FUNCTIONAL ANALYSIS OF HUMAN T CELL SUBSETS DEFINED BY MONOCLONAL ANTIBODIES

IV. Induction of Suppressor Cells within the OKT4+ Population*

By YOLENE THOMAS, LINDA ROGOZINSKI, OSCAR H. IRIGOYEN, STEVEN M. FRIEDMAN, PATRICK C. KUNG, GIDEON GOLDSTEIN, AND LEONARD CHESS

From the Division of Rheumatology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York 10032; and The Division of Immunosciences, Ortho Pharmaceutical Corporation, Raritan, New Jersey 08869

Previous studies (1-9) have suggested that peripheral human T cells can be divided into two mutually exclusive functional subsets by the two monoclonal antibodies, OKT8 and OKT4. The OKT8+ subset does not provide helper activity but contains cells capable of suppressing B cell differentiation. Importantly, the suppression observed with OKT8+ cells requires the presence of radiosensitive OKT4+ cells. Potent helper activity is found in a radiosensitive OKT4+ subset. Irradiated OKT4+ cells also induced B cell differentiation, but only at high ratios of T cells to B cells. In our previous studies (7), we also found that the addition of graded numbers of radiosensitive OKT4+ cells to B cells eventually decreased the net helper activity observed. These experiments raised the possibility that precursors of suppressor cells may be contained within the OKT4+ population.

The current study was undertaken to further investigate the functional heterogeneity within the OKT4+ population. In particular, we asked whether OKT4+ cells could be induced to differentiate into immunoregulatory cells capable of suppressing B cell differentiation. In the experiments reported here, we observed that although in vitro pokeweed mitogen (PWM)-activated OKT4+ cells can function as radioresistant helper cells, these activated OKT4+ cells could also exert potent feedback suppression. This suppression mediated by activated OKT4+ cells required the presence of radiosensitive cells contained within the resting OKT4+ population. These data emphasize the potential role of interactions of T cell subsets contained exclusively within the OKT4+ population in the immunoregulation of B cell differentiation.

Materials and Methods

Lymphocyte Preparation and Isolation of Human T and B Cells. Fresh peripheral blood lymphocytes were isolated from consenting healthy human volunteers by Ficoll-Hypaque density gradient centrifugation. Highly enriched population of T and B cells were then isolated by methods previously described in detail (10). In brief, human mononuclear cells were washed in minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 5% fetal calf serum (FCS; Microbiological Associates, Bethesda, Md.) and then separated into surface Ig"
and surface Ig* populations using Sephadex G200 anti-F(ab)2 columns. The surface Ig* populations were further purified by complement (C)-mediated lysis of residual T cells using the OKT3 monoclonal antibody (1). The resulting B cell fraction was <1% E rosette positive (E*). Functional evidence for B cell purity was obtained by the lack of activation of these B cells by PWM in the absence of added T cells. The Ig* population was further fractionated into highly purified T cell populations by the formation of E rosettes with sheep erythrocytes. This population, which represents the unselected T cell population in the current studies, is >95% E* and <1% surface membrane Ig*.

Isolation of Human T Cell Sets by Complement-mediated Lysis Using the Monoclonal Antibodies OKT4 and OKT8. Production, growth, and characterization of the hybridomas-secreting monoclonal antibodies OKT3, OKT4, and OKT8 were previously described in detail (3). In brief, the monoclonal OKT3, OKT4, and OKT8 antibodies were shown to be highly specific for human T cell subsets. The OKT3 antibody reacts with ~85-90% on E* cells. The OKT4 antibody reacts with 50-60% of peripheral T cells, whereas the OKT8 antibody reacts with 30-40% of human T cells. In experiments designed to isolate T cell populations, 50 x 10^6 unfractionated T cells were resuspended in 1 ml of ascitic OKT4 or OKT8 diluted 1:250 in RPMI-1640 media containing 10% FCS and incubated for 45 min at room temperature. Fresh rabbit C was added at a final dilution of 1:10 and incubation was further carried out for 1 h at 37°C in a humid atmosphere. Analysis of the resulting populations showed that the OKT4-treated population contained >90% OKT3* cells, >90% OKT8* cells, and <2% OKT4* cells, whereas the OKT8-treated population contained >90% OKT3* cells, >90% OKT4*, and <2% OKT8* cells. Because of these results, we used the notation OKT4* to signify a population of T cells remaining after treatment with OKT8 plus C, and OKT8* to signify the reciprocal population remaining after treatment with OKT4 plus C.

PWM Activation of Purified OKT4+ T Cells (First Culture). The final medium for all cultures consisted of RPMI-1640 (Grand Island Biological Co.) supplemented with 1% penicillin-streptomycin, 20 mM l-glutamine, 12.5 mM Hepes buffer, 0.05% sodium bicarbonate (Microbiological Associates, Walkersville, Md.) and 12% heat-inactivated FCS. Isolated OKT4* cells (see above) were activated with PWM (Grand Island Biological Co.) (10 μg/ml) at a concentration of 2 x 10^6 cells/ml in 25-cm² surface area tissue culture flasks (Falcon Labware, Oxnard, Calif.) for 60-70 h at 37°C in a humid atmosphere containing 5% CO₂. Control OKT4* cells were cultured in identical fashion without PWM. Subsequently, cells from these first cultures were recovered, washed extensively, and added in graded numbers to appropriate secondary cultures.

Assay Cultures (Secondary Cultures). The ability of the PWM-activated OKT4+ T cells (first culture) to exert helper activity was determined by adding graded numbers of these cells to 2 x 10^6 fresh autologous B cells. After 5 d of in vitro sensitization in the presence of 10 μg PWM, the cultures were harvested and assayed for plaque-forming cell (PFC) activity using the reverse hemolytic plaque assay (7, 11, 12). Each population from the first culture was also tested for suppressor activity by addition to fresh autologous OKT4* cells and B cells. The cell mixtures were then cultured with PWM for 5 d and assayed for PFC activity. In some experiments, activated OKT4* cells or freshly isolated OKT4* cells were irradiated with 1,250 rad by using the Model M 38-1 gammator emitter (Isomedix, Parsippany, N. J.).

Reverse Hemolytic Plaque Assay for the Enumeration of Antibody-secreting Cells. The assay for the measurement of total Ig-producing hemolytic PFC was described elsewhere (11, 12). Briefly, on the day of assay, cells were thoroughly washed in RPMI-1640 medium and resuspended. 50-100-μl aliquots were added to 0.9 ml of 0.5% liquid agarose (Seakem Agarose; Marine Colloids, Rockland, Maine) containing 100 μl of an 11% suspension of antibody-coated sheep erythrocytes. This mixture was layered on a 60- x 15-mm petri dish previously coated with 5 ml of 0.5% liquid agarose and allowed to gel. The dishes were incubated for 1 h at 37°C, in a humid atmosphere containing 5% CO₂, 95% air. 1 ml of a 1:100 dilution of rabbit anti-human IgG antisera was then added for an additional hour of incubation. Finally, the antisera was removed and 1 ml of a 1:10 dilution of absorbed guinea pig C (Cedarlane Laboratories, Hicksville, N. Y.) was added for an additional hour. Plaques were enumerated in triplicate and the results were expressed as the mean PFC/10^6 B cells in the original culture. The standard
error of the mean was always <20%. In addition, cell counts and viability (by dye exclusion) were performed on all cell cultures at the time of assay.

**Cytofluorographic Analysis.** Phenotypic analysis of all cell populations was performed by indirect immunofluorescence using the monoclonal OKT antibodies and a fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories, Springfield, Va.) using a Model 30-H Cytofluorograf (Ortho Instruments, Westwood, Mass.). In brief, 5 x 10⁶ cells were treated with OKT3, OKT4, or OKT8 at a 1:10,000 final dilution and incubated at 4°C for 30 min. After washing, 0.1 ml of a 1:40 dilution of fluorescein-conjugated goat anti-mouse IgG was added to the cell pellet (1:80 final dilution), mixed well, and incubated at 4°C for 30 min. After this, the cells were washed twice and resuspended in 1 ml of phosphate-buffered saline with 0.1% sodium azide. Mouse ascites fluid was included as a negative control. The cells were analyzed on the Cytofluorograf; the intensity of fluorescence per cell was recorded on a pulse height analyzer and those cells with fluorescence intensity greater than that of the ascites control were considered positive.

**Results**

**Radioresistant Activated OKT4⁺ Cells Mediate Helper Function.** In initial experiments, we asked whether the radiosensitivity of OKT4 immunoregulatory functions was dependent on the state of activation of the cells. To activate cells, purified OKT4⁺ populations were cultured with PWM for 60-70 h. Controls consisted of identical populations cultured in medium alone (first culture). Graded numbers of either nonirradiated or irradiated cells from the first culture period were then added to a secondary culture containing PWM-driven autologous B cells. PFC activity was measured 5 d later. As shown in Fig. 1, activated OKT4⁺ cells were capable of inducing B cell differentiation, and this helper function was relatively radioresistant. In contrast, control unactivated OKT4⁺ cells remained radiosensitive over a broad range of concentrations. These data support the idea that the radiosensitivity of OKT4⁺ cell-mediated helper function is in part dependent on the state of activation of these cells. In addition, it should be noted that nonirradiated PWM-activated OKT4⁺ cells were less efficient in inducing B cells than unactivated OKT4⁺ cells.

![Graph](image)

**Fig. 1.** Activated OKT4⁺ cells are enriched in radioresistant helper cells. (A) Purified OKT4⁺ cells were cultured with PWM for 60-70 h (first culture). Graded numbers of either untreated OKT4⁺ cells (—) or irradiated OKT4⁺ cells (---) from the first culture period were then added to a secondary culture containing PWM-driven autologous B cells (2 x 10⁶). (B) Control culture consisted of purified OKT4⁺ cells cultured with medium alone for 60-70 h. Graded numbers of either untreated OKT4⁺ cells (——) or irradiated OKT4⁺ cells (—–) from the first culture period were then added to the secondary culture containing PWM-driven autologous B cells (2 x 10⁶). 5 d later, cultures were harvested and assayed for PFC activity.
Activated OKT4+ Cells Suppress B Cell Differentiation Induced by Fresh OKT4+ Cells. We next asked whether the decreased helper activity observed in the PWM-activated OKT4+ populations was due to active suppression. To address this question, graded numbers of unactivated or activated OKT4+ cells were added to secondary autologous cultures containing 2 x 10⁶ B cells plus 0.1 x 10⁶ OKT4+ cells in the presence of PWM. As shown in Fig. 2, the addition of small numbers of activated OKT4+ cells suppressed B cell differentiation. In contrast, control unactivated OKT4+ cells cultured in medium alone had minimal inhibitory activity. Furthermore, the addition of fresh OKT4+ cells enhanced B cell PFC production. These data strongly support the idea that suppression can be generated by polyclonal activation of the OKT4+ subset.

Suppression Mediated by Activated OKT4+ Cells Required the Presence of Radiosensitive Cells within the Resting OKT4+ Populations. In previous studies, we demonstrated that a radiosensitive subset of OKT4+ cells is required for OKT3+-mediated suppression of B cell differentiation. We next asked whether the suppression induced by activated OKT4+ cells also requires radiosensitive cells within the resting OKT4+ populations. Graded numbers of activated OKT4+ cells were added to secondary cultures containing 2 x 10⁶ B cells and either nonirradiated fresh OKT4+ (Table I, part A) or fresh irradiated OKT4+ cells (Table I, part B). Consistent with Fig. 1, the addition of activated OKT4+ cells to cultures containing nonirradiated fresh OKT4+ cells resulted in a reduction of the PFC response (Table I, part A). In contrast, the addition of identical populations of activated OKT4+ cells to cultures containing fresh irradiated OKT4+ cells failed to decrease the PFC response (Table I, part B). In fact, under these latter conditions, a substantial enhancement was noted. However, the addition of a small percentage of nonirradiated fresh OKT4+ cells (0.1 x 10⁶) allowed for reexpression of suppression (Table I, part C). These observations indicate that the
expression of suppression mediated by activated OKT4+ cells required the presence of radiosensitive cells within the resting OKT4+ population.

Irradiation of Activated OKT4+ Cells Abrogates Suppression Function. To determine whether the regulatory function mediated by activated OKT4+ cells is radiosensitive, graded numbers of nonirradiated or irradiated activated OKT4+ were added to a constant number of fresh autologous B cells plus OKT4+ cells in the presence of PWM (Table II). Consistent with results in Fig. 2, the addition of nonirradiated activated OKT4+ cells suppressed PFC formation. Irradiation of these activated OKT4+ cells (1,250 rad) ablates this regulatory function and resulted in rather marked helper effects. Thus, the suppressor function mediated by activated OKT4+ cells is radiosensitive.

Activated OKT4+ Cells Maintain the OKT3*, OKT4+, and OKT8− Surface Phenotype. Isolated OKT4+ cells were cultured with medium alone or PWM. After 60–70 h, activated and control unactivated OKT4+ cells were harvested and subsequently reanalyzed for surface phenotype by immunofluorescence on the cytofluorograph. The results shown in Table III demonstrate that upon activation with PWM, the OKT4+ cells maintain the original OKT3*, OKT4+ and OKT8− surface phenotype. Similar results were obtained using a C-dependent microcytotoxicity assay. In

### Table I

| Numbers of fresh OKT4+ cells present in the secondary culture* | Activated OKT4+ cells (first culture) added | PFC/10⁶ cells | Percent suppression$ ‡$ |
|---|---|---|---|
| 0 | 13,890 ± 1,800 | 0 |
| (A) 0.1 × 10⁶ nonirradiated | 0.2 × 10⁶ | 8,000 ± 420 | 42 |
| | 0.4 × 10⁶ | 7,500 ± 750 | 47 |
| | 1.0 × 10⁶ | 5,190 ± 900 | 63 |
| (B) 1 × 10⁶ irradiated | 0 | 8,640 ± 608 | 0 |
| | 0.2 × 10⁶ | 18,450 ± 2,404 | 0 |
| | 0.4 × 10⁶ | 12,600 ± 1,680 | 0 |
| | 1.0 × 10⁶ | 8,310 ± 180 | 4 |
| (C) 1 × 10⁶ irradiated + 0.1 × 10⁶ nonirradiated | 0 | 19,880 ± 300 | 0 |
| | 0.2 × 10⁶ | 13,240 ± 1,780 | 24 |
| | 0.4 × 10⁶ | 9,440 ± 1,321 | 53 |
| | 1.0 × 10⁶ | 6,960 ± 600 | 65 |

*2.0 × 10⁶ fresh B cells were cultured during 5 d in the presence of 10 μg of PWM. To these cultures various numbers of fresh OKT4+ cells and activated OKT4+ cells (first culture) were added.

‡ Percent suppression was calculated as follows:

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\left(1 - \frac{\text{PFC (experimental culture)}}{\text{PFC (standard culture)}}\right) \times 100.
\]
TABLE II

Irradiation of Activated OKT4+ Cells Abrogates Suppressor Function

| Numbers of activated OKT4+ cells (first culture) added to secondary culture* | Irradiation of activated OKT4+ cells† | PFC/10^6 cells | Percent suppression§ |
|---|---|---|---|
| 0 | − | 14,100 ± 1,640 | |
| 0.1 × 10^6 | − | 15,010 ± 2,000 | 0 |
| 0.2 × 10^6 | + | 23,070 ± 3,480 | 0 |
| 0.4 × 10^6 | − | 8,100 ± 999 | 42 |
| 1.0 × 10^6 | + | 22,500 ± 1,802 | 0 |
| 1.0 × 10^6 | − | 7,850 ± 878 | 47 |
| 1.0 × 10^6 | + | 27,900 ± 3,050 | 0 |
| 1.0 × 10^6 | − | 4,900 ± 641 | 63 |
| 1.0 × 10^6 | + | 30,900 ± 4,030 | 0 |

* Graded numbers of OKT4+ cells previously activated with PWM (first culture) were added to standard cultures containing 2 × 10^6 B cells and fresh OKT4+ cells (0.1 × 10^6).
† Irradiated T cells are designated by (+).
§ Percent suppression was calculated as before (Table I).

TABLE III

Activated OKT4+ Cells Maintain the OKT3+, OKT4+, and OKT8- Surface Phenotype

| Cell populations | Percent reactivity with monoclonal OKT antibody* |
|---|---|---|---|---|
| | Ascites | OKT3 | OKT4 | OKT8 |
| Fresh OKT4+ cells | 1 | 98 | 95 | 2 |
| Unactivated OKT4+ cells | 1 | 91 | 90 | 3 |
| Activated OKT4+ cells | 1 | 87 | 100 | 1 |

* Assessed by indirect immunofluorescence.

TABLE IV

Treatment of OKT4+ Cells by OKT8 plus C after Activation Does Not Abrogate Suppression

| Numbers of activated OKT4+ cells (first culture) added to secondary culture* | Ascites + C | OKT8 + C | OKT4 + C |
|---|---|---|---|
| | PFC/10^6 cells | Percent suppression‡ | PFC/10^6 cells | Percent suppression‡ | PFC/10^6 cells | Percent suppression‡ |
| 0.2 × 10^6 | 9,330 ± 210 | 31 | 8,700 ± 420 | 36 | 11,850 ± 210 | 13 |
| 0.4 × 10^6 | 4,650 ± 630 | 66 | 4,380 ± 240 | 68 | 13,800 ± 1040 | 0 |
| 1.0 × 10^6 | 3,390 ± 330 | 75 | 2,730 ± 280 | 80 | 11,400 ± 1100 | 16 |

* Graded numbers of OKT4+ cells previously activated with PWM (first culture) were added to standard cultures containing 2 × 10^6 B cells and fresh OKT4+ cells (0.1 × 10^6). Standard cultures alone, in the absence of added activated OKT4+ cells, generated 15,500 ± 860 PFC/10^6 cells.
‡ Suppression was calculated as before (Table I).
addition, functional experiments were performed in which activated OKT4+ cells generated in primary culture were treated with ascites, OKT4, or OKT8 in the presence of C. These treated cells were then added to standard cultures of B cells plus fresh OKT4+ cells. As shown in Table IV, OKT8-treated but not OKT4-treated cells maintained their capacity to suppress. Taken together, these data further demonstrate that the suppression induced by this activated OKT4+ population is not secondary to the emergence of OKT8+ cells in culture.

Discussion

The experiments reported above demonstrate functional heterogeneity within the OKT4+ subset of human T cells. We found that although in vitro PWM-activated OKT4+ cells can function as helper cells, these activated OKT4+ cells can also inhibit B cell differentiation induced by fresh OKT4+ cells, and this suppressor function is radiosensitive. Interestingly, the expression of suppression mediated by activated OKT4+ cells requires the presence of radiosensitive populations within the unactivated OKT4+ cells. These data demonstrate the role of T-T interactions among cells contained exclusively within the OKT4+ population in the immunoregulation of B cell differentiation.

In previous studies, we showed that addition of graded numbers of nonirradiated OKT4+ cells to B cells did not result in a linear increase of PFC, which would be predicted if the OKT4+ population were exclusively inducer cells. Instead, we observed a plateauing effect at relatively low OKT4+ cell concentrations and a subsequent decline of PFC at high concentrations. Because of this observation, we proposed the possibility that suppressor cells are generated within the OKT4+ population alone (7). In the experiments reported here, we found that small numbers of activated OKT4+ cells were able to suppress B cell differentiation induced by fresh OKT4+ cells. It is unlikely that the suppression observed was secondary to cell crowding by the activated OKT4+ cells because (a) activated OKT4+ cells did not suppress fresh OKT4+ cells that have been pretreated with 1,250 rad; (b) addition of identical numbers of control cultures or fresh OKT4+ cells did not suppress PFC activity; and (c) the yields of viable cells at the end of the culture period were similar in all the secondary cultures. In addition, the emergence of suppressor function within the activated OKT4+ population was not secondary to PWM-induced expansion of OKT8+ cells because the surface phenotype of activated OKT4+ cells with respect to OKT3, OKT4, and OKT8 did not change.

It is of interest that some mechanisms involved in the suppression mediated by activated OKT4+ cells are in fact similar to those involved in OKT8+-mediated suppression. For example, the suppressor function mediated by these two populations was radiosensitive, and importantly, both required the presence of radiosensitive cells within the unactivated OKT4+ population. Therefore, the data presented here further support the view that cooperative interactions between distinct T cell sets must occur before suppressor activity is expressed and that radiosensitive OKT4+ cells participate in immunoregulatory functions other than induction of B cell differentiation. Furthermore, these data also indicate that the OKT4+ population contains precursors cells that can be induced to further differentiate into suppressor cells independent of OKT8+ cells. The precise regulatory mechanism of this type of T-T interaction cannot be resolved at the present time. One possibility is that radiosensitive OKT4+ cells can
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induce either OKT4+ cells or OKT8+ cells to further differentiate into mature suppressor cells. Alternatively, it is possible that the target of suppression by OKT8+ cells or by activated OKT4+ cells are radiosensitive OKT4+ cells. Clearly, these two models are not mutually exclusive.

Although activated OKT4+ cells can exert potent feedback suppression, activated OKT4+ cells also contain helper cells. Unlike the helper function mediated by unactivated or fresh OKT4+ cells, the help induced by the activated OKT4+ cells is radioresistant over a wide range of cell concentrations. One interpretation of this observation is that the differential radiosensitivity observed within the OKT4+ populations depends in part on their state of activation. This hypothesis is consistent with previous reports suggesting that lectin- or antigen-activated lymphocytes become more radioresistant (13, 14). At the present time, it is unknown whether the radiosensitive and radioresistant helper cells within the OKT4+ population represent two distinct population or reflect the existence of a single population of OKT4+ cells whose radioresistant cells arise from resting OKT4+ cells within the same subset. Additional monoclonal antibodies reacting with a fraction of OKT4+ cells might dissect the putative distinct helper populations and, in addition, may distinguish helper from suppressor cells within the OKT4+ population. Nevertheless, these data indicate that the state of activation of the OKT4+ population dictates the relative degree of help or suppression that will be mediated by this subset. This information should be considered in studies investigating the numbers of OKT4+ and OKT8+ cells in patients with rheumatoid arthritis, systemic lupus erythematosus, and other disorders in which activated T cells have been demonstrated in vivo (15).

Summary

In this report, we explored the functional heterogeneity within the OKT4+ subset of human T cells. Evidence was obtained that although in vitro pokeweed mitogen-activated OKT4+ cells can function as radioresistant helper cells, these activated OKT4+ cells could also exert potent feedback suppression. Despite the induction of suppressor cells after pokeweed mitogen activation, the OKT4+ population maintains its original OKT3+, OKT4+, and OKT8- surface phenotype. The suppressor cells contained within the activated OKT4+ population were found to be radiosensitive. Importantly, the suppression mediated by activated OKT4+ cells required the presence of radiosensitive cells contained within the resting OKT4+ population. Taken together, these results suggest that the OKT4+ subset of human T cells contains cells that can be activated to differentiate into suppressor cells independent of OKT8+ cells.

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