

Px 2018

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Volume 436

**MULTIPLE SCLEROSIS:
EXPERIMENTAL AND
CLINICAL ASPECTS**

Edited by Labe Scheinberg and Cedric S. Raine



*The New York Academy of Sciences
New York, New York
1984*

T Regulator Cell Surface Antigens in Multiple Sclerosis^a

ANTHONY T. REDER, JACK P. ANTEL, AND
BARRY G. W. ARNASON

*Department of Neurology
and
Brain Research Institute
University of Chicago
Chicago, Illinois 60637*

INTRODUCTION

The proportion of various cell subsets within the overall mononuclear cell population of peripheral blood is reported to differ in MS patients compared to age- and sex-matched controls. The differences are usually most apparent in those patients with active (progressive or relapsing) disease. Using monoclonal antibodies (mAbs) which recognize mononuclear cell surface proteins, the most consistently demonstrated changes have been found in the number of T suppressor (T_s) cells (T8⁺, T5⁺, Leu 2a⁺), although differences from controls have also been found in other cell subsets including total T cells (T3⁺) and monocytes (M1⁺). The extent to which the numbers of the above cell subsets in MS patients differ from non-MS individuals has, however, varied markedly from study to study.

In our study, we have analyzed some of the properties of T_s (T8⁺) cells from MS patients that may contribute to the discrepancies existent among enumeration studies of T_s cells in MS and may be associated with the aberrant suppressor-cell function that has been rather consistently observed in MS patients.¹⁻³ Our data indicate that the density of T8⁺ antigen on cells from MS patients with active disease is reduced.^{4,5} Using immune modulation with OKT8 as a model of reduced T8 antigen density, we can demonstrate that distinguishing cells with low antigen density from nonfluorescent cells can be difficult, a factor that in turn increases the variability of results in T_s cell enumeration studies in MS. In addition, OKT8-modulated mononuclear cells exhibit reduced functional suppressor activity.

DONORS

The MS patients included in this study were clinically rated as having either active or stable disease. The active group was comprised of patients with gradually but continually increasing disability in the preceding one year (progressive MS) or with an exacerbation in the prior week (MS relapse). Stable patients had no clinically apparent change in function over the preceding year. In all assays, MS patients' cells were studied together with cells from an age (± 10 years)- and sex-matched control.

^aThis work was supported by grants from the National Multiple Sclerosis Society and the Kroc Foundation.

METHODS

Isolation and mAb Labeling of Mononuclear Cells

Peripheral blood mononuclear cells (PBM) were separated on ficoll-hypaque (Pharmacia) density gradients and washed $\times 3$ in Hanks' Balanced Salt Solution (HBSS: GIBCO, Grand Island, NY) at 4°C. Cells ($n = 1 \times 10^6$) were pelleted in 2 ml Eppendorf tubes and incubated on ice for 30 minutes with mAbs recognizing peripheral T cells (OKT3), helper cells (OKT4), or suppressor/cytotoxic cells (OKT8, Ortho Pharmaceuticals, Raritan, NJ). Stock solutions of mAbs containing 0.1% Na azide were suspended in HBSS containing 10% heat inactivated and ultracentrifuged normal goat serum (NGS), previously absorbed with mouse Ig, at a dilution of 1:20. This dilution had been previously determined to be a saturating concentration. Fifty μ l of diluted mAb were added to each tube. Cells were incubated for 30 minutes on ice and then washed $\times 3$ in HBSS with 0.1% bovine serum albumin (BSA) and 0.01% sodium azide by cycles of centrifugation (300 g $\times$ 10 min at 4°C) and resuspension. Cell pellets were then incubated with 50 μ l of goat anti-mouse IgG conjugated with FITC (FITC G α -M IgG from Cappel, Cochranville, PA, 1:100 dilution of the $\sim$ 8 mg/ml stock solution in HBSS with 10% NGS, previously determined to be a saturating concentration). Cells were again washed $\times 3$ and resuspended in 1 ml of indicator-free HBSS with 0.1% BSA and 0.01% Na azide.

Cell Subset Enumeration and Fluorescence Intensity Determination Using FACS Analysis

Ten thousand cells exposed to a given mAb were recorded at a rate of 500 cells/second on a fluorescence-activated cell sorter (FACS: Becton Dickinson, Sunnyvale, CA) equipped with a 2-watt argon laser (Spectra-Physics, Inc., Mountain View, CA). The fluorescence intensity of each cell was assigned to one of the 128 available fluorescence channels. In preliminary experiments, fluorescence detector gain settings (amplification) were determined for each mAb so that the median fluorescence for cells from control donors fell approximately at the midpoint of the 128 channels. Monoclonal antibody-labeled cells were considered positive if their fluorescence fell to the right of the midpoint of the trough (approximately channel 17) between autofluorescent unlabeled cells and fluorescent labeled cells. Almost all ($>95\%$) positive cells were found below channel 88.

The median fluorescence of the cells considered as positive was determined from cell-frequency histograms. For each mAb evaluated, MS patients and controls were compared with regard to the channel representing the median of fluorescence intensity. The distribution of fluorescence intensity for T8 $^+$ cells could be further compared between MS patients and controls by determining the number of T8 $^+$ cells found below or above the channel number (52.5) representing the median between the usual channel numbers containing cells with the lowest (channel 17) and highest (channel 88) fluorescence intensity.

Cells positive for OKT8 staining were brightest and the gain setting was set at 2.0. OKT3 $^+$ cells were counted with amplification at 4.0. OKT4 $^+$ cells were dim even at optimal staining conditions and it was necessary to increase electronic amplification of the fluorescence signal to 8.0. The laser output was set at 150 mW at 488 nm and the fluorescence detector at 520 V, with filters of 520 nm and 530 nm long pass and 0.45 neutral density in front of the fluorescence detector. A standard preparation of glutaraldehyde-fixed chicken red blood cells (G-CRBC) and occasionally fluorescent

microspheres were used to adjust the laser output intensity to comparable levels on different days to reduce day-to-day variation. The strongly fluorescent G-CRBC exhibited a fluorescence peak at channel 42 under these conditions with the gain setting at 1.0.

Modulation Studies with OKT8

Washed and pelleted lymphocytes ($n = 4 \times 10^6$) were incubated either with 20 μ l of OKT8 diluted 1:20 in HBSS plus 10% NGS (treated cells) for 30 minutes on ice or with HBSS plus 10% NGS only (untreated cells) and then resuspended in 4 ml of RPMI 1640 (GIBCO) + 10% newborn calf serum, 6 mg/ml L-glutamine, and 0.1 mg/ml gentamycin. Treated and untreated cells were incubated in 25 cm² tissue culture flasks (Corning, Corning, NY) for 18 hours at 37°C in a humidified 5% CO₂ atmosphere. Following incubation, 10⁶ treated or 10⁶ untreated cells were pelleted and then stained with OKT8 followed with FITC G α -M IgG as described above. Treated

TABLE 1. FACS Analysis of Peripheral Blood MNCs in MS*

mAb	Enumeration of Cell Subsets			Median Fluorescence Intensity		
	Percent $\pm$ SEM			Channel Number $\pm$ SEM		
	Stable	Progressive	Relapse	Stable	Progressive	Relapse
T3	50.7 $\pm$ 3.7 <i>n</i> = 9	49.1 $\pm$ 2.8 <i>n</i> = 7	60.1 $\pm$ 3.8 <i>n</i> = 7	46.0 $\pm$ 3.1 <i>n</i> = 9	43.0 $\pm$ 2.9 <i>n</i> = 7	41.9 $\pm$ 4.0 <i>n</i> = 7
	60.3 $\pm$ 2.5	58.6 $\pm$ 1.6 ^b	61.6 $\pm$ 2.6	44.4 $\pm$ 2.3	42.6 $\pm$ 2.8	42.2 $\pm$ 2.8
T4	33.3 $\pm$ 2.9 8	37.6 $\pm$ 1.9 8	44.0 $\pm$ 1.5 4	55.3 $\pm$ 4.3 8	60.8 $\pm$ 4.6 6	47.5 $\pm$ 6.9 6
	41.1 $\pm$ 3.4	33.3 $\pm$ 1.9	39.5 $\pm$ 3.6	54.4 $\pm$ 2.4	51.5 $\pm$ 3.4	48.3 $\pm$ 3.8
T8	17.3 $\pm$ 2.5 8	14.5 $\pm$ 7 ^b 8	17.8 $\pm$ 3.0 6 ^c	58.5 $\pm$ 7.6 8	48.0 $\pm$ 4.4 ^b 8	45.2 $\pm$ 3.2 ^c 6
	18.6 $\pm$ 1.9	20.9 $\pm$ 2.0	20.7 $\pm$ 1.9	54.3 $\pm$ 9.4	54.5 $\pm$ 3.7	50.5 $\pm$ 3.3

*Control values are shown in italics.

^b $p < 0.05$.

^c $p < 0.001$.

cells ($n = 10^6$) were also stained with FITC G α -M IgG alone as a control. In some studies, treated and untreated cells were initially cultured with or without concanavalin A (Con A), 3 μ g/ml for 48 hours. Cells were then treated with mitomycin and their suppressor activity assayed by culturing them with fresh responder cells plus Con A for 72 hours as previously described.⁸ Suppressor effects of Con A activated OKT8-treated and nontreated cells were compared.

RESULTS

Enumeration Studies Using FACS Analysis in MS

As shown in TABLE 1, the proportion of T8⁺ cells was significantly lower in progressive MS patients, suggestively lower in patients during relapse, and not

significantly different in stable MS patients compared to paired controls. The mean percent T3⁺ cells for both progressive and stable MS groups was lower than for the matched control group. The percent T3⁺ cells in relapsing MS did not differ from controls. The proportion of T4⁺ cells did not differ between any MS groups and controls.

Median Fluorescence Intensity Studies

The median fluorescence intensity (MFI), expressed as the median fluorescence channel number of OKT8⁺ cells stained, was lower in both progressive (48.0 ± 4.4) and relapsing MS (45.2 ± 3.2) when compared to same-day controls (54.5 ± 3.7 , $p < 0.05$ and 50.5 ± 3.3 , $p < 0.02$, respectively). For the active MS patients overall, MFI was significantly lower than for controls (46.8 ± 2.9 versus 53.2 ± 2.7 , $p < 0.001$). The ratio of low:high density cells, as defined on the basis of fluorescence intensity being less than or greater than 52.5, i.e., the median between channels 17 and 88, was increased in the active MS patients (1.80) compared both to the control (1.25)

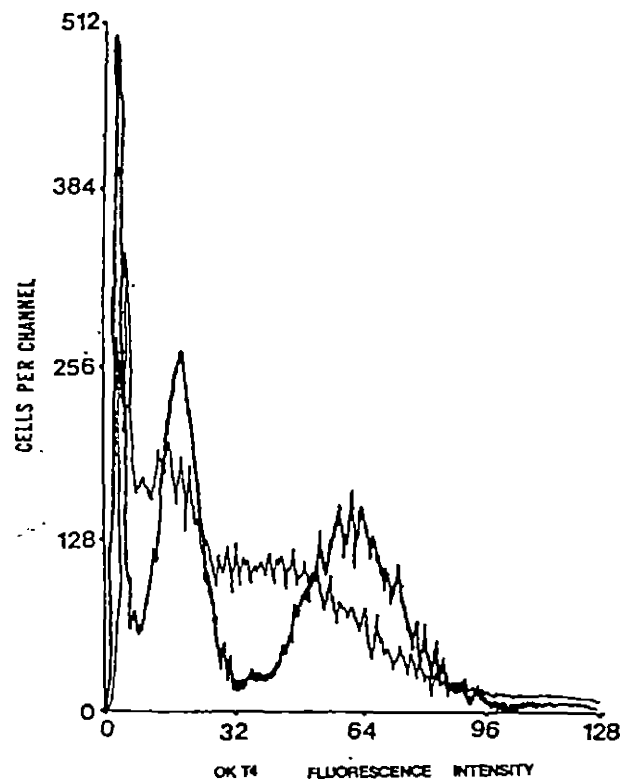


FIGURE 1. T4 antigen expression on mononuclear cells from two control donors. For donor (—), relatively bright T4⁺ cells are clearly separated from dim autofluorescent cells. In contrast, for donor (---), the weakly staining T4⁺ cells are difficult to distinguish from autofluorescent cells.

FIGURE 1. T4 antigen expression on mononuclear cells from two control donors. For donor (—), relatively bright T4⁺ cells are clearly separated from dim autofluorescent cells. In contrast, for donor (---), the weakly staining T4⁺ cells are difficult to distinguish from autofluorescent cells.

and: num (subg diffe there addi diml was T4⁺ T4-r defic elect prof

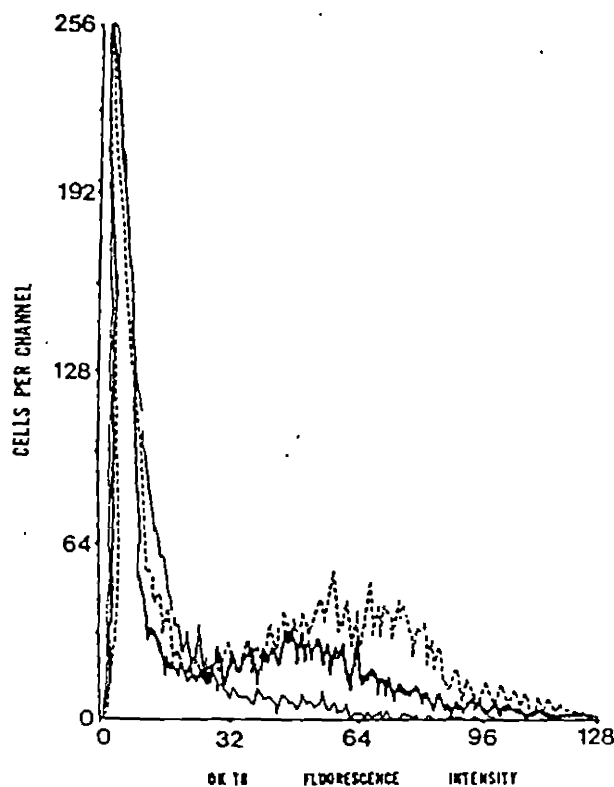


FIGURE 2. T8 antigen expression on cells of a representative patient with active MS (—) and on control OKT8-modulated (—) and nonmodulated (---) cells. After 18-hour OKT8 modulation, mononuclear cells were stained with OKT8 followed by FITC G α -M IgG. MS T8⁺ cells are less numerous and have lower median fluorescence than nonmodulated controls. Modulated cells show a marked reduction in both the number of fluorescence-positive cells and in median fluorescence intensity.

and stable disease patient (0.75) groups. There was no internal correlation between the number of T8⁺ cells and the MFI in either MS or in controls ($r = 0.08$).

OKT3-labeled cells did not differ, with respect to MFI, between any of the MS subgroups and their matched controls. OKT4-labeled cells were also not consistently different between MS subgroups and controls. With OKT4-labeled cells, however, there was considerable variation in the maximal fluorescence recorded (FIGURE 1); in addition, overlap with autofluorescent T4-negative cells sometimes obscured the more dimly fluorescent T4⁺ cells, preventing accurate determination of MFI. This problem was a consequence of the high gain settings necessitated by the weak fluorescence of T4⁺ cells which in turn resulted in amplification of the weak signal given off by T4-negative cells. This overlap of cell populations interfered with the accurate definition of T4⁺ cells even if lymphocytes alone (low scatter cells) were counted after electronically gating out large cells (generally macrophages with a high scatter profile).

Modulated Cell Studies

Following 18 hours of incubation with OKT8, the mean proportion of cells in the mononuclear cell population considered as being T8⁺ using the FACS was 7% compared to 23% for mononuclear cells cultured without antibody. The MFI of these OKT8-modulated cells was markedly reduced compared to control cells. FIGURE 2 depicts a representative FACS profile of OKT8-stained mononuclear cells from a control before and after modulation along with OKT8-stained mononuclear cells from an MS patient with progressive disease. When the same OKT8-stained modulated cells as above were enumerated using a fluorescence microscope, the mean percent positive cells was found to be 18%. For nonmodulated cells, in contrast, a close correlation existed between results of enumeration performed with the microscope compared to the FACS.

As previously reported,⁴ we have found that Con A-induced suppressor activity directed toward a mitogen-induced T cell response by OKT8-modulated mononuclear cells was significantly reduced compared to the suppressor activity of nonmodulated cells ($29 \pm 5\%$ versus $43 \pm 6\%$, $p < 0.02$). Using a "panning" technique to prepare enriched T4⁺ or T8⁺ cell populations, we have shown that this suppressor activity is mediated by the T8⁺ cell population.

DISCUSSION

Our enumeration data on T cell subsets in MS do indicate that during clinically active phases of disease, there is an underrepresentation of T8⁺ cells in the MNC population. The findings seem most apparent in patients with chronic progressive disease. Considerable overlap, however, is found in T cell subset values between stable and active MS patients, precluding the use of a given value for T8⁺ cell number as a clear-cut indicator of active disease. Our studies did not address whether serial determinations of T_H numbers for a given individual can predict a pending exacerbation of disease. In prior studies, we have shown that the amount of *in vitro* pokeweed mitogen-induced immunoglobulin production by mononuclear cells of either MS patients or controls is not directly correlated with the absolute number of T_H (T8⁺) cells present but rather with the functional properties of the T8⁺ cells.⁴

The analysis of the fluorescence intensity of MS patients' mononuclear cells after staining with OKT mAbs suggests several potential mechanisms that may contribute to the functional immunoregulatory defects found in MS and provide some explanation for the apparent discrepancies in results obtained in the various studies enumerating T cell subsets. We find that in active MS, particularly with progressive disease, the proportion of cells with a high density of T8 antigen is reduced. This observation could reflect either a selective depletion of a T8⁺ subset with high density of surface antigen, a reduction in T8 antigen expression (modulation), or masking of the T8 antigen. With regard to the first possibility, specific functional properties of T8⁺ cells have been associated with different antigen densities. Titus *et al.*⁹ reported that high antigen density T8⁺ cells mediated high levels of cytolytic activity.

With regard to antigen modulation or masking, several lines of evidence indicate that the expression of the T8 antigen may be linked to cell function and that removal or blocking of the antigen can alter functional activity. Reinherz *et al.* found that incubation of T8-bearing cells with OKT8 blocked their cytolytic activity.¹⁰ Interaction between the T8 antigen and the mitogen wheat germ agglutinin results in cell activation.¹¹ Our data indicate that modulation of the T8 antigen from the cell surface results in reduced Con A-activated suppressor activity.

The data derived from enumeration of OKT8-modulated cells using both a FACS and a fluorescence microscope further indicate difficulties that arise in using immunofluorescence techniques to enumerate cells expressing low amounts of surface antigen. When the density of the T8 antigen on cells is reduced by modulating the cells, significant differences are found between the two techniques, with higher numbers being found using the microscope.⁴ Dim cells are less likely to be counted as positive with a FACS than with a microscope because cells retaining even a few flecks of fluorescence are read as positive under the microscope but are not distinguished from autofluorescent cells with the FACS. Furthermore, with reduced cell fluorescence, the cutoff between positive and negative cells on the FACS becomes less sharply defined, resulting in further difficulties in segregating the cells. This difficulty is comparable to that encountered when enumerating OKT4-stained cells (see Results section). The reduced MFI which we find for T8-bearing cells from active MS patients may thus contribute to the discrepancies in results among studies, particularly if positive and negative are the only criteria used in the analysis of fluorescence.

REFERENCES

1. ANTEL, J. P., B. G. W. ARNASON & M. E. MEDOFF. 1979. Suppressor cell function in multiple sclerosis—correlation with clinical disease activity. *Ann. Neurol.* 5: 338–342.
2. GONZALEZ, R. L., P. C. DAU & L. E. SPITLER. 1978. Altered regulation of mitogen responsiveness by suppressor cells in multiple sclerosis. *Clin. Exp. Immunol.* 36: 78–84.
3. NEIGHBOUR, P. A. & B. R. STONE. 1979. Absence of virus-induced lymphocyte suppression and interferon production in multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 76: 476–480.
4. REDER, A. T., J. P. ANTEL, J. OGER, T. A. MCFARLAND, M. ROSENKOETTER & B. G. W. ARNASON. 1984. Low T8 antigen density on lymphocytes in active multiple sclerosis. *Ann. Neurol.* 16: 242–249.
5. REDER, A. T., T. A. MCFARLAND, J. OGER & J. P. ANTEL. 1983. Cytotoxic/suppressor cell population in active multiple sclerosis: Reduction in T8 antigen density. *Ann. Neurol.* 14: 116.
6. ANTEL, J. P., J. OGER, S. JACEVICIUS, H. H. KUO & B. G. W. ARNASON. 1982. Modulation of T lymphocyte differentiation antigens: Potential relevance for multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 79: 3330–3334.
7. ANTEL, J. P., D. M. PEEPLES, A. T. REDER & B. G. W. ARNASON. 1984. Analysis of T regulator cell surface markers and functional properties in multiple sclerosis. *J. Neuroimmunol.* 6: 93–103.
8. ANTEL, J. P., M. ROSENKOETTER, A. REDER, J. OGER & B. G. W. ARNASON. 1984. Multiple sclerosis: Relation of *in vitro* IgG secretion with T suppressor cell number and function in multiple sclerosis. *Neurology* 34: 1155–1160.
9. TITUS, J. A., S. O. SHARROW & D. M. SEGAL. 1983. Analysis of Fc (IgG) receptors on human peripheral blood lymphocytes by dual fluorescence flow microfluorometry. II. Quantitation of receptors on cells that express the OKM1, OKT3, OKT4, and OKT8 antigens. *J. Immunol.* 130: 1152–1158.
10. REINHIERZ, E. L., R. E. HUSSEY, K. FITZGERALD, P. SNOW, C. TERHORST & S. F. SCHLOSSMAN. 1980. Antibody directed at a surface structure inhibits cytolytic but not suppressor function on human T lymphocytes. *Nature* 294: 168–170.
11. BOLDT, D. H. & S. A. DORSEY. 1983. Interactions of lectins and monoclonal antibodies with human mononuclear cells. *J. Immunol.* 130: 1646–1653.