (18, 19). On the other hand, Roszman found that the late addition of Con A to rabbit spleen cells was less effective in reducing the response than early addition and concluded that Con A was acting only on the early phase(s) of the response (20). A similar interpretation could be placed on our studies if only the final number of PFC on day 4 or 5 is examined. However, a more complete analysis of the kinetics of the response indicates that the suppressive effect of Con A is still active much later in the response than is true for the mouse. Therefore, either the rabbit has relatively more or longer-lived suppressor cells, or the B-cell targets are more sensitive to suppression. The data are more consistent with the former explanation.

Several findings in this and other studies can be more easily explained by proposing that a direct relationship exists between the number of proliferating B-target cells and the number of suppressor cells required to produce a given degree of inhibition. Cells prestimulated by Con A can suppress both primary and secondary responses but more activated cells are required for the latter (Tables IV-VI). Primed cell cultures may contain at least 10-fold more antigen-specific cells than do normal spleen cells (1). Appendix cells suppress in vitro responses by primed spleen cells even though the appendix cells are not stimulated before addition. However, very large numbers of these appendix cells are required to produce an effect (21). This concept of a suppressor cell-B-cell
relationship in control of PFC proliferation also explains why direct Con A addition is more efficacious than prestimulated suppressor cells. After direct Con A addition more suppressor cells could conceivably be generated as the response progresses, thus keeping pace with the addition of recruited cells. However, when prestimulated cells are added, the number of suppressors is relatively fixed while more specific B cells arise via "recruitment" of precursors. This effect can be seen in Fig. 3, which depicts cultures that eventually "escaped" suppression after Con A-prestimulated PBL were added. This hypothesis may also explain why mouse spleen cultures are more difficult to suppress as the time after antigen stimulation increases (10, 11). The studies of SIRS by Pierce and co-workers have shown it to be effective only if added relatively early during culture (16, 17). However, it is possible that late suppression could be obtained with higher doses of the suppressive factor if the problems of nonspecific toxicity could be overcome (16).

The nature of the effect of activation upon suppressor cells is not well understood, but the activation of suppressor cells by Con A is very rapid. Our results, in addition to those of Rich and Pierce (16) and Roszman (20) demonstrate that suppressor cells become active before the induction of DNA synthesis by Con A, i.e., during the G1 phase of blastogenesis. The data shown in Fig. 2 indicate that Con A added to antigen-stimulated cultures affects the PFC response before Con A-induced DNA synthesis could have occurred. Rich and Pierce (16) showed that SIRS was generated within 12 h of Con A stimulation, and Roszman (20) reported that only a 2 h exposure to Con A was sufficient to induce a suppressive effect. Therefore, suppressor cells become activated during the G1 phase of Con A-induced transformation, i.e. while protein and RNA synthesis are being initiated, but before DNA synthesis and mitosis occurs (22). SIRS have been shown to be sensitive to peptic enzymes, and Ambrose (23) has reported a suppressive factor (antibody inhibitory material) sensitive to RNase degradation. These are properties consistent with a factor produced early in the cell cycle after activation.

If such soluble factors are the major mediators of suppression then they must be somehow inactivated or exhausted in vivo.Suppressor factors could be inherently labile. However, we have found that active supernates survive freezing and thawing, and Pierce has reported SIRS to be relatively stable (16, 17). A possible catabolic degradation is suggested by the finding that SIRS activity is lower in supernates of 48-h prestimulated cells than in 12-h stimulated supernates (16). The possibility that suppressive factors may become attached or internalized within their target cells will be examined in future experiments. Such a fate most likely occurs in vivo, otherwise suppressive factors could accumulate sufficiently to shut down all immune responses.

Although the term suppressor cell has achieved wide usage, it is not clear that suppressor cells are actually a separate functional population or merely one possible activity of a T-cell population which may perform other functions under different circumstances. The results of Scavulli and Dutton (24) show that suppressor activity is not simply "too much help." In addition, we did not find that low numbers of Con A-activated suppressor cells enhanced the response (Table IV). Thus help and suppression are probably mediated by separate
Suppressor cell populations. Suppressor cells are shorter lived in the mouse (25) and the rabbit (26) and apparently decline faster after thymectomy than do helper cells. The elegant studies of Cantor and Boyse (27) indicate that different antigens exist on helper and suppressor populations. The conclusion derived from the above data is that help and suppression are mediated by different populations which may be distinct cell lines or differentiative states of the same cell line.

Summary

The late B-cell proliferative phase of the in vitro antibody response by rabbit spleen cells is highly susceptible to suppression by activated T cells. The in vitro antiserum plaque-forming cell (PFC) response by spleen cells from normal or primed rabbits can be suppressed by adding concanavalin A (Con A), Con A-prestimulated peripheral blood or spleen lymphocytes, or supernates from Con A-prestimulated peripheral blood lymphocytes. The suppression is not mediated by a direct interaction of Con A with responding cells as shown by the effectiveness of prestimulated cells. Primed spleen cultures remain sensitive to Con A suppression as late as 72 h after initiation, and the addition of Con A after 24-72 h rapidly stops the increase in the number of PFC. T cells are required for Con A addition to be effective but the suppression can be induced at a time when T-helper cells are no longer necessary. Further, the suppressive effect of Con A addition is abrogated by specific antisera to rabbit T cells. We propose that Con A activates suppressor T cells which then exert their effects on proliferating PFC or their immediate precursor B cells. The early inductive or recruitment phase of the response is probably not blocked by suppressor cells. Also, there is an apparent relationship between the number of proliferating B cells and the number of suppressor cells required. Finally, the difficulties in inducing a stimulatory effect by Con A and the prolonged period that Con A addition is suppressive suggests that the rabbit has relatively more and/or longer-lived suppressor cells than the mouse and may be a particularly useful species for studying suppressive phenomena and their mechanisms.

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