T lymphocytes can be activated specifically with antigens (e.g. 1, 2) or nonspecifically with mitogens such as concanavalin A (Con A)\(^1\) (3) to express helper and suppressor activities. Such activities have been convincingly shown to be mediated by distinct subpopulations of T cells. Not only can Con A-activated helper and suppressor cells be physically separated (4), they are also shown to bear different Ly surface markers (5). Helper cells are Ly\(^1\)+ while suppressor cells are Ly\(^23\)+. In addition, suppressor cells, but not helper cells, are also found to carry an I-region determinant that maps to the I-J subregion of the major histocompatibility complex (6, 7). More recently, Tada et al. (8, 9) showed that extracts from high dose keyhole limpet hemocyanin-primed thymocytes contained both suppressive and enhancing T-cell factors which carry antigens of the I-J and I-A subregions, respectively.

T-cell mitogens such as Con A have been conveniently used to study T-cell activation and functional expression. DNA synthesis as measured by labeled thymidine incorporation is usually used as an indication of mitogenesis. By using anti-Ly antisera, Jandinski et al. (10) suggested that both helper and suppressor cells responded to Con A by DNA synthesis and proliferation. A substantial amount of thymidine incorporation was observed when either Ly\(^1\)+ or Ly\(^23\)+ cell populations were cultured with Con A. However, a number of recent observations by Niederhuber et al. (11) and Frelinger et al. (12) suggested further complications. It was first shown that T cells that responded to Con A were Ia\(^+\) (11). Once activated, these "promotor" cells were capable of recruiting other bystander Ia\(^-\) cells to proliferate. Treatment of spleen cells with anti-Ia antisera and complement (C\(^+\)) prevented the generation of Con A suppressor cells. Con A-activated suppressor cells, however, were not eliminated by anti-Ia plus C\(^+\). The promotor cells were later demonstrated to bear I-J determinants (12). The relationship between proliferation and the expression of helper and/or suppressor activities is thus unclear. The possibility that the proliferating cells may represent a subset of T cells distinct from those that functionally help or suppress has not been ruled out.

We have previously used a Ficoll velocity sedimentation gradient to separate physically the Con A-activated helper and the suppressor cells (4). We have shown that the cell population that mediates helper activity contains the small, slowly sedimenting cells. These are also the cells that incorporate very little, if
any, "H thymidine in the 20- to 40-h period. It would appear that the generation of Con A helper activity does not require DNA synthesis or cell division.Suppressor cells, on the other hand, cosediment with actively DNA-synthesizing blast cells towards the bottom of the gradients. In this report we have continued to use the cell separation technique to identify the Ly phenotypes of these helper and suppressor cells. We have also used the technique in combination with DNA synthesis inhibitors to study the relationship between proliferation and the generation of helper and/or suppressor activities. We show that the slowly sedimenting helper cells are Ly1+ and the fast sedimenting suppressor cells are Ly23+. We also double thymidine label cells in the pre- and post-gradient cultures and show that cells that are in the same pool as the helper cells incorporate neither label. Furthermore, neither mitomycin C treatment nor irradiation of cells before they are cultured with Con A blocks the generation of helper activity. More surprisingly we find that the generation of suppressor cells was also not inhibited. This suggests the possibility that the cells that incorporate the bulk of labeled thymidine upon activation by Con A may be distinct from either the helper or the suppressor cells. The function and significance of the proliferating cells have yet to be clarified.

Materials and Methods

Mice. C57BL/6J female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. (C57BL/6 female × DBA/2 male)F1 hybrid mice were bred in our own colony. 10- to 14-wk-old male and female BDF1 mice were used. C57BL/6J mice were usually between 6- and 8-wk old.

Chemicals and Reagents. Twice crystallized Con A (lot 111) was obtained from Miles Laboratories, Inc., Kankakee, Ill. Ficoll 400 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. RPMI 1640 from the Core Facilities at UCSF supplemented with 5% fetal calf serum (FCS) was used as culture medium. The same lot of FCS was used throughout these experiments and together with colcimid in Hanks' balanced salt solution (HBSS), were obtained from Grand Island Biological Co., Grand Island, N. Y. Mitomycin C was obtained from ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio. "H-thymidine (sp act 20 Ci/mmol) was from New England Nuclear, Boston, Mass. [14C]thymidine (sp act 57 mCi/mmol) was from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. Whatman GF/A glass fiber filter paper (2.4 cm) was obtained from W & R Balston Ltd., England.

Cultures. The pregradient cultures were carried out in Falcon No. 2005 polypropylene tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Mouse spleen suspensions were cultured at 5 × 10^6 cells/ml in RPMI by a modification of the method of Mishell and Dutton (13). Con A at a final concentration of 2 μg/ml was added at time 0. Cells were cultured for 40 h before being subjected to separation procedures. The second culture period was done on Falcon No. 3040 Microtest II culture plates. 0.05 ml of fractionated cells plus 0.05 ml of normal spleen cells were used per well. Six replicates were used. One drop of 0.04% sheep erythrocyte (SRBC) suspension from a 22 gauge needle was added to each well. The response was assayed by the direct hemolytic plaque assay 4 days later.

Irradiation. Cells were irradiated with 1,000 R from a Cobalt-60 Irradiator at the Salk Institute, San Diego, Calif.

Mitomycin C Treatment. Cells were first resuspended to 2-5 × 10^6 cells/ml in HBSS. Mitomycin C, at a final concentration of 25 μg/ml was added. The cells were then incubated for 20 min at 37°C. After treatment, cells were washed three times with HBSS plus 5% FCS before being cultured.

Removal of Dead Cells and Ficoll Velocity Sedimentation Gradient. The procedures for cell separation have previously been reported (4).

Anti-Ly Treatment. Cells were treated with anti-Ly antisera after gradient separation. Anti-Ly-1.2 and anti-Ly-2.2 antisera were the generous gifts of Doctors H. Cantor, (Department of Medicine, Harvard Medical School, Sidney Farber Cancer Center, Boston, Mass.)  and E. A. Boyse,
Fig. 1. Thymidine incorporation profile and functional differences of fractionated cells. Normal spleen cells from a 40 h culture with Con A (final concentration 2 \( \mu \)g/ml) were separated on a 5-20% Ficoll gradient and fractionated (Materials and Methods). (A) \(^{3}H\)Tdr uptake was present during the last 20 h of culture. The gradient fractions were assayed for \(^{3}H\)Tdr uptake. (\(\triangle\)) \(^{3}H\) counts in the absence of Con A; (\(\triangle\)) \(^{3}H\) counts in the presence of Con A. (B) The gradient fractions were made into four pools and graded number of cells from each pool were titrated into 10\(^{6}\) normal spleen cells plus sheep erythrocytes (SRBC). Plaque assay to SRBC was determined 4 days later. Each point represents the arithmetic mean of six replicates. Pool I, fractions 1-13; pool II, 14-17; pool III, 18-23; and pool IV, 24-34. PFC, plaque-forming cells; UF, unfractionated.

**Thymidine Labeling.** 5 \( \times \) 10\(^{6}\) cells/ml were cultured in tubes. For tritiated thymidine (\(^{3}H\)TdR), 1/\(\mu\)Ci (diluted to a sp act of 0.24 Ci/mmol) in 0.1 ml of HBSS was added to each tube. For \(^{14}C\)TdR, 0.2 \(\mu\)Ci (sp act 57 mCi/mmol) in 0.1 ml HBSS per tube was used. At appropriate times, cells were collected over GF/A glass filters, TCA precipitated, and washed with cold 95% ethanol. The filters were collected, dried, and counted in a liquid scintillation counter.

**Results**

**Cell Separation on the Gradient.** The protocols used in the following experiments are essentially the same as reported in a previous publication (4) and here in the Materials and Methods. Here we present, in Fig. 1, another set of experiments that shows that Con A-induced helper activity for the humoral response to SRBC is confined to a population of small, slowly sedimenting and nonthymidine incorporating cells. Pool I (Fig. 1B) corresponds to cells that remain at the top of the 5-20% Ficoll velocity sedimentation gradient. As can be seen from Fig. 1A, these cells incorporated very little of the \(^{3}H\)thymidine which was present during the last 20 h of the 40-h culture period. Nevertheless,
these cells enhanced the response of a constant number of normal fresh cells. On the other hand, cells that incorporated the bulk of the label cosedimented with cells that exhibited suppressor activity to the bottom of the gradient in pool IV. The possibility that pool I cells may have gone through one round of DNA synthesis and stopped was checked. Thymidine was added as early as 6 h in culture, but there was still no indication of any [3H]thymidine incorporation by pool I cells (data not shown).

Ly Phenotypes of Helper and Suppressor Cells. Having shown that pool I contained helper cells and pool IV suppressors we investigated the Ly phenotype of the cells mediating these activities. In this experiment, pool I cells, pool IV cells, and unfractionated cells were each divided into three portions corresponding to untreated control, αLy-1.2, and αLy-2.2-treated populations. Cells recovered from these treatments were resuspended to the same volume as the untreated control and were titrated into 10⁶ normal spleen cells plus SRBC. Plaque-forming cells (PFC) were determined 4 days later.

Double-Labeling Experiment. As indicated above, pool I cells, when assayed
HARLEY Y. TSE AND RICHARD W. DUTTON

DOUBLE-LABELING EXP.

Spleen Cells + ConA

\[ +^{3}H-TdR \]
\[ 36 \text{ h} \]
\[ \text{dead cells removed} \]

Ficoll Gradient

\[ \text{I} \]
\[ + \text{ConA} \]
\[ - \text{ConA} \]

\[ \text{IV} \]
\[ + \text{ConA} \]
\[ - \text{ConA} \]

\[ +^{14}C-TdR \]
\[ 24 \text{ h} \]

Assay for TdR Incorporation

Fig. 3. Protocol for double-labeling pre- and post-gradient cultures.

**TABLE I**

Results of Double-Labeling Experiment (Fig. 3)

<table>
<thead>
<tr>
<th>Pools</th>
<th>Con A in post-gradient cultures</th>
<th>(^{3}H) cpm</th>
<th>(^{14}C) cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>618</td>
<td>1,100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>677</td>
<td>1,504</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>27,287</td>
<td>20,846</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20,794</td>
<td>19,003</td>
</tr>
</tbody>
</table>

Counts were corrected for overlapping between the \(^{3}H\) and \(^{14}C\) channels of the scintillation counter.

for thymidine incorporation were found to be unlabeled; yet these cells exhibited the helper activity. The possibility that pool I cells might have proliferated in the postgradient culture was investigated next. The protocol is shown in Fig. 3 and the results in Table I. We use \([^{3}H]TdR\) to label cells that proliferate in the pregradient cultures and \([^{14}C]TdR\) to label cells in the postgradient cultures. As expected, pool I cells incorporated very little \([^{3}H]TdR\) in the first culture period. Nor did these cells incorporate any \([^{14}C]TdR\) in the second culture period after gradient fractionation. On the other hand, pool IV cells carried most of the \(^{3}H\) label after the first culture period and the cells continued to proliferate in the postgradient cultures regardless of whether Con A is present or not.

**Inhibition of DNA Synthesis.** To confirm that DNA synthesis is not required for the induction of helper activity, we used mitomycin C or irradiation to inhibit DNA synthesis and showed that these agents have no effect on the generation of helper cells. Thus cells were irradiated or were treated with mitomycin C before culture with Con A. \([^{3}H]TdR\) was added after 20 h. At 40 h the cultured cells were gradient fractionated and assayed for the various activities. In one set of experiments, the 34 1-ml fractions were not pooled, but were...
individually assayed for TdR incorporation. As shown in Fig. 4, in which cells were treated with mitomycin C, the amount of label in the bottom fractions was very much reduced although not to the background level. This may represent an incomplete blockade of DNA synthesis. In another set of experiments, fractionated cells were pooled and titrated into normal cells. In addition, aliquots of unfractionated cells from each experiment were assayed for TdR incorporation. Figs. 5 and 6 show the results of irradiation and mitomycin C experiments, respectively. In either case, the treatment had no effect on the generation of helper activity, a third piece of evidence showing that Con A helper cells can be activated without concomitant DNA synthesis. However, the observation that there were still suppressor activity present in pool IV cells was somewhat unexpected. Experiments in which 0.1 μg/ml of colcimid was included in the culture or in which the hot-pulse (\[3H\]TdR) technique was used (15) showed similar results (data not shown). In previous studies of the effect of irradiation on the generation of Con A suppression (16, 17) it had been found that Con A suppressor cells are radiosensitive if irradiated before induction. It is interesting to observe that the (irradiated) unfractionated cells in Fig. 5 are not in the least suppressive and those (mitomycin treated) in Fig. 6 are much less suppressive than untreated cells. Thus it appears that in this more sensitive system we are able to pick up suppressor activity which may otherwise be left undetected in an unfractionated suspension of spleen cells.

Discussion

It is clear that Con A-activated helper and suppressor cells belong to two physically distinct subclasses. The former are the small cells while the latter are more rapidly sedimenting blast cells. In a single experiment, treatment of the small cells from pool I of the Ficoll gradient with anti-Ly-1 antisera reduced two-
Fig. 5. Effect of irradiation on the generation of Con A helper and suppressor activities. Normal spleen cells were irradiated with 1,000 R before being cultured with Con A. Experimental procedures were the same as in Fig. 1 (B). DNA synthesis as measured by \(^*\text{H}^\text{TdR}\) incorporation by both treated and untreated populations were also determined. PFC, plaque-forming cells; UF, unfractionated.

Fig. 6. Effect of mitomycin C treatment on the generation of Con A helper and suppressor activities. Normal spleen cells were treated with mitomycin C before being cultured with Con A. Experimental procedures were the same as in Fig. 5. PFC, plaque-forming cells; UF, unfractionated.

thirds of the helper activity. Conversely, anti-Ly-23 antisera abrogated the suppressor activity of pool IV cells. Because of the limited availability of Ly antisera, the reasons for the failure to eliminate completely the helper activity
with anti-Ly-1 has not been determined. With this reservation, we conclude that the helper and suppressor cells examined here have the same Ly phenotypes as those already characterized in other systems (5, 9, 10).

Spleen cells normally respond to Con A by massive blast transformation and proliferation. It is customarily believed that these processes reflect the activation and differentiation of functional subsets of T cells. Our experiments, however, clearly define a population of Con A-activated helper cells distinct from the proliferating cells. First, Con A helper activity for the humoral response to SRBC is always associated with a population of small, gradient fractionated pool I cells. These cells do not incorporate any labeled thymidine up to 3 days even in the continuous presence of Con A. Furthermore, irradiation and mitomycin C treatment, although they block most of the thymidine incorporation activity, are unable to inhibit the generation of these helper cells. The possibility that these small cells merely passively carry Con A into the second culture on their surface had been previously eliminated by showing that the helper activity was not inhibited by high concentrations of α-methyl-mannopyranoside (4). These observations are consistent with the previously reported radioresistance of Con A helper cells (17). In fact, irradiated spleen cells cultured with Con A have been conveniently used as a source of nonspecific helper cells for the anti-SRBC response (e.g., 18). These cells are thus activated by Con A in the absence of DNA synthesis. This lack of DNA synthesis requirement may reflect the already fully differentiated nature of the cell type involved, perhaps a T2 memory cell. Supporting this thesis is the observation that long-term primed and boosted helper activity for the secondary anti-hapten humoral response is also insensitive to mitomycin C treatment (19). Perhaps pertinent to this point is the finding of Stout and Herzenberg (20) that the cells that help secondary IgG humoral response are Ly1+ Fc receptor (FcR) negative and that FcR- cells do not respond to Con A by mitogenesis in the absence of FcR+ promotor cells (21).

If primed mice are boosted on day 3 and their spleen cells fractionated in the gradient, carrier-specific helper activity for the anti-hapten response is found associated with the fast sedimenting pool IV cells (S. L. Swain and H. Y. Tse, unpublished observation). One explanation for this difference could be that Con A and specific antigens induce the same cell but the cell follows different differentiation pathways. Alternatively, Con A and specific antigens might activate subsets of cells at different stages of differentiation. This has, in fact, been proposed by Kappler and Marrack (22) who used adult thymectomy and anti-thymocyte serum to show that Con A-activated T help for SRBC was found mainly in the T2 subpopulation and priming for helper activity involved precursors within both T1 and T2 subpopulations.

The relationship between Con A suppressor cells and proliferating cells is more difficult to establish. In the Ficoll gradient system, both thymidine incorporation and suppressor activity are found in the same pool IV. However, inhibition of thymidine incorporation does not inhibit the generation of Con A suppressor cells (Figs. 5 and 6). This would imply that Con A can activate a class of suppressor cells without the concomitant requirement for DNA synthesis or proliferation. This observation, though rather surprising at first look, is not an isolated one. Working with Con A in the mouse system, Rich and Pierce (16)
showed that an inhibitory factor can be isolated by culturing spleen cells with Con A for just 12 h. In a rabbit model, Redelman et al. (23) could also demonstrate the existence of suppressor activity before the induction of DNA synthesis by Con A. Roszman (24) also observed that a brief 2-h pulse of rabbit spleen cells with Con A was sufficient to activate suppressor cells without DNA synthesis. Taken together, it seems that Con A activation of suppressor cells again involves a T₂ subpopulation which can be effective without further differentiation. It must be pointed out, however, that cell recovery from irradiated or mitomycin C-treated cultures is usually much lower than that from untreated cultures, especially for pool IV cells. In order to get an equivalent number of pool IV cells, three times as many cells are often used in the initial cultures. On a per culture basis, the activity of mitomycin-treated cultures is indeed lower than that of the untreated. When allowance is made for this fact it is found that the total suppressor activity is one-third of the untreated control while the total proliferation is one-tenth of the untreated control. On this basis, the notion that Con A suppressor cells arise from both T₁ and T₂ subpopulations (22) fits our data better. Analogous situations in the study of cytotoxic cells have been reported. Thus cytosine arabinoside has not been able to inhibit the induction of Con A cytotoxic cells (25) and there are both DNA synthesis-dependent and independent phases in the activation of secondary antigen-specific cytotoxic cells (26). (Although suppressor and cytotoxic cells sediment to the same pool IV on the Ficoll gradient [H. Y. Tse and R. W. Dutton, unpublished observation], whether they are the same or distinct subsets has yet to be determined.)

Previously, the induction of suppressor cells by Con A was found to be radiosensitive (16, 17). Irradiation of spleen cells before culture with Con A eliminated the subsequent suppressor effect. It must be noted that in the whole population there are two counteracting sets of cells, i.e. helper vs. suppressors, and that lack of apparent suppression does not necessarily imply the absence of suppressor cells. Rather it reflects a balance between the two cellular events. The unfractionated cells shown in Fig. 5 are indeed not suppressive; but gradient fractionation is capable of resolving this counterbalance into its positive and negative components. It is conceivable that irradiation may affect the T₁ subsets so that the residual suppressor activity derived from the T₂ subset can easily be counterbalanced by the helper cells in the same culture. Gradient fractionation, on the contrary, concentrates these suppressor cells to one fraction void of helper cells so that its activity can be revealed.

The nature and function of Con A-proliferating cells remains unclear. Theoretically, precursor cells of any effector functions can participate in this proliferation. There is no a priori reason to assume that this is a homogeneous population. On the basis of Ly and FcR phenotypes, Stout et al. (27) described four functionally distinctive T-cell subpopulations which may be tabulated as follows:

<table>
<thead>
<tr>
<th>Ly₁⁺</th>
<th></th>
<th>Ly₂⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcR⁻</td>
<td>FcR⁺</td>
<td>FcR⁻</td>
</tr>
<tr>
<td>Helpers for secondary IgG response</td>
<td>Cytotoxic amplifiers</td>
<td>Cytotoxic precursors</td>
</tr>
</tbody>
</table>

Downloaded from jem.rupress.org on July 13, 2017
It has been suggested that only a population of FcR⁺ cells can directly respond to Con A by incorporating labeled thymidine. FcR⁻ cells, however, can also participate in mitogenesis if FcR⁺ cells are present. This cellular interaction is reminiscent of the often observed cooperation of a Ly1⁺ and Ly23⁺ cell types. In this case, the cooperating cells are Ly1⁺ FcR⁺ and Ly23⁺ FcR⁻, without the involvement of Ly1⁺ FcR⁻ cells. If this is true, Ly1⁺ FcR⁻ cells can still remain nonproliferating and may be equivalent to the Con A helper cells we report here. Nevertheless, until the relationship between suppressor and cytotoxic cells can be clarified, it would seem premature to draw any conclusions regarding the nature and functions of cells that respond to Con A by proliferation.

**Summary**

Using a Ficoll velocity sedimentation gradient, we have been able to fractionate concanavalin A (Con A)-induced helper and suppressor cells into separate pools. Cells activated by Con A to mediate helper activity are Ly1⁺, do not require DNA synthesis for induction, and remain as small cells after activation. Suppressor cells are Ly23⁺, are found in the blast cell fraction and their induction is not inhibitable by prior treatment with mitomycin C or irradiation, both of which inhibit DNA synthesis. The implications of such findings are discussed.

We thank our colleagues for critical discussions and valuable suggestions. We also thank Doctors E. A. Boyse and H. Cantor for sending us the aLy antisera.

Received for publication 18 April 1977.

**References**


