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IMPORTANCE OF IMMUNOGLOBULIN ISOTYPE IN THERAPY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS WITH MONOCLONAL ANTI-CD4 ANTIBODY[1]

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Experimental allergic encephalomyelitis (EAE) is an autoimmune disease mediated by CD4+ T cells. Prior studies have established that monoclonal anti-CD4 antibodies can reverse EAE. To determine whether immunoglobulin isotype plays a role in the therapy of EAE with anti-CD4 antibody, an isotype switch variant family of the mouse IgG1 anti-rat CD4 antibody W3/25 was isolated with the fluorescence-activated cell sorter. The IgG1, IgG2b, and IgG2a W3/25 isotype variants all had identical binding capacities for rat CD4+ T cells. Although all three W3/25 isotypes showed some beneficial effects in the amelioration of EAE, the IgG1 and IgG2a W3/25 antibodies were superior to the IgG2b W3/25 in the treatment of EAE. Multiparameter fluorescence-activated cell sorter analysis of T cell subpopulations from treated rats showed that none of the antibodies of the W3/25 isotype switch variant family substantially depleted CD4+ target cells in vivo. These experiments demonstrate that immunoglobulin isotype is important in the monoclonal antibody therapy of autoimmune disease. They indicate that therapy of EAE may be successful without a major depletion of CD4+ lymphocytes. Immunotherapy may be optimized by selecting an appropriate isotype of a monoclonal antibody.

Experimental allergic encephalomyelitis (EAE)[3] represents the prototype of an autoimmune disease mediated by T lymphocytes that bear CD4 cell surface molecules (1-5). Autoreactive $CD4^+$ T lymphocytes restricted to Ia antigens which recognize portions of the myelin basic protein molecule induce EAE (5). Because the major clinical manifestation of EAE, namely, paralysis, is so clear, it has served as a useful model for testing therapy of autoimmune disease with monoclonal antibodies (6-9).

Among the various monoclonal antibodies to T cells that have been tested, anti-CD4 antibodies have been particularly effective in treating EAE. Brostoff and Mason (2) showed that the mouse anti-rat CD4 monoclonal antibody, W3/25, ameliorated EAE in rats. In murine EAE, the rat antimouse CD4 antibody, GK1.5, was similarly effective in reversing paralysis (3). Antibodies to pan T cell antigens such as monoclonal anti-Ly-1 and anti-Thy-1 antibodies in the mouse (9), and W3/13 and OX19 in the rat (2) were ineffective in the treatment of EAE. Clearly, the specificity of an antibody molecule, determined by the variable regions of its heavy and light chains, plays a critical role in establishing the therapeutic potential of an antibody.

The constant region of the heavy chain molecule determines immunoglobulin isotype and the effector functions of an antibody molecule. The biologic activity of different antibody isotypes is highly variable. For example, among the subclasses of mouse IgG, IgG1, IgG2a, IgG2b, and IgG3, major differences exist in their ability to fix complement (10), or to direct antibody-dependent cell-mediated cytotoxicity (11). The importance of antibody isotype in the immunotherapy of tumors has been established (12-14). The technology for selecting isotype variants of a monoclonal antibody allowed us to compare a family of monoclonal antibodies, all with the identical specificity for CD4 present in the parent W3/25 antibody, but with different heavy chain isotypes, for the treatment of EAE.

We have used the fluorescence-activated cell sorter (FACS) to isolate isotype switch variants that arise spontaneously in hybridomas secreting monoclonal antibodies (10). The detectable rate of spontaneous isotype switching in hybridomas ranges from 10^{-4} to 10^{-7}/cell. Rare spontaneous IgG2a and IgG2b heavy chain class isotype variants were selected and cloned from the W3/25 parental hybridoma that produces an IgG1 monoclonal antibody. This family of isotype switch variants was utilized to define the influence of immunoglobulin isotype on the therapy of an autoimmune disease.

MATERIALS AND METHODS

Animals. Female Lewis rats, 10 to 12 wk old, were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA.

Preparation of myelin basic protein. Guinea pig myelin basic protein (GPMBP) was purified from guinea pig spinal cord (Pel-Freez Biologicals, Rogers, AR) according to the method of Smith (15).

Induction of EAE. Female Lewis rats were immunized with 100 μg of GPMBP in 0.1 ml of an emulsion of three parts complete Freund's adjuvant to two parts phosphate-buffered saline with 4 mg/ml of *Mycobacterium tuberculosis H37Ra* (Difco Laboratories Inc., Detroit, MI) in the hind footpads.

Scoring system for clinical assessment of EAE. An observer who did not know what treatment any given rat had received graded the

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[3] Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; GPMBP, guinea pig myelin basic protein; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; FPLC, fast performance liquid chromatography.

severity of the rats' EAE symptoms according to the following scale: (0) no signs of disease; (1) loss of tail tone; (2) hind limb paresis; (3) hind limb paralysis; and (4) hind limb paralysis accompanied by sufficient weakness to prevent movement.

Monoclonal antibodies. The hybridomas OX19 (16), Ox8 (16), and W3/25 (17) which secrete murine antibodies against the rat T cell differentiation antigens equivalent to CD5 (a pan T cell marker), CD8 (a suppressor/cytotoxic T cell marker), and CD4 (a helper/inducer T cell marker), respectively, were kindly provided by Dr. A Williams. Antibodies 8.3 (18), reactive with Igh-1a, 21-48.3 reactive with Igh-1a,3a (18), mouse anti-rat κ antibody MAR 18.5 (19), and rat anti-mouse κ antibody 187.1 (20), were described previously.

Monoclonal antibodies were purified from ascites fluid by ammonium sulfate precipitation followed by ion exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Hardy (21).

The preparation of fluorescein isothiocyanate (FITC) and biotin conjugates of monoclonal antibodies was as described by Goding (22).

Multiparameter FACS analyses. Preparation and staining of cells for FACS was as described by Hayakawa et al. (23). Immunofluorescence analysis of peripheral blood lymphocytes and lymph node cells was performed on a modified Becton Dickinson dual laser FACS IV equipped with logarithmic amplifiers for the fluorescence and large angle light scatter measurements as described by Parks and coworkers (24, 25). Two-color staining data are presented as contour plots that are representations of three-dimensional surfaces in which the levels of green and red fluorescence per cell define locations on a two-dimensional surface and the frequency of cells with that value of fluorescence defines the elevation of that location. After this surface is smoothed contour lines are drawn to divide the sample into equal fractions.

Isolation of isotype switch variants. The FACS was used to isolate IgG2b and IgG2a isotype variants of the IgG1-secreting mouse anti-rat CD4 hybridoma W3/25. Fluorescein-conjugated isotype-specific goat anti-mouse IgG2b and IgG2a antisera were used to select variants (26, 27). A device that permits the deposition of a defined number of cells into the wells of microtiter plates was used in combination with a modified FACS IV for pauci-clonal sorting (25). Care was taken to insure that the cell population used for sorting was greater than 95% viable before staining. In addition, propidium iodide was used to stain dead cells so that they could be excluded from FACS analysis and sorting.

A combination of FITC-conjugated goat anti-mouse IgG2b and goat anti-mouse IgG2a was used to sort potential IgG2 variants of the parent IgG1 W3/25. Cells were deposited at 50 cells/well into microtiter plates (Falcon Labware, Oxnard, CA). FITC-conjugated anti-mouse IgG2 antisera was used to screen the supernatants from these potential variants for binding to rat thymocytes. Supernatants from positive wells were rescreened in a similar fashion with antisera that was specific for IgG2b or IgG2a. Only IgG2b variants were isolated from this initial sort. The IgG2b-secreting variants were cloned from the positive wells using the FITC-conjugated anti-IgG2b antisera for sorting. The clones were expanded and then tested for IgG2b expression. Murine Igh allotype nomenclature was used to name the IgG2b variants of W3/25. Thus, the first IgG2b variant was named W3/25-3.1.

IgG2a variants were isolated from W3/25-3.1 using an FITC-conjugated goat IgG2a antisera to sort potential variants at 25 cells/well into microtiter plates. A solid phase sandwich radioimmunoassay (28) using an anti-Igh 1a,3a antibody and an iodinated anti-Igh 1a antibody was used to screen for the presence of IgG2a antibody in the supernatants from the sorted cells. Supernatants from positive wells were rescreened with FITC-conjugated anti-Igh 1a for binding on rat thymocytes. FITC-conjugated anti-Igh 1a was then used to clone the IgG2a variants. Clones were expanded and tested for Igh 1a expression. IgG2a variants were named W3/25-1.1.

Characterization of the switch variants. Fast performance liquid chromatography (FPLC) was performed on a Superose 6-column (Pharmacia) in 50 mm Tris, 50 mm NaCl, pH 7.8. A total of 12% polyacrylamide gel electrophoresis with sodium dodecyl sulfate-containing buffers was prepared according to Jones (29). The gels were stained with Coomassie brilliant blue. The binding of the W3/25 isotype variants to CD4+ cells was evaluated with a fluorescence-binding assay. Lewis rat lymph node cells were incubated with various amounts of each of the W3/25 isotype variants. After washing, the mouse antibody bound to CD4+ lymph node cells was detected with a FITC-conjugated rat anti-mouse κ antibody, 187.1. Fluorescence data were collected using a modified FACS IV equipped with a logarithmic amplifier. The mean fluorescence of each sample was then converted to linear units to allow comparisons of the fluorescence intensity between samples.

Treatment of EAE with W3/25 isotype switch variants. Twelve days after immunization with GPMBP, rats were divided into four groups. One group received no treatment and each of the other three groups was treated with one of the three W3/25 isotype variants. A coin toss was used to determine what treatment any group received. Treated rats received i.p. infections with 1.5 mg of antibody in phosphate-buffered saline on day 12 after GPMBP immunization and with 1.0 mg on days 15 and 18.

RESULTS

Characterization of W3/25 isotype switch variants. The IgG2b isotype variant of the IgG1 W3/25, designated W3/25-3.1, and the IgG2a variant, designated W3/25-1.1, were isolated by the selection of rare spontaneous isotype switch variants with the FACS. The apparent m.w. of the IgG1, IgG2b, and IgG2a W3/25 isotype variants were all approximately 150,000 as assessed by FPLC. Electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of the variants showed that the light chains of each of the variants had identical m.w. and that there were slight differences in the apparent m.w. of the heavy chains (Fig. 1). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the FPLC analyses of the W3/25 isotype variants indicated that the isotype switch from IgG1 to IgG2b or to IgG2a yielded intact antibody molecules.

The binding activities of purified IgG1, IgG2b, and IgG2a W3/25 antibodies were assessed with a fluorescence immune cell binding assay. The titration curves for the binding of each of the variants to a fixed number of Lewis rat lymph node cells were identical (Fig. 2). The identity of these three curves suggests that the switch in the heavy chain constant region from IgG1 to IgG2b or IgG2a had no detectable effect on the antigen-combining site of these anti-rat CD4 antibodies.

Reversal of EAE. At 12 to 14 days after immunization with GPMBP, 90 to 100% of Lewis rats developed symptoms of EAE. Generally there is a progression of symptoms from an initial loss of tail bone to hind limb paresis and paralysis. The disease is monophasic and symptoms persist for 3 to 6 days and then the animals usually make a rapid and complete recovery.

We attempted to prevent the manifestations of EAE by

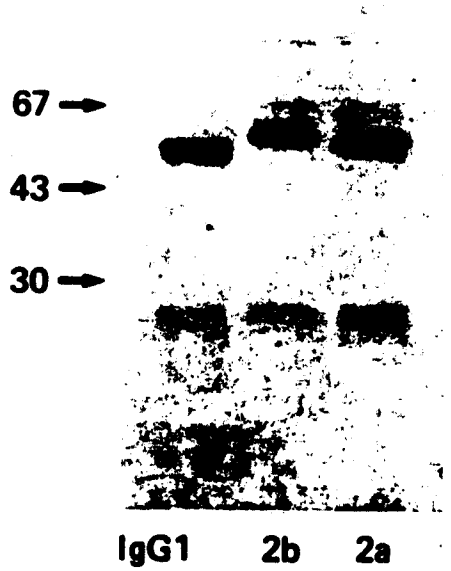

Figure 1. Twelve percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of W3/25 isotype variants. The antibodies were purified from ascites.

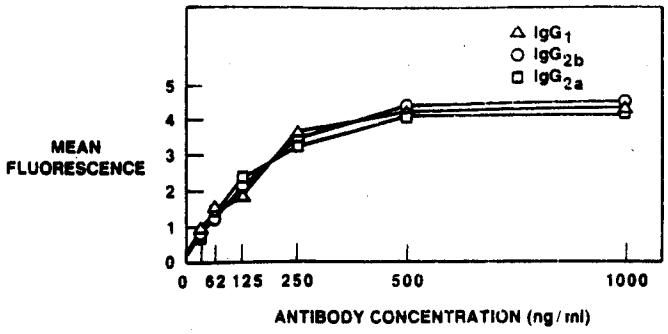

Figure 2. Titration of the binding of the W3/25 isotype variants to rat lymph node cells. Bound antibody was detected with FITC-conjugated rat anti-mouse κ antibody.

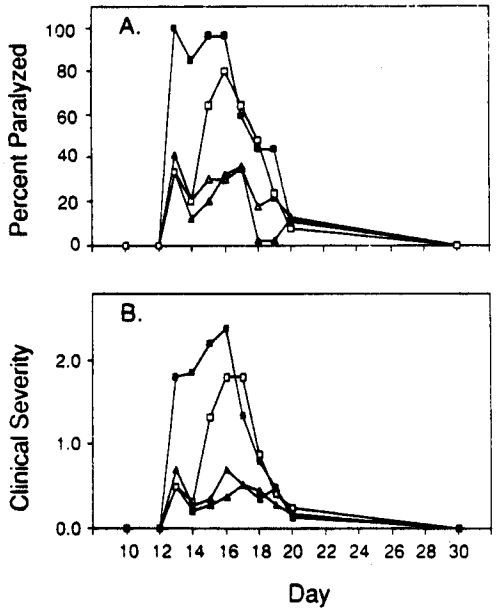

Figure 3. A and *B*, Treatment of EAE with W3/25 isotype variants. Rats were immunized with GPMBP on day 0. Antibody treatment was given on days 12, 15, and 18. An observer who did not know what treatment any given rat received graded the severity of the rats' EAE. *A* shows the percentage of rats in each of the groups that exhibited EAE. *B* shows the mean clinical severity of the rats in each group. There were 23 to 27 rats per group. *Closed squares* represent untreated rats; *open squares* represent rats receiving IG2b (W3/25-3.1); *open triangles*, rats receiving IgG2a (W3/25-1.1); and *closed triangles*, rats receiving IgG1 (W3/25).

passive immunotherapy with administration of W3/25 or one of the two isotype variants beginning 12 days after immunization with GPMBP. By day 12, autoreactive CD4+ T lymphocytes that can transfer EAE to naive recipient rats have already been generated (30). Antibodies were again administered to the treated rats on days 15 and 18.

Differences in the effectiveness of the various anti-CD4 antibodies could be detected. With this protocol, the IgG1 and IgG2a W3/25 antibodies prevented the development of EAE (Fig. 3*A* and *B*). The IgG2b W3/25 antibody, however, was only partially effective. Compared with the IgG1- and the IgG2a W3/25-treated groups, there was both an increased frequency and severity of EAE in the IgG2b W3/25-treated rats (Fig. 3*A* and *B*). The number of rats with EAE symptoms in the IgG2b-treated group (20 of 25) differed significantly from the number of rats with EAE in the IgG2a- and IgG1-treated groups (7 of 23 and 8 of 25, respectively) on day 16 (χ^2 for IgG1 or IgG2a vs IgG2b > 11.68, $p < 0.001$; IgG1 or IgG2a vs control $\chi^2 > 23.7$, $p < 0.001$). Similarly, on day 16, in terms of degree of clinical severity, the IgG2b-treated group differed from the IgG1- and IgG2a-treated groups (either IgG1 or IgG2a vs IgG2b, $p < 0.001$ with the Wilcoxon test for scored samples). Although the IgG2b W3/25 treatment was not nearly as effective as either the IgG1 or IgG2a W3/25 treatment, there was a small benefit of IgG2b treatment compared with untreated rats. On day 16, the IgG2b-treated group differed from the untreated group ($p < 0.02$ with the Wilcoxon test). There was no significant difference between the IgG1- and IgG2a-treated groups.

Frequencies of lymphocyte subsets in W3/25-treated rats. The frequencies of T cell subsets were investigated using multiparameter FACS analyses after in vivo treatment with W3/25 or one of the two isotype variants. Twenty-four hours after injection of 1.5 mg, the injection antibody could be detected on cell surfaces with an FITC-conjugated anti-mouse κ antibody (Tables I and II). Because of this there were no lymph node or peripheral blood cells that could be stained with FITC-conjugated W3/25 (Tables I and II). Two-color immunofluorescence analysis allowed the enumeration of the CD4+ cells without having to rely on antibodies to the CD4 glycoprotein. Because all rat T cells are OX19+ and W3/25 and OX8 divide peripheral T cells into distinct subsets (16), the CD4+ T cells are $OX19^+/OX8^-$ and CD4− cells are $OX19^+/OX8^+$.

TABLE I

Frequency of lymphocyte subsets in peripheral blood of rats treated with W3/25 isotype variants

Lymphocyte Subset	Cell Frequency (%)			
	Untreated	Treated (W3/25 isotype)[a]		
		IgG1	IgG2b	IgG2a
$OX19^+8^-$	39.9	53.8	58.4	55.5
$OX19^-8^+$	3.6	3.2	3.2	3.3
$OX19^+8^+$	14.2	19.2	17.6	19.8
$W3/25^+8^-$	49.9	<1.0	<1.0	<1.0
$W3/25^-8^+$	16.5	21.0	19.6	21.4
Rat κ^+ $OX19^-$	11.6	7.6	6.3	5.5
Mouse κ^{+b}	<1.0	47.8	52.0	56.7

[a] Treated rats received i.p. injections with 1.5 mg of one of the W3/25 isotype variants on the day before being killed for FACS analysis. There were two rats in each treatment group and four control rats. The data shown are the mean value for each group.

[b] An anti-mouse κ antibody was used to detect cells with W3/25 on their surfaces.

TABLE II

Frequency of lymphocyte subsets in lymph nodes of rats treated with W3/25 isotype variants

Lymphocyte Subset	Cell Frequency (%)			
	Untreated	Treated (W3/25 isotype)[a]		
		IgG1	IgG2b	IgG2a
$OX19^+8^-$	44.7	35.9[b]	37.4[b]	31.1[b]
$OX19^+8^+$	17.6	18.1	20.9	21.5
$W3/25^+8^-$	49.1	<1.0	<1.0	<1.0
$W3/25^-8^+$	16.4	18.3	20.5	22.5
Rat κ^+ $OX19^-$	16.1	22.7	22.1	20.9
Mouse κ^{+c}	<1.0	35.6	36.6	34.9

[a] Treated rats received i.p. injections with 1.5 mg of one of the W3/25 isotype variants intraperitoneally on the day before sacrifice for FACS analysis. There were two rats in each treatment group and four control rats. The data shown are the mean value for each group.

[b] Significantly different from the control value ($p < 0.05$), Student's *t*-test.

[c] An anti-mouse κ antibody was used to detect cells with W3/25 on their surfaces.

Twenty-four hours after treatment with each of the W3/25 isotype variants there was a small increase in the frequency of the $OX19^+8^-$ T cell subset in the peripheral blood (Table I). In the lymph nodes, there was a small decrease in the frequency of $OX19^+8^-$ cells (Fig. 4; Table II). The frequency of the $OX19^+8^-$ cells in both the lymph node and the peripheral blood was approximately equal to the frequency of cells that stained with the anti-mouse κ antibody (Tables I and II). Forty-eight hours after treatment, the frequency of $OX19^+8^-$ lymph node cells was unchanged from the levels at 24 hr, and by 4 days after treatment, there were no longer cells with W3/25 antibody on their surfaces and the antibody was no longer detectable in the serum (data not shown).

DISCUSSION

Heavy chain class switch variants of the IgG1 mouse anti-rat CD4 antibody W3/25 were isolated in order to assess the importance of antibody isotype in the anti-CD4 treatment of EAE. The IgG1, IgG2b, and IgG2a variants all had identical binding capacities for rat CD4+ cells. However, although all three isotypes showed some beneficial effects in the amelioration of EAE, the IgG1 and IgG2a W3/25 antibodies were superior to the IgG2b W3/25 in the treatment of EAE.

The finding that substantial depletion of CD4+ cells is not necessary for successful treatment of EAE confirms the observation of Brostoff and White (31). These authors showed that W3/25 and OX35, and IgG2a antibody that recognizes a different epitope on CD4 than W3/25, were equally efficacious in reversing EAE. While W3/25 did not lower the frequency of circulating CD4 cells, OX35 reduced the frequency of peripheral blood CD4 cells by about 25% (31). They also showed that $F(ab')_2$ fragments of OX35 were effective in reversing EAE in two rats. Our observations, and those of Brostoff and White (31), indicate that it is sufficient to bind CD4+ cells without major depletion of circulating CD4+ cells in vivo in order to successfully treat EAE. It has also been shown that the administration of $F(ab')_2$ fragments of the anti-L3T4 antibody GK1.5 inhibited humoral immunity in mice without depletion $L3T4^+$ cells (32). It is important to note, however, that in our study there was a small depletion of CD4+ cells in the lymph nodes of treated rats and it is possible that this small reduction in $CD4^+$ cells was important in the amelioration of EAE in the rat.

Other studies of murine heavy chain class switch variant families have definitively shown that murine antibody isotype plays an important role in the fixation of human complement (26) and in the direction of antibody-dependent cell-mediated cytotoxicity (11). Similar studies using rat complement and rat K cells with murine isotype switch variants have not yet been done.

The importance of antibody isotype in the therapy of murine tumors has been established with heavy chain class switch variant families in three different tumor systems (12–14). IgG2a antibodies were found to be superior to the IgG1 and IgG2b antibodies in these systems. Differences in the clearance rates and the abilities of the different isotypes to interact with host effector mechanisms were proposed to explain the different tumoricidal capabilities of murine isotypes.

Regardless of the mechanisms that result in the superiority of IgG1 and IgG2a W3/25 isotypes compared with the IgG2b W3/25 in the treatment of EAE, the importance of antibody isotype in the therapy of autoimmune disease is clearly established by these experiments. In addition to EAE, a variety of autoimmune diseases including the spontaneous lupus-like disease in NZB/NZW F_1 (33), collagen-induced arthritis (34), and experimental myasthenia gravis (35) has been successfully treated with anti-CD4 antibody. If anti-CD4 antibody therapy of the human analogues of any of these diseases is attempted, the selection of the appropriate isotype of the anti-CD4 antibody may be critical for a beneficial response.

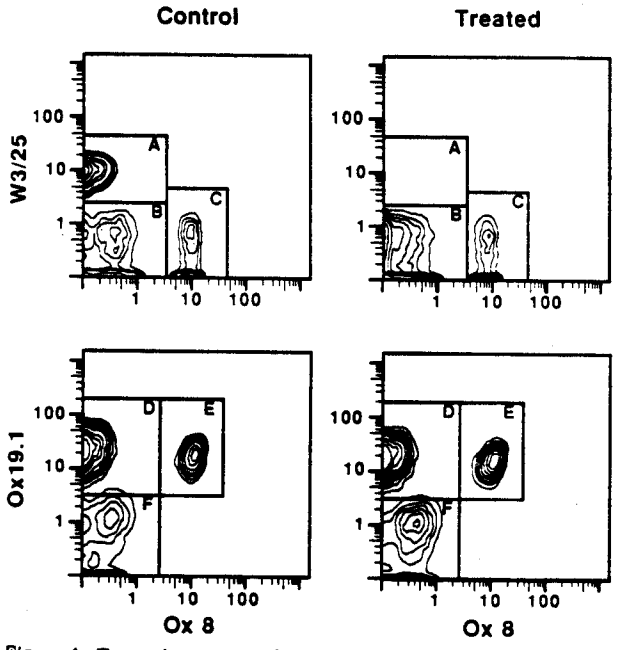

Figure 4. Two-color immunofluorescence analysis of lymph node T cell subsets after treatment with IgG1 W3/25. Lewis rats received i.p injections with 1.5 mg of W3/25 24 hr before being killed for FACS analysis. Cells were stained with FITC-conjugated OX8 and either biotin-conjugated W3/25 or OX19 followed by Texas red avidin and analyzed on a dual laser FACS equipped with logarithmic amplifiers. *Axes* represent the relative amounts of the respective antigens as revealed by fluorescence intensity. The integration boundaries used to determine the frequencies of $W3/25^+OX8^-$ (*A*), $W3/25^-OX8^-$ (*B*), $W3/25^-OX8^+$ (*C*), $OX19^+OX8^-$ (*D*), $OX19^+OX8^+$ (*E*), and $OX19^-OX8^-$ (*F*) cells are marked on the plot.

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