SUPPRESSOR CELLS: DEPENDENCE ON ASSAY CONDITIONS FOR FUNCTIONAL ACTIVITY*

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T cells can regulate the response of other immunocompetent cell populations (1-3). The method generally used for detecting the regulatory effect of suppressor cells is to mix them with cells capable of a positive immune response. If the response of the mixed population is less than appropriate controls, the added cells are called "suppressor cells." We have shown, however, that the behavior of the responder or assay population may be of critical importance in determining whether regulatory cells act to suppress or augment the response (4).

In a previous report (5) we showed that spleen cells could be educated in vitro to induce thymus-dependent help or suppression of the immune response to sheep red blood cells (SRBC). Low doses of SRBC induced helper cells and high doses of SRBC induced suppressor cells, as tested by addition of educated cells to fresh spleen cultures (assay cultures). These results suggested that induction of different types of regulatory cells is clearly dependent on antigen dose. Experiments reported below indicate the situation is more complex because adjustment of experimental conditions in the assay culture significantly affects the regulatory effect produced by spleen cells educated with a high dose of SRBC. When the response of the target population is low, the educated cells augment the response and vice versa, supporting the hypothesis that the effect regulator cells produce depends on the activity of the cells they regulate (5).

Materials and Methods

Mice. 6-8-wk-old male BDF1 (C57BL/6 × DBA/2) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Antigen. SRBC were obtained from Colorado Serum Company, Denver, Colo.

Spleen Cell Cultures. Spleen cells were educated and assay cultures prepared as previously described. Briefly, spleen cells were educated using the tissue culture techniques of Mishell and Dutton (6) in medium containing 5 × 10⁻⁴ M 2-mercaptoethanol. Educated cells were cultured in

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3.5 ml in Falcon 3002 tissue culture dishes (Falcon Plastics, Oxnard, Calif.) at 10^7 viable spleen cells/ml in the presence of 0.02 ml of a 1% suspension of SRBC/ml (approximately 2 x 10^9 SRBC) of spleen cells, incubated 4 days with gentle rocking, and fed daily with a nutritional cocktail. After harvest of these cultures, various numbers of viable educated cells were added to fresh spleen cultures (assay cultures; 10^7 cells/culture in Falcon 3001 tissue culture dishes) and challenged with 0.02 ml of 1% SRBC unless otherwise indicated. The cultures were assayed for plaque-forming cells after 4 more days of culture. All assay cultures were gently rocked except where indicated.

Plaque Assay. The number of antibody-forming cells per culture was determined by the Cunningham modification of the Jerne plaque assay (7). The mean number of plaque-forming cells was calculated from triplicate cultures.

Preparation of B Cells. B cells were prepared by incubation of spleen cells (5 x 10^7 cells/ml) in an anti-0 serum at 4°C for 30 min, washed, and then incubated with guinea pig complement (2 x 10^7 cells/ml) for 5 min at 4°C and then 37°C for 30 min. The viability was determined by trypan blue dye exclusion. Anti-0 treatment killed 99% of normal thymocytes and 30-40% of normal spleen cells.

Results

Culture of Optimal or Suboptimal Numbers of Spleen Cells. The effect of spleen cells educated in vitro on assay cultures of optimal or suboptimal numbers of spleen cells was investigated. As seen in Fig. 1 the addition of 3.3 x 10^4 or more educated spleen cells significantly suppressed the response of assay cultures of 10^7 spleen cells. The response of control cultures (10^7 spleen cells without educated cells added) was 5,560 ± 570 plaques per culture. Culture of 3 x 10^6 spleen cells alone yielded only 1,680 ± 360 plaques per culture, and addition of the same population of educated cells to these cultures enhanced rather than suppressed the response, when less than 10^7 educated cells were added. Suppression was observed when more than 10^7 educated cells were added. However, doses of educated cells (6.6 x 10^4 or 3.3 x 10^4) which suppressed the response of assay cultures of 10^7 spleen cells augmented the response of 3 x 10^6 spleen cells. In fact, with the addition of 6.6 x 10^4 educated cells the response of 3 x 10^6 normal spleen cells (3,270 ± 124 plaques per culture) was higher than the response of 1 x 10^7 normal spleen cells (1,040 ± 691 plaques per culture).

Antigenic Challenge. We also examined the effect of varying the dose of antigenic challenge on the response of the assay cultures to which educated spleen cells were added. Varying numbers of educated spleen cells were added to assay cultures of 10^7 spleen cells and challenged with 10, 1, and 0.1% SRBC. The results, shown in Fig. 2, indicate that challenge of assay cultures with high doses of SRBC (10 or 1%) caused educated cells to suppress the subsequent response while challenge with a low dose of SRBC (0.1%) resulted in augmentation of the response by 10^4 or 10^5 educated cells and suppression by 10^6 educated cells. The response of control cultures (no educated cells added) to challenge with 10, 1, or 0.1% SRBC was 3,600 ± 156, 5,160 ± 156, and 660 ± 180 plaques per culture, respectively. Thus, challenge with optimal or supraoptimal antigen doses in cultures with low numbers (10^4-10^5) of educated cells resulted only in suppression, while challenge with suboptimal antigen doses led to a marked augmentation of the response. Higher doses (10^6) of educated cells suppressed all cultures.

Addition of Educated Spleen Cells to B Spleen. To determine the role that the normal T cells in the assay cultures played in suppression of the response by
Assay Culture

- $10^7$ Spleen Cells; control = 5560 ± 570
- $3 \times 10^6$ Spleen Cells; control = 1680 ± 360

**Fig. 1.** Spleen cells were educated with 0.02 ml of 1% SRBC/10^7 spleen cells for 4 days. $10^4$ - $10^6$ viable educated cells were added to assay cultures of $10^7$ spleen cells (●—●) or $3 \times 10^6$ (○—○) spleen cells and cultured 4 more days with 0.02 ml of 1% SRBC/ml culture. Each point represents the mean plaque-forming cell response of triplicate cultures expressed as a percent of response of control cultures which received no educated cells. The response of control cultures of $10^7$ spleen cells was 5,560 ± 570 plaques per culture and of $3 \times 10^6$ spleen cells was 1,680 ± 360 plaques per culture.

Educated cells, we added educated spleen cells to whole spleen cells ($10^7$ cells) or an equivalent number of B cells ($5 \times 10^6$ anti-0-treated cells). The educated cells suppressed the response of whole spleen assay cultures as seen in Fig. 3. The same number of educated cells ($10^4$ or $10^5$), which suppressed assay cultures of whole spleen, helped the response of the B-spleen cells which were unable to respond on their own. To confirm the role of normal spleen cells in suppression mediated by educated cells, $5 \times 10^6$ normal T cells were added back to cultures of $10^4$ educated spleen cells and $5 \times 10^6$ B spleen cells. The results are shown in Table I. Normal B cells which could not respond on their own cooperated with educated spleen cells to give 4,480 plaques per culture. The addition of normal T cells to these cultures reduced the response almost 20-fold, while it augmented the response of the "pure" B-cell culture to almost the same extent as the educated cells. Thus, educated spleen cells and normal T cells which could each cooperate with B cells on their own led to a suppressed response when mixed together.

**Nonrocked Assay Cultures.** Optimal antibody responses in Mishell-Dutton cultures require gentle rocking, presumably to allow optimal cell interactions. We examined the effect rocking had on the suppression of assay cultures by spleen cells educated in vitro. As seen in Fig. 4, rocked assay cultures responded well to SRBC and addition of educated spleen cells suppressed the response. The
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Fig. 2. Varying numbers of spleen cells educated with 0.02 ml of 1% SRBC/10^7 spleen cells were added to assay cultures of 10^7 spleen cells which were then challenged with 0.02 ml of 10, 1, or 0.1% SRBC per 1 ml culture. Each point represents the mean plaque-forming cell response of triplicate cultures expressed as a percent of response of control cultures which received no educated cells. Response of control cultures challenged with 10, 1, or 0.1% were 3,600 ± 156, 5,160 ± 156, and 660 ± 180 plaques per culture, respectively.

effect of the same number of educated cells on nonrocked assay cultures was just the opposite. Nonrocked assay cultures responded very poorly, and educated spleen cells augmented the response.

We also examined the effect rocking had during education of spleen cells (data not shown). Both rocked and nonrocked educated cells suppressed rocked assay cultures, indicating that rocking is not necessary for generation of cells that mediate suppression.

Activity of the Regulator Cells. Under the conditions of immunization in cultures used for education, there was never a response in secondary cultures if no assay cells were added. This indicates that the antibody response made in the assay cultures was regulated but not made by the added educated cells. This contention is supported by other studies using purified T cells devoid of antibody-forming capacity which reproduce the effects we have described using educated whole spleen cells (unpublished observations).

Discussion

A number of reports have described the conditions required for generation of antigen-specific suppressor T cells (reviewed in 2, 8, and 9). It is a general practice to look for suppressive effects by adding putative suppressor cells to an assay system where a good immune response is taking place. We previously described a system in which the addition of splenic T cells, educated in vitro with a dose of SRBC which is optimal for antibody production, produced a
specific suppression of the antibody response of fresh assay cultures. In the course of examining the mechanism of suppression mediated by the T cells educated in vitro, we altered the conditions of the assay cultures. We found that the effect obtained by the addition of educated cells to assay cultures was extremely dependent on the environment within the assay cultures. The environmental factors examined included cell density, dose of antigenic challenge, amount of cell-cell contact, and the presence of normal T cells. In general we found that any change that reduced the response of the control cultures either reduced the amount of suppression the educated cells produced or actually changed their activity from suppression to help. These results suggest that feedback interactions between the educated T cells and the cells in the assay cultures are an important factor in suppressor cell generation and/or activity.

There was one possible exception to the general rule. In the studies on antigen dose (Fig. 2) we found that the response of primary spleen cell assay cultures to a 10% suspension of SRBC (supraoptimal dose) was less than the response to 1% suspension (optimal dose); yet the suppressor cells were more active in the cultures containing the higher dose of antigen. This observation can be interpreted to mean that although feedback signals may be of great importance in determining whether suppression or help is seen, other factors are also important and may be overriding.

A similar conclusion can be reached from the fact that with high numbers
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**Table I**

| Educated spleen cells* | Assay culture | Plaques per culture |
|------------------------|---------------|---------------------|
| 10^4                   | 5 x 10^6 normal B cells‡ | 4,480 ± 320         |
| 10^4                   | 5 x 10^6 normal B cells + 5 x 10^6 normal T cells§ | 240 ± 0            |
|                        | 5 x 10^6 normal B cells | 0                   |
|                        | 5 x 10^6 normal B cells + 5 x 10^6 normal T cells | 3,640 ± 280        |

* Spleen cells were educated 4 days before mixing with assay cultures of fresh B or T cells.
‡ B cells were prepared by treatment of whole spleen with anti-∅ plus complement.
§ T cells were prepared by passage of whole spleen over nylon wool.

(i.e., 10^6 or greater) of educated cells suppression was almost always seen, independent of the activity of the assay culture. The one exception was in the studies on rocking of cultures (Fig. 4), where 10^6 educated cells produced a good helper effect in the nonrocked cultures. However, in other experiments (data not published), we have been able to suppress nonrocked cultures with higher doses of educated cells.

The fact that a population of educated cells, which contains known specific suppressor cells, can be made to be either nonsuppressive or actually augmentative simply by altering the assay conditions indicates that the failure to demonstrate suppressor cells in any given assay must be interpreted cautiously. It cannot be definitively taken to indicate that suppressor cells are not present. This is particularly true when one takes cells out of an animal and assays them under conditions that are perforce different than they were in the donor animal. Many workers have found it difficult to demonstrate suppression with cells taken from animals and put in vitro or into adoptive transfer experiments although the donors from which the cells were taken were able to inactivate cells transferred into them (reviewed in 3), possibly another example of changing conditions which change suppressor cell activation.

What changes in cell interactions could be produced by changing the assay conditions? There are three distinct populations of T cells which have been characterized by the use of antisera against Ly differentiation antigens (10, 11); an Ly 1+ cell, an Ly 2,3+ cell, and an Ly 1,2,3+ cell. Since we have shown that an immune and a nonimmune T cell interact to generate suppression (Table I), there are at least six potentially different T-cell subpopulations which might be involved in producing the suppression we have described. Determining which ones are actually doing the suppressing and which ones are regulating the generation of the suppressor will be a difficult problem to solve. There is an impressive amount of preliminary data to indicate that the Ly 1+ cell is the helper cell, and that the Ly 2,3+ cell is both the killer cell and the suppressor cell.
(see 12). Since the Ly 1+ cell can help the Ly 2,3+ cell become a killer cell (11), perhaps it can also help it to become a suppressor. The role of the Ly 1,2,3+ cell has not been clearly delineated, but it certainly could be involved in the interactions we have described. We are presently using anti-Ly serum to try and delineate the respective roles of the six different T-cell types.

Another question that must be answered is whether the change from suppression to help, produced by reducing the activity of the assay cultures, is due to a change in activity of one regulatory cell type or to differential activation of different cell types. We know, for example, that when we educate cells with very low doses of antigen, the educated cells yield specific helper effects, even when the assay cultures are responding very well. Only when we use higher doses of antigen is it required that we alter the conditions of the assay cultures to see helper effects. Are there blocked helper cells in the population educated with high doses of antigen which are deblocked when the assay culture conditions are changed or is the change from suppression to help caused by a cell with bidirectional regulatory capacities which is sensitive to the feedback signals in the primary culture?

Another question one must consider is the possible role of the B cell in the assay culture. These cells also emit feedback signals as we have previously shown (13), and some of the T-dependent effects might actually be mediated via a T to B to T-type signal. Hopefully, with the appropriate use of Ly antisera and
the use of assay systems which do not require the presence of B cells, some of these perplexing questions may be answered.

At the present time, we consider it most likely that the educated cells contain both helper and suppressor cells, and that the conditions in the assay culture determine which of these two types of cells will have a dominant effect. However, whatever the mechanism turns out to be, our results add to a growing body of evidence that suggests that the regulatory effect produced by a mixed T-cell population is highly dependent upon the activity of the cells being regulated. When the response being regulated is relatively high, the regulatory cells tend to act as suppressor cells; when the response is low, they tend to act as helpers. It has been suggested that, by use of a Newtonian transplant (14), this observation might be called the "second law of thymodynamics" (5).

Summary

Spleen cells educated in vitro with sheep red blood cells (SRBC) suppressed the plaque-forming cell response of Mishell-Dutton assay cultures challenged with optimal doses of SRBC. Changing conditions in the assay cultures changed the effect educated cells had on the assay culture response. For example, educated cells helped rather than suppressed assay cultures of suboptimal numbers of spleen cells. Similarly, augmentation resulted upon addition of educated cells to assay cultures challenged with suboptimal doses of SRBC. Such a reversal of regulatory effects was not observed when assay cultures were challenged with supraoptimal antigen doses.

Educated cells helped assay cultures of B spleen cells, and the addition of normal T cells reinstated suppression. Furthermore, maintenance of assay cultures under stationary rather than the usual rocking conditions allowed educated cells to help rather than suppress the antibody response of assay cultures. These results show that when the response of the target population (assay cultures) is low, the regulator (educated) cells augment the response, and vice versa, supporting the hypothesis that the effect regulator cells produce depends on the activity of the cells they regulate.

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