Functional Heterogeneities among Concanavalin A-activated OKT4$^+$ and OKT8$^+$ Cells by Using Autologous Erythrocyte Rosette Technique

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ABSTRACT Normal human peripheral blood T lymphocytes activated by concanavalin A (Con A) were fractionated into OKT4$^+$ and OKT8$^+$ populations by complement-dependent cell lysis using OKT8 and OKT4 antibodies, respectively. By using the preferential ability of some, but not all, Con A-activated T cells to form rosettes with autologous erythrocytes, each population was further divided into autorosetting cells and nonautorosetting cells, and thus Con A-activated OKT4$^+$ autorosetting, OKT4$^+$ nonautorosetting, OKT8$^+$ autorosetting, and OKT8$^+$ nonautorosetting cells were obtained. The immune regulatory function of these populations was then investigated using a pokeweed mitogen-driven B cell plaque-forming cell system. These studies demonstrated that (*a*) autorosetting cells can exert potent suppressor activity regardless of their phenotypes of OKT4$^+$ and OKT8$^+$ antigens, and fail to help B cell differentiation; suppressor function mediated by these cells is radiosensitive; moreover, receptors for autologous erythrocytes may constitute either the interleukin 2 (IL2) receptors themselves or a component of an IL2 receptor-effector complex involved in modulating the growth signal that IL2 transmits to T cells; (*b*) OKT4$^+$ nonrosetting cells serve adequately as radioresistant helper cells, but are devoid of suppressor cells; and (*c*) OKT8$^+$ nonrosetting cells are found to lack either suppressor or helper activity, suggesting that they may belong to a T lymphocyte subset distinct from the subsets related to immune regulation. The results lead us, therefore, to the conclusion that there may exist functional heterogeneities among both the OKT4$^+$ and OKT8$^+$ populations; these heterogeneities can be dissected by virtue of the autologous erythrocyte rosette technique.

INTRODUCTION

Human peripheral T lymphocytes can be subclassified on the basis of differential reactivity to monoclonal antibodies, OKT4 (inducer/helper) and OKT8 (cytotoxic/suppressor). Functional studies indicate that OKT4$^+$ T cells can induce B cell differentiation in a pokeweed mitogen (PWM)[1]- or antigen-driven system, but do not provide suppressor activity, whereas the reverse is true of OKT8$^+$ T cells (1–3).

In our previous studies (4), we found that concanavalin A (Con A)-induced suppressor cells can be identified and separated from Con A-induced helper cells by the autologous erythrocyte rosette technique; suppressor and helper cells are confined to the autorosetting and nonrosetting T cell populations, respectively. Moreover, OKT4$^+$ and OKT8$^+$ cell types were equally distributed among either autorosetting or nonrosetting T cells activated by Con A (4). These experiments raised the possibility that suppressor cell populations among the autorosetting cells are limited to OKT8$^+$ cells, or alternatively, that the autorosetting OKT4$^+$ cells would also act as mediators of suppression induced by Con A. It also remained uncertain whether among the nonrosetting cell population, only the cells bearing OKT4$^+$ antigen can function as helper cells, or the OKT8$^+$ cells may also mediate helper function.

In the present study, Con A-activated T cells were divided into OKT4$^+$ autorosetting, OKT4$^+$ nonrosetting,

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[1] *Abbreviations used in this paper:* C, complement; Con A, concanavalin A; IL2, interleukin 2; PFC, plaque-forming cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SLE, systemic lupus erythematosus; SRBC, sheep erythrocyte.

$OKT8^+$ autorosetting and $OKT8^+$ nonrosetting cell populations by means of monoclonal antibodies and autorosette technique, and the regulatory effects of each separate population were investigated on the B cell immunoglobulin production in the system driven by PWM. We observed that Con A-induced suppressor cells can belong to the autorosetting populations, regardless of the phenotype of either $OKT4^+$ or $OKT8^+$ antigens. In contrast to the function of autorosetting cells, activated nonrosetting $OKT4^+$ cells are specific for the function of help. Of interest is that nonrosetting $OKT8^+$ cells are related to neither helper function nor suppressor function. Furthermore, relationship between Con-A activated T cell subpopulations and population bearing Tac antigen will be discussed.

METHODS

Isolation of T cells, non-T cells, and monocytes. Peripheral blood mononuclear cells of normal individuals were isolated, and T and non-T cells separated by the sheep erythrocyte (SRBC) rosette technique as described previously (5, 6). Double-purified rosetting T cells were recovered after lysis of SRBC by hypotonic shock. These T cell preparations consisted of >95% T cells as determined by rerosetting. Double-purified nonrosetting cells were incubated in a 60 × 55-mm petri dish (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) at 37°C in a 5% CO_2/95% air humidified environment for 2 h, and the cells that did not adhere to the dish and those that adhered firmly were collected separately. The latter cells are referred to as monocytes. Monocyte preparations consisted of 95% cells that were identified as monocyte after Giemsa staining (5, 6). The nonrosetting cells that did not adhere were further treated with OKT3 monoclonal antibody (Ortho Pharmaceutical Corp., Raritan, NJ) and rabbit complement (C; Behringwerke AG, Marburg, FRG). This C-dependent lysis was performed twice. The resultant population was <0.2% SRBC rosette positive and <0.5% cells reactive with OKT3 antibody. This population is designated non-T cells. The reappearance of the OKT3-defined antigen in cells of the non-T cell population (7) did not occur (<1%) when the cells were incubated for 24 h in vitro in the culture medium; moreover, this population did not respond at all to phytohemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, England).

Activation of T cells with Con A (first culture). Con A-activated cultures were established in 17 × 100-mm plastic tubes (Falcon Labware) and consisted of 4 ml of culture medium, RPMI 1640 (Flow Laboratories Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories Inc.) containing 4×10^6 purified T cells and 3×10^5 mitomycin (Sigma Chemical Co., St. Louis, MO)-treated monocytes. These cultures were incubated at 37°C in a 5% CO_2/95% air humidified environment in the presence or absence of 40 μg of Con A (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, NJ). 48 h later, the cells were harvested, treated with 0.1 M α-methyl-D-mannoside (Sigma Chemical Co.) in Hanks' balanced salt solution (HBSS) for 30 min at room temperature and were followed by successive washes in 0.1 M α-methyl-D-mannoside.

C-dependent lysis of T cells with the monoclonal antibodies OKT8 and OKT4. 1×10^7 T cells (fresh T cells, T cells from the first culture) were incubated with 100 μl of either OKT8 or OKT4 monoclonal antibody (Ortho Pharmaceutical Corp.) on ice for 1 h. Thereafter 25 μl of rabbit C was added and further incubation carried out for 1 h at 37°C. To obtain highly purified T cell subpopulations, this entire procedure was repeated twice (8). After washing, the numbers of the viable cells were determined by dye exclusion. Analysis of T cell subsets obtained by the OKT8-treated population contained >92% $OKT4^+$ cells and <2% $OKT8^+$ cells, whereas the OKT4-treated population contained <3% $OKT4^+$ cells and >90% $OKT8^+$ cells. In addition, the surface phenotypes of these depleted T cell subsets held in culture for 24 h did not change. These OKT8- and OKT4-depleted cell populations will be referred to as $OKT4^+$ and $OKT8^+$ cell populations, respectively, because there is evidence so far that OKT4 and OKT8 antigens can be expressed independently of one another (1, 2, 9).

Rosette formation of Con A-activated T cells with autologous erythrocytes and separation of autorosetting cells from nonrosetting cells. To assess the ability of Con A-activated T cell subpopulations to form rosettes with autologous erythrocytes, 2×10^6 Con A-activated $OKT4^+$ and $OKT8^+$ cells in 200 μl HBSS were placed in 200 μl of fetal bovine serum and 500 μl of 2.5% autologous erythrocytes in HBSS in 12 × 75-mm tubes (Falcon Labware), where they were incubated at 37°C for 10 min. After the incubation, the mixtures were centrifuged at 150 *g* for 5 min and further allowed to react on ice for 2 h. The cells were gently resuspended for either counting the autorosettes or for their separation on Ficoll-Hypaque gradients from nonrosetting cells (4). For counting, at least 300 lymphocytes were enumerated and T cells binding three or more autologous erythrocytes were regarded as autorosette-forming cells. For separation, the autologous erythrocyte-T cell suspension was layered over Ficoll-Hypaque, centrifuged at 400 *g* for 20 min at 4°C, and rosetting cells and nonrosetting cells were collected separately. The pelleted rosette-forming cells were resuspended in cold HBSS and further purified on another Ficoll-Hypaque gradient. The rosetting fraction obtained had ≧95% autorosettes. Autologous erythrocytes in this fraction were lysed by hypotonic shock. The nonrosetting population was rerosetted with autologous erythrocytes exactly as described above and recentrifuged over Ficoll-Hypaque. This purification procedure gave a fraction containing only 1% autorosettes. Thus, Con A-activated $OKT4^+$ autorosetting, $OKT4^+$ nonrosetting, $OKT8^+$ autorosetting, and $OKT8^+$ nonrosetting cells were obtained.

Assay cultures for regulatory activities of Con A-activated T cell subpopulations (second culture). Responder non-T cells to be used in the second helper and suppressor assay cultures, and helper $OKT4^+$ cells to be used in the second suppressor assay cultures were obtained 2 d later from the same normal individuals who originally provided the Con A-activated regulatory T cells (10). The ability of activated T cell subpopulations to exert helper activity was determined by adding graded numbers of each cell type to 2×10^5 fresh autologous responder non-T cells in a 1-ml volume in 12 × 75-mm plastic tubes. Polyclonal immunoglobulin production was stimulated by the addition of 1 μg of PWM (Gibco Laboratories, Grand Island, NY) to these cultures, which were incubated at 37°C in an atmosphere of 5% CO_2 in air for 5 d. Thereafter the cells were harvested, washed extensively, and assayed for plaque-forming cell (PFC) activity by a reverse hemolytic plaque assay (see below). Activated T cell populations from the first culture were also tested for their suppressor activity in the second assay culture system. Graded numbers of the activated T cell subpopulations were added to the second autologous cultures containing 2×10^5

fresh responder non-T cells and 1×10^5 fresh $OKT4^+$ cells as helper cells, stimulated for 5 d with PWM, and assayed for PFC activity. In some experiments, activated T cell subpopulations were irradiated with 1,300 rad before the addition to the second culture.

The percent suppression was calculated with the following formula:

$$\% \text{ suppression} = \left(1 - \frac{(\text{PFC activity of non-T cells + fresh } OKT4^+ \text{ cells + regulatory T cell subsets + PWM}) - (\text{PFC activity of non-T cells + fresh } OKT4^+ \text{ cells + regulatory T cell subsets})}{(\text{PFC activity of non-T cells + fresh } OKT4^+ \text{ cells + PWM}) - (\text{PFC activity of non-T cells + fresh } OKT4^+ \text{ cells})} \right) \times 100.$$

Cell counts and viability (by dye exclusion) were also performed on all the second cultures at the time of assay. The yields of viable cells at the end of the culture period were similar in all the second assay cultures.

Reverse hemolytic plaque assay for the enumeration of antibody-secreting cells. The assay for the measurement of immunoglobulin M-producing hemolytic PFC was performed by using a modification of the procedure described by Gronowicz et al. (11). Briefly, on the day of assay, PWM-stimulated cells were thoroughly washed in HBSS and resuspended. 100-μl aliquots ($5–10 \times 10^4$ PWM-stimulated cells) were added to 300 μl of 0.5% agarose in HBSS, 20 μl of protein A (Pharmacia Fine Chemicals)-coupled SRBC (25% vol/vol), and 20 μl of a 1:50 dilution of the IgG fraction of a rabbit anti-human IgM (Miles Laboratories, Inc., Miles Research Products, Elkhart, IN). This mixture was poured onto a 60 × 15-mm petri dish and allowed to gel. The dishes were incubated for 8 h at 37°C in a humidified atmosphere containing 5% CO_2/95% air. They were then covered with a 1:20 dilution of absorbed guinea pig C (Toshiba Chemical Co., Tokyo) and incubated for additional 2–3 h at 37°C. Hemolytic plaques were enumerated in triplicate and the results were expressed as the mean PFC/10^6 non-T cells in the original culture (12). The standard error of the mean was always <20%.

RESULTS

Autorosette levels of Con A-activated T cell subpopulations. After Con A stimulation, normal T cells were fractionated into $OKT4^+$ and $OKT8^+$ cells, and then each cell population was further rosetted with autologous erythrocytes. Both the $OKT4^+$ and $OKT8^+$ cell populations formed rosettes with autologous erythrocytes to roughly the same extent (mean percentages of autorosettes with SEM in the activated $OKT4^+$ and $OKT8^+$ cell populations were 39.2±0.8 and 35.8±1.8%, respectively). In contrast, ~1% of control unactivated $OKT4^+$ or $OKT8^+$ cells cultured in medium alone formed rosettes with autologous erythrocytes.

Suppressor activity of Con A-activated T cell subpopulations. Con A-activated $OKT4^+$ and $OKT8^+$ cell populations were further fractionated into autorosetting and nonrosetting cells. To evaluate the ability of T cell subsets to mediate suppression of B cell PFC activity, graded numbers of each subtype of Con A-activated cells were added to second autologous cultures containing 2×10^5 non-T cells plus 1×10^5 fresh $OKT4^+$ cells (source of helper cells) in the presence of PWM. The results of a representative experiment are depicted in Fig. 1 *A*, and clearly show that the addition of small numbers of activated autorosetting cells suppressed B cell immunoglobulin production, regardless of their phenotype of $OKT4^+$ or $OKT8^+$ antigen. In marked contrast, both $OKT4^+$ and $OKT8^+$ nonrosetting cells had only minimal inhibitory activity. Thus, these data suggest that suppressor cells within the Con A-activated cells are restricted to a distinct subset of either $OKT4^+$ or $OKT8^+$ cells; this subset is capable of forming rosettes with autologous erythrocytes after Con A activation.

To determine whether the above experiment was unique to lymphocytes for this single normal individual, lymphocytes from additional 11 normal individuals were studied and the results summarized in Fig. 2. In this experiment, 1×10^5 cells of Con A-activated or unactivated T cell subsets were introduced into the second autologous cultures. Consistent with Fig. 1, activated autorosetting cells, either $OKT4^+$ or $OKT8^+$, led to potent suppression of PFC activity. In contrast, activated nonrosetting $OKT4^+$ as well as $OKT8^+$ cells gave no demonstrable suppression. The suppressor T cell activity mediated by Con A-activated autorosetting cells seemed to be Con A dependent. As can be seen in Fig. 2, when fresh $OKT4^+$ cells or unactivated control $OKT4^+$ cells cultured in medium alone were added to the second cultures, the suppression of immunoglobulin production failed to occur. The addition of fresh or unactivated $OKT8^+$ cells to the second cultures resulted in significant suppression of the PFC response; these $OKT8^+$ cell populations were, however, far less efficient in suppressing B cell differentiation than activated autorosetting $OKT8^+$ cells. The suppression observed in the fresh and unactivated $OKT8^+$ cells would occur due to the activation of these cells by PWM-activated $OKT4^+$ cells present in the second cultures.

It should be noted that the emergence of suppressor function within the activated $OKT4^+$ population was not secondary to Con A-induced expansion of cells that lost $OKT8^+$ antigens and acquired new antigens, $OKT4^+$ antigens during the culture period; when $OKT4^+$ cells were first isolated and then activated for 2 d with Con A, the potent suppression could be also generated by Con A activation of $OKT4^+$ populations (data not shown); in addition, the surface phenotype of these activated $OKT4^+$ cells (which had been first isolated

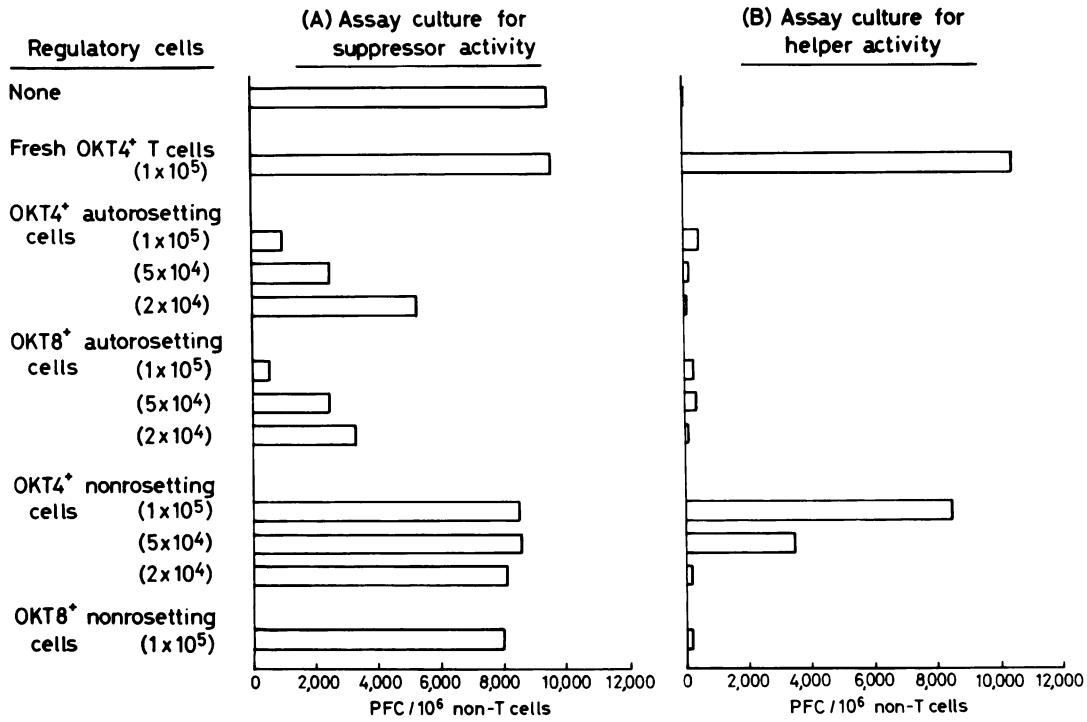

FIGURE 1 Suppressor and helper activities of Con A-activated T cell subpopulations: a representative experiment. T cells activated with Con A for 48 h (first culture) were fractionated into $OKT4^+$ and $OKT8^+$ cells, followed by further fractionation by using autologous erythrocyte rosette technique. These fractionated T cell subsets were used as regulatory cells in the second culture; graded numbers of cells from Con A-activated T cell subsets were added to the second autologous cultures containing PWM-driven non-T cells (2×10^5) plus fresh $OKT4^+$ cells (1×10^5) for assay on suppressor activity (*A*), or those containing PWM-driven non-T cells (2×10^5) for assay on helper activity (*B*). 5 d later, cultures were harvested and assayed for PFC activity.

and activated by Con A) with respect to OKT3, OKT4, and OKT8 did not change (>95% $OKT3^+$ cells, >90% $OKT4^+$ cells, and <3% $OKT8^+$ cells). It is also unlikely that such suppression was mediated by contaminated $OKT8^+$ cells in the non-T cell preparations through their activation by Con A-activated $OKT4^+$ populations that would function as suppressor-inducer cells (13, 14); because in the present study, only highly purified non-T cell populations were used, $OKT8^+$ cells that might act as suppressor-effector cells would not have been present in sufficient numbers to mediate suppression (Methods). Moreover, it is very unlikely that such suppression mediated by $OKT4^+$ cells represents merely a shift in time at which the maximal PFC response occurs; when activated autorosetting $OKT4^+$ cells were added to the second autologous cultures of 2×10^5 non-T cells and 1×10^5 $OKT4^+$ cells, the results of IgM-PFC generation showed profound suppression throughout the second culture period with no evidence of altered kinetics of response (Fig. 3 *A*). Moreover, any PFC activity of second assay cultures was not induced by activated $OKT4^+$ autorosetting cells, regardless of the time of harvest (Fig. 3 *B*). Finally, the suppression is not due to cytotoxic effect by the activated autorosetting cells, because both $OKT4^+$ and $OKT8^+$ autorosetting cells did not produce a cytotoxic effect at all on the non-T cells (responder cells of the second helper and suppressor assay cultures), or on the fresh $OKT4^+$ cells (helper cells of the second suppressor assay cultures) as judged by ^{51}Cr-release assay system (data not shown). Thus, Con A-activated autorosetting $OKT4^+$ cells could mediate suppressor function observed.

Helper activity of Con A-activated T cell subpopulations. In earlier studies (4), we found that activated nonrosetting cells contain helper cells. We next investigated whether the autologous erythrocyte rosette technique could dissect the helper and suppressor cell functions within the activated $OKT4^+$ or $OKT8^+$ cells. To address this question, graded numbers of Con A-activated T cell subpopulations from the first culture were added to the second cultures containing PWM-

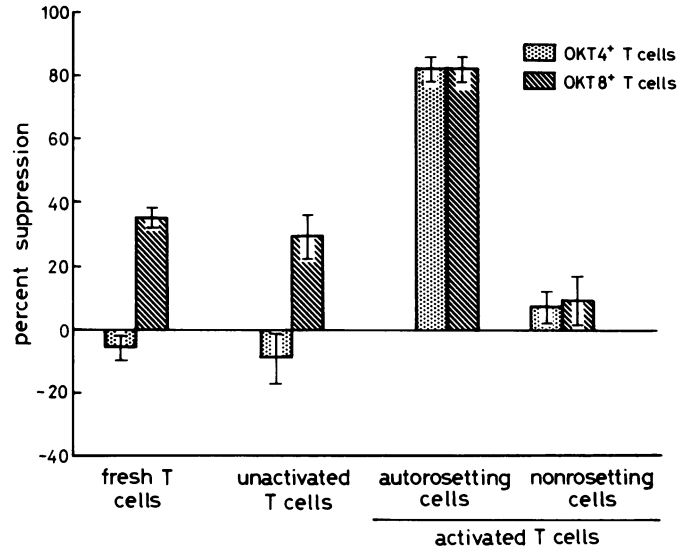

FIGURE 2 Differential suppressor activity of Con A-activated T cell subpopulations: summary of the results obtained from 12 normal individuals. The standard culture contained 1×10^5 fresh OKT4$^+$ cells and 2×10^5 non-T cells in addition to 1 μg of PWM. To this system were added 1×10^5 cells from either Con A-activated or unactivated T cell subpopulations. After 5 d, cultures were harvested and assayed for PFC activity. The mean percent suppression and the range encompassed by ±1 SEM are shown.

driven autologous non-T cells. As shown in Fig. 1 *B* (a representative experiment) and in Fig. 4 (summary of such experiments), only nonrosetting OKT4$^+$ cell population that had been activated by Con A could function in the role of helper cells for B cell differentiation; the degree of helper activity by these cells was as great as

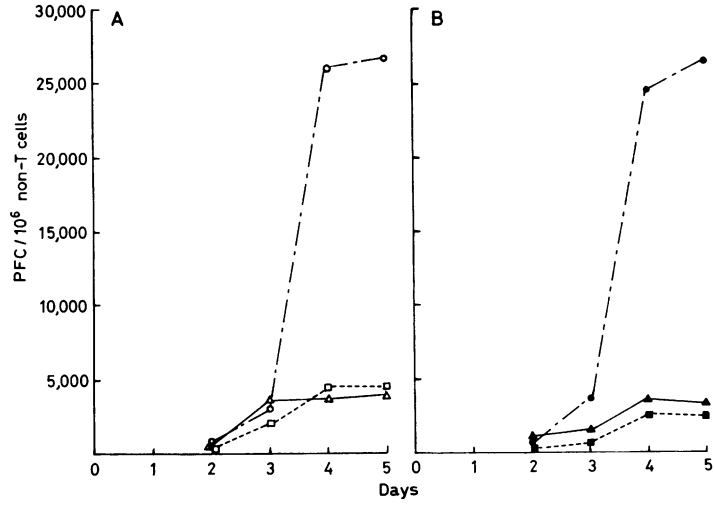

FIGURE 3 Kinetics of suppressor and helper activities of Con A-activated autorosetting OKT4$^+$ or OKT8$^+$ cells. (*A*) 1×10^5 of either activated autorosetting OKT4$^+$ (——), OKT8$^+$ (· · ·) cells or fresh OKT4$^+$ (— · —) cells were added to the second autologous culture containing 2×10^5 non-T cells and 1×10^5 fresh OKT4$^+$ cells in the presence of 1 μg of PWM. (*B*) 1×10^5 of either activated autorosetting OKT4$^+$ (——), OKT8$^+$ (· · ·) cells, or fresh OKT4$^+$ (— · —) cells were added to the second culture containing PWM-driven autologous non-T cells (2×10^5). At daily intervals, cultures were harvested and assayed for PFC activity. Additional two experiments were performed and similar results were obtained.

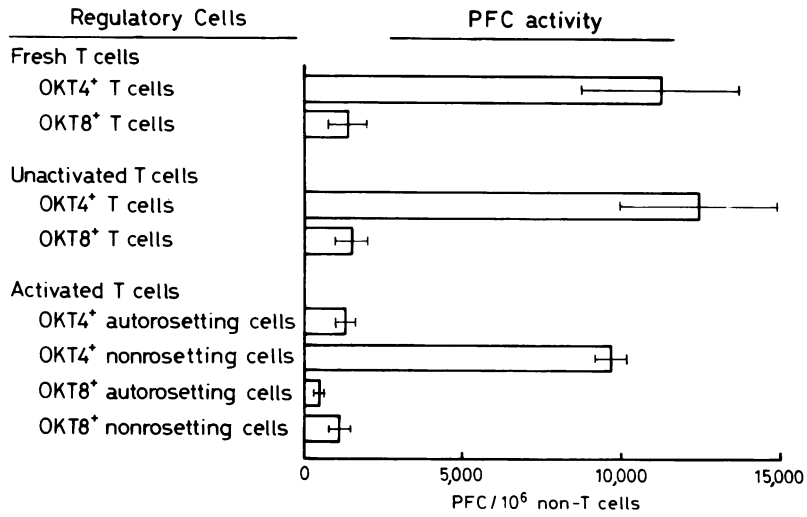

FIGURE 4 Differential helper activity of Con A-activated T cell subpopulations: summary of the results from 12 normal individuals. The standard culture contained 2×10^5 non-T cells in addition to 1 μg of PWM. To this system were added 1×10^5 cells from Con A-activated or unactivated T cell subpopulations. After 5 d, cultures were harvested and assayed for PFC activity. The mean PFC response±1 SEM is shown.

the activity by either fresh $OKT4^+$ cells (Figs. 1 *B* and 4) or by control unactivated $OKT4^+$ cells (Fig. 4). In contrast, activated autorosetting $OKT4^+$ and $OKT8^+$ cells (that exerted potent suppressor activity) and activated nonrosetting $OKT8^+$ cells (which did not exhibit any suppressor activity) failed to induce B cell differentiation at all (Figs. 1 *B* and 4). As expected, there was no effect of fresh $OKT8^+$ cells and control unactivated $OKT8^+$ cells on inducing PFC activity.

Radiosensitivity of the regulatory functions mediated by Con A-activated T cell subpopulations. We asked whether the regulatory activities induced by Con A-activated T cell subpopulations are radiosensitive. To this end, the activated T cell subpopulations were first irradiated (1,300 rad) before addition to the second cultures. 1×10^5 cells of nonirradiated or irradiated activated T cell subpopulations were then added to a constant number of fresh autologous $OKT4^+$ cells and non-T cells (for assay on suppressor activity) or fresh autologous non-T cells (for assay on helper activity) in the presence of PWM (Fig. 5). The addition of nonirradiated autorosetting cells regardless of their $OKT4^+$ or $OKT8^+$ phenotype, suppressed the PFC response (Fig. 5 *A*). The activated autorosetting cells that had been irradiated, however, no longer suppressed (Fig. 5 *A*). In addition, these irradiated cells failed to promote the PFC response of B cells (Fig. 5 *B*). Thus, the suppressor function mediated by activated $OKT4^+$ and $OKT8^+$ cells is radiosensitive, and helper cells (at least radioresistant ones) are not included in these cell populations.

Contrary to the activated autorosetting cells, the helper activity of activated $OKT4^+$ nonrosetting cells was radioresistant (Fig. 5 *B*). These irradiated cells exhibited a helper function similar to that of the nonirradiated $OKT4^+$ nonrosetting cells (Fig. 5 *B*).

Response of Con A-activated suppressor T cell subpopulations to interleukin 2 (IL2). We have next examined whether Con A-activated T cell subpopulations proliferate in response to IL2 stimulation. As shown in Table I, Con A-activated autorosetting T cells regardless of their phenotypes, responded well to standard IL2 (Bethesda Research Laboratories, Bethesda, MD), whereas minimal response was observed in the activated nonrosetting $OKT4^+$ and $OKT8^+$ cell populations. These results indicate that Con A-activated suppressor cells (that are capable of forming autorosettes) are susceptible to further stimulation by this factor, probably by expressing receptors for IL2 during the course of Con A stimulation (15, 16). The results further suggest that the suppressor activity of Con A-activated autorosetting cells observed would occur due to the following processes: (*a*) Con A-activated autorosetting cells may absorb IL2 produced by the fresh $OKT4^+$ cells present in the second assay cultures; (*b*) this process would deprive the $OKT4^+$ helper cells of the necessary growth factor, thereby not causing the PWM-induced expansion of $OKT4^+$ helper cells (16); and (*c*) thus, a decreased PFC response of B cells induced by PWM would occur.

To test this hypothesis, standard IL2 was added at a final concentration of 20% to the second suppressor

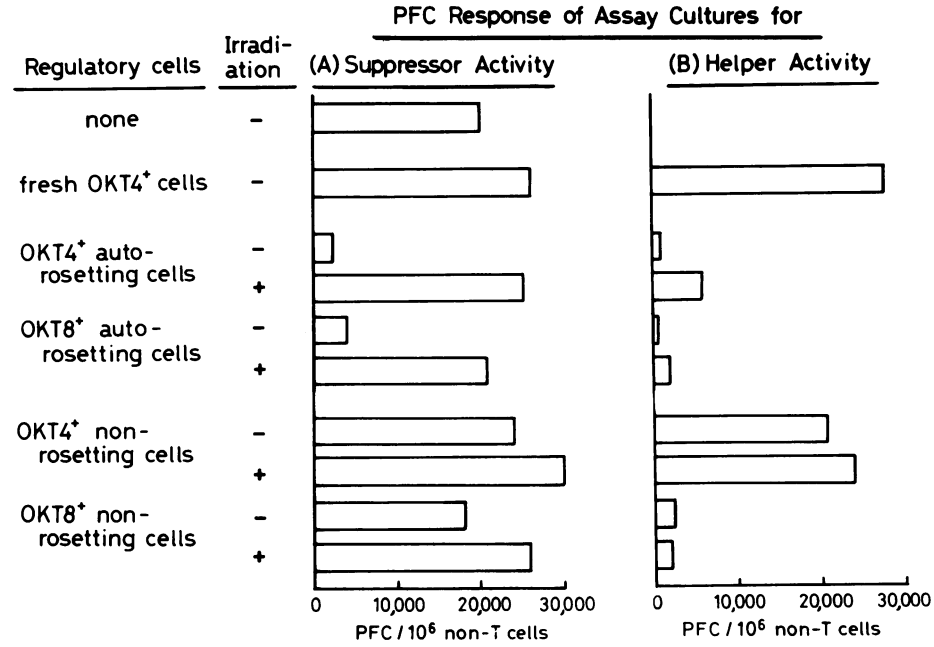

FIGURE 5 Effects of irradiation to Con A-activated T cell subpopulations on their regulatory activities. (*A*) Con A-activated T cell subpopulations were irradiated (1,300 rad) before the addition to the second culture. 1×10^5 cells from the irradiated or nonirradiated T cell subpopulations were added to standard autologous cultures containing 2×10^5 non-T cells and 1×10^5 fresh $OKT4^+$ cells in addition to PWM. (*B*) 1×10^5 cells from the irradiated or nonirradiated Con A-activated T cell subpopulations were added to the standard cultures containing 2×10^5 non-T cells in the presence of PWM. Additional three experiments were performed and similar results were obtained.

assay cultures containing graded numbers of the autorosetting cells. After 5 d of incubation, the cells were harvested, and the suppressor activity evaluated by PWM-induced PFC activity. If the hypothesis is valid, then, addition of exogenous IL2 to the second culture should result in saturation of IL2 receptors expressed on the surface of Con A-activated cells, thereby causing them to lose their suppressive capacity. The results of such experiments are shown in Fig. 6. When the sufficient numbers (1×10^5 cells/culture) of $OKT4^+$ or $OKT8^+$ autorosetting cells were used as suppressor cells, a potent suppressive effect was observed, regardless of the addition of IL2. However, when the number of autorosetting cells to be added was reduced to 2×10^4 cells/culture, the suppressor activity was enhanced evidently in the presence of IL2, both with $OKT4^+$ and with $OKT8^+$ cells. This means that when the suppressor cells sufficient to inhibit the induction of B cells are present in the second culture, IL2-induced expansion of the suppressor cells is not necessary for the emergence of their suppressor function. In contrast, when the number of suppressor cells added to the second cultures is small, it is necessary that IL2 interacts with the activated autorosetting cells to promote their proliferation and differentiation; their number then reaches an amount sufficient to maximally inhibit the assay cultures. Therefore, the suppressor activity by activated autorosetting cells does not occur because of their absorption of, and the decreased available level of IL2 produced in the second cultures; the Con A-induced autorosetting cells are presumed to contain "genuine" suppressor cells, which have a suppressive effect on the immune response.

Relationship between Con A-induced autorosetting cells and cells with Tac antigen. Tac antigen has been demonstrated to be expressed in conjunction with acquisition of IL2 receptors on the activated T cells (17, 18); the Tac antigen is associated with ~20% of Con A-activated T cells; these Con A-activated T cells bearing the Tac antigen act as suppressor cells (19, 20). Con A-activated cells bearing receptors for autologous erythrocytes also respond vigorously to IL2, indicating that expression of receptors for autologous erythrocytes may be related with expression of Tac antigen. To test this possibility, the procedure for autologous erythrocyte rosette formation was performed in the presence of anti-Tac antibody (kindly provided by Dr. Takashi Uchiyama, Kyoto University School of Medicine, Kyoto,

TABLE I
*Response of Cells from Con A-activated T Cell Subpopulations to IL2**

Cells	IL2 added	[^{3}H]Thymidine incorporated
		cpm‡
Fresh T		
OKT4$^+$	−	120±50
	+	1,298±466
OKT8$^+$	−	85±26
	+	1,800±517
Unactivated T		
OKT4$^+$	−	154±85
	+	1,969±256
OKT8$^+$	−	223±129
	+	2,119±104
Con A-activated T		
OKT4$^+$ autorosetting	−	93±26
	+	15,037±1,015
OKT4$^+$ nonrosetting	−	72±15
	+	3,845±239
OKT8$^+$ autorosetting	−	93±19
	+	16,107±2,195
OKT8$^+$ nonrosetting	−	63±15
	+	4,545±983

* Standard IL2 was added at a final concentration of 20% to 1 × 10^4 cells/well of Con A-activated T cell subpopulations and incubated for 72 h at 37°C. Proliferation was determined by measuring [^{3}H]thymidine incorporation during the last 6 h of culture.
‡ Mean±SEM cpm of four separate experiments, each in triplicate.

Japan). Table II illustrates the effects of varying concentrations of anti-Tac antibody (1:500 to 1:10,000 dilution) on inhibition of autologous erythrocyte binding to Con A-activated T cells. Anti-Tac antibody had no significant effect on autologous erythrocyte binding to Con A-activated OKT4$^+$ cells, and similarly, this antibody had no significant effect on the binding to Con A-activated OKT8$^+$ cells. Thus, it is unlikely that the anti-Tac antibody may interact directly with the receptor for autologous erythrocytes.

If anti-Tac antibody and autologous erythrocytes both preferentially bound to the same cell subclass, then the percentage of cells reactive with anti-Tac antibody should be increased or decreased in the populations enriched for or depleted of autorosetting cells, respectively. To investigate this point, cells reactive with anti-Tac antibody were determined in the Con A-activated autorosetting or nonrosetting populations by indirect immunofluorescence under a fluorescence microscope. In this experiment, anti-Tac antibody was used at a dilution of 1:5,000, because our preliminary titration studies demonstrated that this concentration was optimal to visualize positive cells. The results of such experiment are shown in Table III. Tac$^+$ cells were mostly distributed in autorosetting cell populations regardless of the surface phenotype of OKT4$^+$ or OKT8$^+$ antigens. By contrast, there was small overlap between nonrosetting cells and cells with Tac antigen. Taken together, the data indicate that substantial overlap exists between cells bearing receptors for autologous erythrocytes and those bearing Tac antigen, although au-

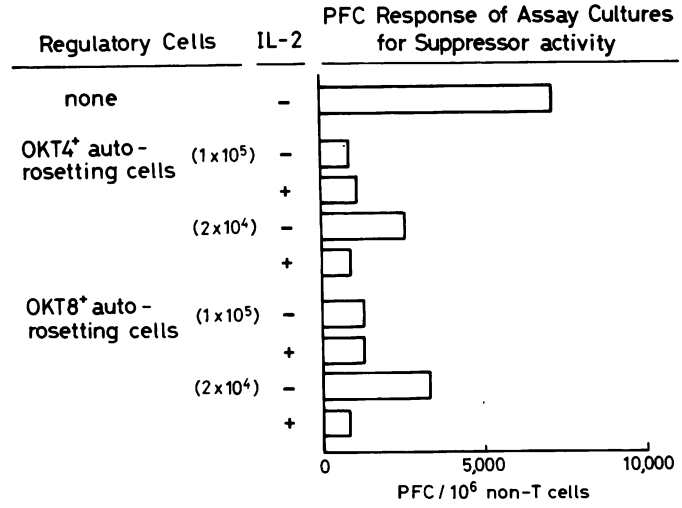

FIGURE 6 Effect of IL2 on the suppressor function mediated by Con A-activated autorosetting T cells. The standard culture contained 1 × 10^5 fresh OKT4$^+$ cells and 2 × 10^5 non-T cells in the presence of PWM. To this system were added graded numbers of either activated autorosetting OKT4$^+$ or OKT8$^+$ cells and incubated for 5 d with or without IL2 at a final concentration of 20%. Additional three experiments were performed and similar results were obtained.

TABLE II
*Effect of Anti-Tac Antibody on Ability of Con A-activated T Cell Subsets to Form Autorosettes**

Dilution of anti-Tac antibody	Con A-activated T cell subsets	
	OKT4$^+$	OKT8$^+$
	% autorosettes‡	
0	33.3±2.9	31.0±4.2
1:500	31.3±1.8	33.3±3.2
1:1,000	31.7±3.8	30.0±4.5
1:5,000	33.7±4.3	33.3±4.2
1:10,000	30.0±4.0	31.0±3.8

* Con A-activated OKT4$^+$ or OKT8$^+$ cells were rosetted with autologous erythrocytes in the presence of anti-Tac antibody at various concentrations.
‡ Mean percentage (±SEM) of three separate experiments.

tologous erythrocytes and anti-Tac antibody do not bind to the same antigenic determinants on the same cells.

DISCUSSION

The experiments reported above demonstrate functional heterogeneities within both the OKT4$^+$ and OKT8$^+$ subsets of human T cells. We found that although in vitro Con A-activated OKT4$^+$ cells can function as helper cells, these activated OKT4$^+$ cells can also inhibit PWM-induced B cell differentiation; the helper function is relatively radioresistant and the suppressor function is radiosensitive. Interestingly, the suppressor OKT4$^+$ cells are separable on the basis of their preferential ability to form rosettes with autologous erythrocytes from the helper OKT4$^+$ cells; Con A-activated OKT4$^+$ autorosetting cells are specific for the function of suppression, and do not include cells manifesting helper activity. The reverse is true of the activated OKT4$^+$ nonrosetting cell population.

TABLE III
*Percentage of Tac$^+$ Cells in Con A-activated T Cell Subsets**

Con A-activated T cell subsets	Tac$^+$ cells
	%‡
OKT4$^+$ autorosetting	62.0±4.0
OKT4$^+$ nonrosetting	16.3±1.2
OKT8$^+$ autorosetting	56.7±3.0
OKT8$^+$ nonrosetting	12.3±3.8

* Con A-activated T cell subsets were analyzed by the use of anti-Tac monoclonal antibody (1:5,000) and indirect immunofluorescence with the fluorescence microscope.
‡ Mean percentage of positive cells±SEM in the Con A-activated T cell subsets from three normal individuals.

Con A-activated OKT8$^+$ cells also contain radiosensitive cells important in the suppression of B cell differentiation. Moreover, these suppressor cells are also restricted to the population forming autorosettes; the autorosetting population is depleted of helper function. With regard to OKT8$^+$ nonrosetting cells, neither helper nor suppressor activity can be observed in this population, so that these cells are likely a cell population with other immune functions rather than immunoregulatory T cells.

With regard to the heterogeneity of OKT4$^+$ cells, it has been already reported that helper cells with the phenotype of OKT4$^+$17$^+$, those with the phenotype of OKT4$^+$17$^-$, and suppressor cells with the phenotype of OKT4$^+$17$^+$ are derived from PWM-stimulated OKT4$^+$ cells (21). They reported that the OKT4$^+$17$^+$ suppressor cells are radiosensitive, but the OKT4$^+$17$^+$ and OKT4$^+$17$^-$ helper cells are radioresistant and radiosensitive, respectively (21). We also demonstrate in the present study that Con A-activated suppressor cells reside in both OKT4$^+$ and OKT8$^+$ cells, and both these suppressor populations are radiosensitive and capable of forming autorosettes. Damle and Gupta (25) also found that Con A-induced suppressor T cells reside in both OKT4$^+$ and OKT8$^+$ cell populations. With regard to helper activity mediated by OKT4$^+$ cells, radiosensitive helper activity could not be detected among these cells in the present study. However, even if radiosensitive helper cells would exist in the activated autorosetting cell population, helper activity could not be detected in this population regardless of irradiation, because suppressor cells would dominate overwhelmingly in numbers in the autorosetting T cells. Similarly, the nonrosetting OKT4$^+$ population would also contain radiosensitive helper cells. Thus, we can not rule out the possibility that these populations may contain radiosensitive helper cells induced by Con A. More recently, OKT4$^+$ cells have also been shown to contain cytotoxic T lymphocytes specific for class II HLA antigens (22–24).

It is important to emphasize that the functional heterogeneity dissected by virtue of the autologous erythrocyte rosette technique is possible only after activation, when a subset of OKT4$^+$ cells and that of OKT8$^+$ cells acquire or express much more of the receptors for autologous erythrocytes. Regarding this point, Palacios et al. (26) found that the proportion of autorosetting cells is ~30% in a fresh T cell population obtained from normal peripheral blood; we and other investigators (4, 27) could observe, however, considerable proportions of the autorosetting T cells only in the activated, but not resting, T cells. In the resting T cell population, only 3% of the cells formed rosettes with autologous erythrocytes (4, 27). These differences between our studies and those of Palacios et al. may be

explained by technical difference in the method of autorosette formation; their procedure entailed incubating T cells with autologous erythrocytes in the presence of autologous serum (26); our autorosette procedure was carried out in the presence of fetal bovine, but not human, serum. Distinct T cell populations might form autorosettes under specific conditions.

There is accumulating evidence for the idea that T cells can express different surface molecules or receptors in relation to a particular state of activation (19–21, 28–30). Furthermore, various studies have described several monoclonal antibodies directed at determinants expressed on or lost from activated, but not resting, T lymphocytes (19–21). For example, Uchiyama et al. (19, 20) have described the expression of Tac antigen on most of activated T cells; this antigen is expressed in connection with the functional activities. More recently, the Tac antigen has been demonstrated to be expressed in conjunction with acquisition of IL2 receptors on the activated T cells (17, 18). Thomas et al. (18), on the other hand, describe that the defined subgroup of T cells loses $OKT17^+$ antigen during activation with PWM. In this study, we have demonstrated that most autorosetting cells bear IL2 receptors, and thus cell populations capable of expressing receptors for autologous erythrocytes and cells bearing Tac antigen mostly overlap each other. However, Tac antigen and receptors for autologous erythrocytes are not identical, rather present indifferently on the same cell surface of activated T cells, because anti-Tac antibody cannot inhibit autorosette formation at all. Furthermore, Tac^+ subsets after the PWM stimulation contain T cells with a radioresistant helper activity, while autorosetting cells do not include radioresistant helper cells as far as we have investigated.

Recently, Palacios et al. (16) have demonstrated that the suppressor activity of the Con A-activated T cells is abrogated by the addition of exogenous IL2 to the assay cultures. They concluded therefore that after Con A activation, the resting T cells have been rendered sensitive to IL2, and thus Con A-activated cells absorb IL2 activity produced by responder cells in the assay cultures, thereby leading to their apparent suppressive effect. In our present study, however, when 1×10^5 autorosetting cells were present in the second cultures, addition of exogenous IL2 did not affect the suppressor activity. When the number of autorosetting cells was reduced to 2×10^4 cells/culture, the suppressor activity was rather enhanced by the addition of exogenous IL2 (Fig. 6). These different results between our studies and those of Palacios et al. could be explained in part by differences in technique. For example, Palacios et al. used unfractionated Con A-activated T cells, and thus the $OKT4^+$ nonrosetting cells (helper cells) that could have been included in them would further produce IL2 as well as both B cell growth factors and B cell differentiation factors (31) in the assay cultures. Further addition of exogenous IL2 (which would also contain both B cell growth factors and B cell differentiation factors) to the assay cultures could cause relative predominance of helper activity, which resulted in the apparent abrogation of suppressor function. Another cause might be the difference in the PWM-induced assay culture system. Although it cannot be ruled out that Con A-induced suppressor activity is, at least in part, an IL2-dependent inhibition, much of the suppressive effect is likely an inhibition caused by "genuine" suppressor cells.

We should investigate further in detail how and at which stage of the expression of suppressor activity by Con A-induced autorosetting cells IL2 affects the activity. Moreover, if monoclonal antibodies directed at these activated T cell subpopulations could be established, such antibodies could represent attractive candidates for in vivo use as immunomodulating agents. Although OKT3 antibody, the anti-human pan T cell reagent, has been used to produce prompt reversal of rejection in recipients of renal allografts (32), monoclonal antibodies to the specific functional, and activated T cell subsets may be far better suited for certain clinical situations in which the goal of treatment would be to diminish or enhance an ongoing response. Such studies are currently in progress.

Previous studies demonstrated that patients with active systemic lupus erythematosus (SLE) have a loss of suppressor T cell function (10, 33–37). In several studies, the importance of anti-T cell antibodies in altering T cell number and function in patients with active SLE was emphasized (38, 39). Indeed, SLE anti-T cell antibodies found in the sera of active SLE patients have been shown to preferentially react to T cells that bear the $OKT8^+$ phenotype characteristic of suppressor cells (40, 41). Moreover, such active patients in fact have a decreased proportion of $OKT8^+$ subsets (42). When in the inactive state, the patients did not have anti-T cell antibodies (6, 40), a reduction in the suppressor T cell function or in proportion of $OKT8^+$ cells was found to be largely corrected (6, 42, 43). It can therefore be postulated that the defect in suppressor function mediated by $OKT8^+$ suppressor T cell subsets in active SLE patients may result from the action of anti-T cell antibodies produced by B cells. On the other hand, it has been shown that patients with active SLE have heterogenous patterns of T cell phenotypes identified by monoclonal antibodies (44). More recently, we have also demonstrated that SLE patients have an intrinsic defect in the suppressor T cell function mediated by $OKT4^+$ cells, which occurs independently of B cell abnormalities (41). In addition, in preliminary experiments we have observed that in patients with active

SLE the autorosette levels are profoundly decreased in both Con A-activated $OKT4^+$ and $OKT8^+$ cell populations. The failure of Con A-activated SLE $OKT4^+$ cells to form autorosettes is still observed during disease quiescence. Since there exists a heterogeneity among suppressor cells, dissection of the defective suppressor cell subsets in various diseases with the use of autorosette technique and monoclonal antibodies may lead to a better understanding of the cellular basis for immune abnormalities in these disorders.

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