In Vivo Treatment With Anti-CD8 and Anti-CD5 Monoclonal Antibodies Alters Induced Tolerance to Adjuvant Arthritis

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Resistance (low dose tolerance) to adjuvant arthritis was induced by intradermal immunization with 10 μg *Mycobacterium tuberculosis* administered 5 and 3 weeks before induction of arthritis. With the purpose of determining phenotypes of cells which participate in the maintenance of the induced resistance to adjuvant arthritis, tolerized rats were treated with two different anti-T-cell monoclonal antibodies. In tolerized rats, it was shown that anti-CD8 (OX8) antibodies, which caused an elimination of $CD8^+$ lymphoid cells as determined by immunofluorescence analysis, made the rats responsive to an arthritogenic challenge with mycobacteria. Nine of 19 (47.4%) rats developed the disease as compared with 2 of 18 (11.1%) ($P < 0.05$) in the control antibody-treated group. Also, in vivo treatment with anti-CD5 (OX19) monoclonal antibodies made the rats responsive to an arthritogenic challenge with mycobacteria. Nine of 15 (60%) anti-CD5-treated rats developed the disease as compared with 2 of 18 (11.1%) ($P < 0.01$) rats in the control group. Immunofluorescence analysis performed after anti-CD5 treatment showed a reduction of staining of $CD5^+$ cells as well as a down-regulation of the staining intensity of CD5 cell surface receptors on the remaining $CD5^+$ cells. These data indicate that $CD8^+$- as well as $CD5^+$ cells participate in the maintenance of low dose tolerance to adjuvant arthritis.

Adjuvant arthritis (AA) is an experimental model of arthritis in rats in which the disease is induced after immunization with *Mycobacterium tuberculosis* (Mt) in adjuvant oil [1]. Several experiments have demonstrated the essential role of T lymphocytes in the induction of this disease. Firstly, it has been shown that nude rats are resistant to the induction of AA [2]. Secondly, concanavalin A (Con A)-activated $CD4^+$ T cells can transfer the disease to naive recipients [3]. Thirdly, treatment in vivo with monoclonal antibody (mab) reactive with antigens present either on all T

cells or on activated lymphoblasts can prevent the development of AA [4,5], whereas treatment with mab to CD8 molecules on suppressor/cytotoxic cells does not abrogate the disease development [4].

Evidence that the immune system of arthritis-prone rats also harbors cells capable of specifically counteracting the disease has been obtained from several experimental systems. Specific resistance to disease can thus be induced both via conventional low-dose immunization with the antigen itself [6] or after injection ("vaccination") with attenuated cells from potentially arthritogenic T cell lines or clones [7,8]. Relatively little is, however, known about which cells are responsible for maintenance of the induced resistance to the disease, although transfer experiments have shown that induced tolerance can be transferred to naive rats via spleen cell populations enriched for T lymphocytes [9,10]. We have in the present investigation further studied this question in the context of induced low-dose tolerance to AA [11]. In vivo treatment with mab to the CD8 and CD5 molecules was used in order to investigate the role of CD8- and CD5-expressing cells, respectively, in the maintenance of specific resistance to AA.

MATERIALS AND METHODS
Animals

Lewis rats originally obtained from Mollegaard Laboratories (Roskilde, Denmark) were kept and bred in our own animal department. In all the experiments the rats were age- and sex-matched.

Induction of AA and Tolerance to AA and In Vivo Treatment With mab

To induce AA, rats were injected intradermally at the root of the tail with 100 μl (2 mg/ml) of a suspension of *Mycobacterium tuberculosum* (Mt), strains C, DT, and PN (a generous gift from the Department of Agriculture, Fisheries and Food, Weybridge, Surrey, UK) suspended in Pristane (2,6,10,14-tetramethylpentadecane) (Aldrich Chemical Company, Inc., Milwaukee, WI). Tolerance to AA was induced with two doses of 10 μg Mt in Pristane given 5 and 3 weeks prior to the induction of AA. Evaluation of arthritis by clinical scoring has been described earlier [4]. Briefly, the rats were scored according to a graded scale from 1 point to 3 points, where 1 point represents detectable swelling in one to three joints, 2 points represents severe swelling in more than one joint, and three points represents severe arthritis in the entire paw.

At days 0, 5, and 10 after the induction of AA, 0.5 mg of the anti-CD8 (OX8) mab, 1 mg of the anti-CD5 (OX19) mab, or 1 mg of the control (HY2:15) mab were injected intraperitoneally.

Production and Characterization of mab Used

The production and purification of mab have been described earlier [4]. Briefly, the hybridomas were grown under standard cell-cultivating conditions and supernatants were collected by centrifugation at 10,000g. Monoclonal antibodies binding to Protein-A Sepharose (Pharmacia, Uppsala, Sweden)—i.e., the OX19, W3/13, OX8, and HY2:15—were purified accordingly. The W3/25 mab is not a Protein-A-binding antibody and was precipitated with 50% ammonium sulphate. All antibodies were dialysed against phophate-buffered saline and sterile filtered before use. The antibody content of the protein-A-binding mab was determined, after the sterile filtration, by

TABLE I. Specificity of mab Used for In Vivo Treatment or Immunofluorescence Analysis*

Antibody	Cell type or antigen recognized	Reference
W3/13	All thymocytes, peripheral T cells, and some neutrophil granulocytes; the antibody recognizes a glycophorin-like membrane molecule	[12]
OX19	CD5 on T cells and on some B cells	[13]
W3/25	CD4 on rat T cells and on some macrophages	[14]
OX8	CD8 on rat T cells and on some natural killer	[14]
HY2:15	Anti-trinitrophenylphosphate, used as control mab	[14]

*The subclass of all the mab used is IgG1.

absorbance at 280 nm and the precipitate of W3/25 in an ELISA-test as described elsewhere [15].

The anti-T-cell mab hybridomas were generously given to us by Dr. Alan Williams (Oxford, UK). The trinitrophenylphosphate-reactive hybridoma HY2:15 was used for production of control mab [4]. The binding characteristics of the mab are outlined in Table I. All antibodies used were of IgG1 isotype [4].

Immunofluorescence Analysis

Splenocytes from rats immunized with Mt in adjuvant oil and treated with mab, as described above, were analyzed at 1, 5, and 14 days after the last injection of mab. The splenocytes were gated using forward and side scatter. The gated cells (small mononuclear cells) were analysed in a Becton&Dickinson FACStar® flow cytometer (Moutain View, CA). The anti-T-cell mab W3/13, OX19, W3/25, and OX8 were used as primary reagents. As a control to measure unspecific binding of antibodies to the splenocytes normal mouse immunoglobulin (Ig) produced in our own laboratory was used. As a secondary reagent we used goat antimouse Ig fluorescein isothiocyanate (K&P Laboratories, West Grove, PA) diluted in 1% normal rat serum.

Statistics

The statistical evaluation was done with the help of the chi-square method (incidence of arthritis) and with the Mann-Whitney test (scoring of arthritis) and counting the standard variation (cytofluorometric analysis).

RESULTS
Effects of Treatment With Anti-CD8 and Anti-CD5 Monoclonal Antibodies on Induced Tolerance to Adjuvant Arthritis

In two experiments tolerized rats were treated with anti-CD8 (OX8) mab. In the first experiment the anti-CD8-treated group developed a higher incidence of AA (3/10) as compared with tolerized rats treated with control mab (1/9), even though this difference was not statistically significant (Table II). In the second experiment the tolerized rats were treated identically. Also, this time anti-CD8 mab-treated rats developed arthritis with a higher incidence (6/9) than the control group (1/9) $(P < 0.05)$. Treatment with anti-CD5 (OX19) mab also gave rise to a higher incidence of AA. Two identical experiments were performed, and in both experiments the group of rats treated with anti-CD5 mab developed arthritis with a significantly

TABLE II. Development of AA in Rats Resistant to AA Treated With Anti-CD8 (OX8) mab

Experiment no.	mab treatment	Incidence of arthritis (%)	Statistics	Days of onset
1	OX8	3/10 (30)	n.s.	11, 12, 16
	HY2:15	1/9 (11.1)		11
2	OX8	6/9 (66.6)	$P < 0.05$	12, 12, 12, 12, 12, 16
	HY2:15	1/9 (11.1)		13
Σ	OX8	9/19 (47.4)	$P < 0.05$	
	HY2:15	2/18 (11.1)		

($P < 0.05$; 5/8 vs. 2/12 and 4/7 vs. 0/6, respectively) higher incidence than control treated rats (Table III). Onset of disease was seen between day 8 and day 20 in resistant rats challenged with Mt, and no significant difference of time of onset was seen between groups treated with different mab (Tables II, III). No significant differences could be observed between the groups which received different mab when the severity of the disease was scored (data not shown).

Immunofluorescence Analysis of the Effects of Anti-CD8 and Anti-CD5 Monoclonal Antibody Treatment

In a second set of experiments splenocytes from rats immunized with Mt in adjuvant and treated with either anti-CD8, anti-CD5, or control mab were analyzed at 1, 5, and 14 days after the injections of the mab.

Treatment with anti-CD8 mab reduced CD8 staining to levels of unspecific background staining at day 1. The staining for W3/13$^+$ cells was reduced and the CD4 staining enhanced as compared with the staining of cells in the control group (Fig. 1a). Five days after the injection of anti-CD8 mab a few cells stained positive for CD8. A reduction of W3/13 and CD5 staining was also observed, and the staining of CD4$^+$ cells was higher than among cells from control mab-treated rats (Fig. 1b). At day 14 the stainings showed no significant changes (Fig. 1c) as compared with the stainings at day 5 post injection.

Splenocytes from rats treated with anti-CD5 mab were also analysed. One day after the last injection of antibodies, the numbers of CD5-expressing cells were reduced. The staining intensity, i.e., the amount of expressed CD5 antigen, of the remaining CD5$^+$ cells was less than that found among cells from the control antibody-treated rats (Fig. 2a,b). Also, the numbers of cells stained with W3/13 mab and anti-CD4 mab was reduced. The relative number of CD8$^+$ cells was slightly elevated as

TABLE III. Development of AA in Rats Resistant to AA Treated With Anti-CD5 (OX19) mab

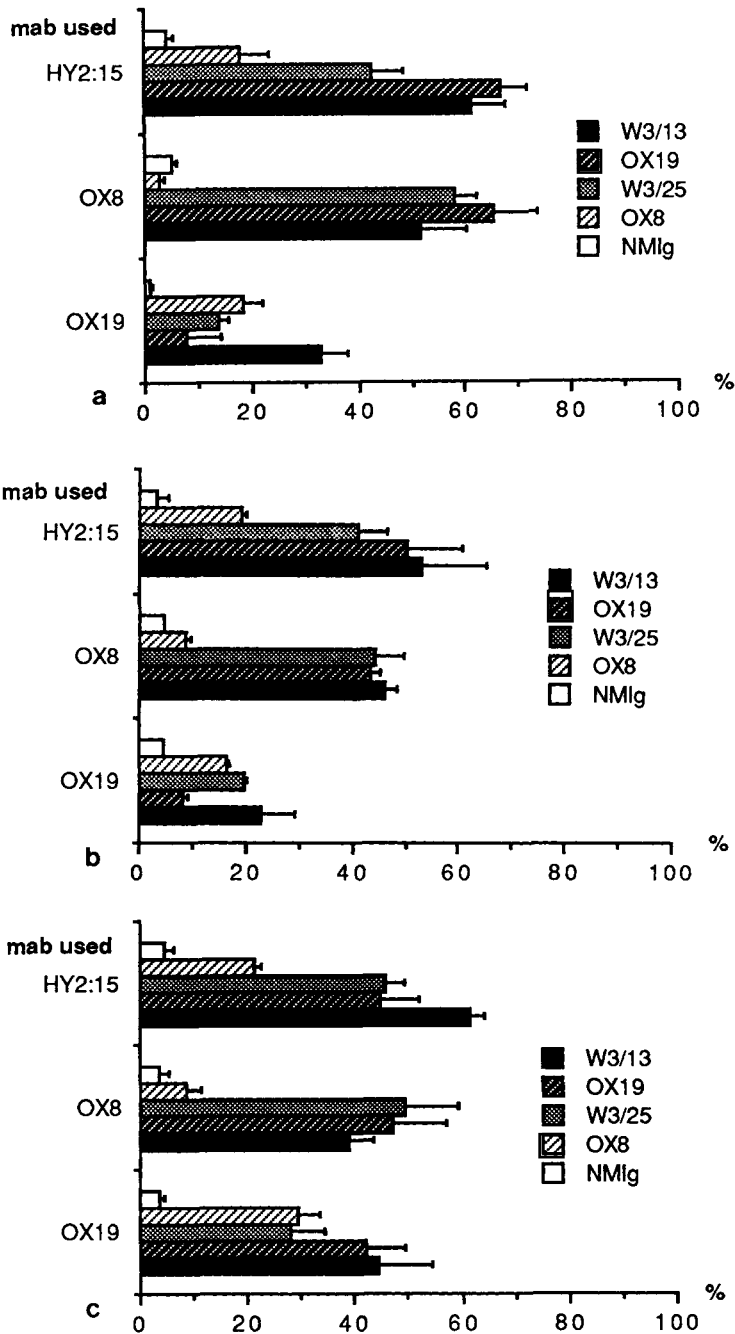| Experiment no.	mab treatment	Incidence of arthritis (%)	Statistics	Days of onset
3	OX19	5/8 (62.5)	$P < 0.05$	10, 13, 17, 17, 17
	HY2:15	2/12 (16.6)		10, 13
4	OX19	4/7 (57.1)	$P < 0.05$	8, 8, 12, 20
	HY2:15	0/6 (0)		
Σ	OX19	9/15 (60)	$P < 0.01$	
	HY2:15	2/18 (11.1)		

Fig. 1.   Immunofluorescence analysis of splenocytes from rats immunized with Mt in adjuvant at 1 (a), 5 (b), and 14 days (c) after the last injection of mab (OX19, OX8, or HY2:15). The cells were gated and the small, mononuclear cell fraction evaluated. Staining was done with W3/13 (anti-pan T), OX19 (anti-CD5), W3/25 (anti-CD4), OX8 (anti-CD8), and normal mouse Ig (control, NMIg) as primary step reagents. As secondary step reagent a goat antimouse FITC conjugate was used.
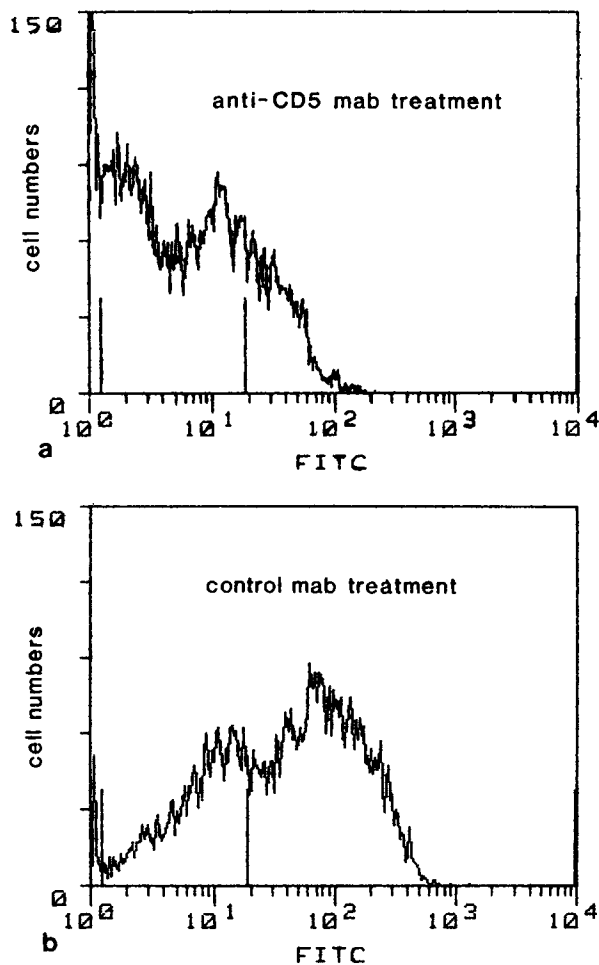
Fig. 2.   Immunofluorescence analysis of the staining intensity of splenocytes from rats treated with either OX19 (a) or with HY2:15 (b). The analysis was done 1 day after the last injection of mab.

compared with what was seen in the control rats (Fig. 1a). At day 5 after the last antibody injection, the numbers of cells stained with anti-CD5 as well as with anti-CD4, anti-CD8, and W3/13 were similar to what was seen at day 1 (Fig. 1b). Fourteen days after the injection of anti-CD5 mab, the staining for CD5 was similar to what was seen in control mab-treated rats both as to the numbers of CD5$^+$ cells (Fig. 1c) and staining intensity (data not shown).

DISCUSSION

The present experiments with anti-CD8 (OX8) treatment were performed in order to take advantage of the efficient depletion capability of this antibody in investigating to what extent CD8$^+$ T cells are essential for the maintenance of tolerance to AA. In previous experiments with the presently used protocol for in vivo anti-CD8

antibody treatment, the cytotoxic capacity of rat spleen cells was shown to disappear in parallel with an almost complete elimination of staining for CD8 on tissue sections of spleen and lymph nodes of antibody-treated individuals. This elimination was shown in these experiments to occur 4 h after injection of the anti-CD8 (OX8) mab [15]. In the present study, flow cytometry analysis was used to analyze the effect of in vivo OX8 treatment at different times after antibody injections. The results confirmed previous evidence that OX8 antibody treatment causes an elimination of most CD8$^+$ T cells [4,15–17] and that only a few CD8$^+$ cells have repopulated the lymphoid organs at 5 and 14 days after the last injection of anti-CD8 mab, i.e., 15 and 24 days after the arthritogenic challenge, hence, over the period when all of the Mt-challenged and mab-treated animals acquire their disease.

The results thus show that CD8$^+$ cells fulfill some function in the maintenance of tolerance to AA as a significantly higher incidence of arthritis was seen in anti-CD8 treated rats as compared with control mab-treated rats. The reciprocal possibility— that also CD8– cells are involved in maintenance of tolerance to AA—is suggestive, but remains—from the experiments with anti-CD8 mab treatment—not fully conclusive, as very low numbers of CD8$^+$ cells may be present in the spleen as well as in lymph nodes even after thymectomy [17].

The experiments with anti-CD5 (OX19) mab treatment were performed partly on the basis of previous evidence that CD5$^+$ cells and/or CD5 receptors may be of importance in the maintenance of tolerance to autoimmune disease. Elimination of Ly1$^+$/CD5$^+$ cells from a lymphoid cell population thus made these lymphoid cells capable of inducing multiple organ-specific autoimmune disease after transfer to nude mice [18]. Addition of OX19 (anti-CD5) mab to in vitro cultures of mitogen-stimulated rat lymphoid cells has also been shown to enhance the mitogenic response without the anti-CD5 mab themselves being mitogenic [13], something that indicates a regulatory role of CD5$^+$ cells and/or CD5 molecules in certain situations.

The presently observed ability of anti-CD5 mab treatment to partially impair induced resistance to AA is compatible with such a regulatory role of CD5$^+$ cells also in AA. The interpretation of the results is, however, complicated by the fact that anti-CD5 treatment, as shown by cytofluorometric analysis, on one hand eliminated some CD5$^+$ spleen cells, and on the other hand reduced (modulated) CD5 antigen expression on the remaining CD5-expressing cells. The interpretation of the data is, however, also complicated by the fact that some B cells, particularly B cells that produce autoantibodies, may express CD5 [19]. Previous experiments have suggested that B cells are of minor importance in both induction and regulation of AA [20], but we cannot exclude the possibility that CD5$^+$ B cells may in some way contribute to the presently observed effects of anti-CD5 mab treatment.

Based on the assumption that T cells are the main regulatory cells in the maintenance of tolerance to AA, the data from the present anti-CD5 mab treatment experiments suggest either that CD5$^+$ T cells are involved in maintenance of the actual resistance to AA and/or that the CD5 molecules themselves are of importance for resistance to AA.

In conclusion, the present results indicate on one hand that different phenotypically defined cells may participate in the maintenance of tolerance to AA. On the other hand they demonstrate that maintenance of resistance to different autoimmune diseases may be maintained via different regulatory mechanisms since the induced resistance to another autoimmune disease in rats, experimental allergic neuritis

(EAN), is not at all affected by depletion of CD8$^+$ but is completely eradicated by anti-CD5 mab injections [21,22]. The study also points to general applicability of in vivo mab treatment as a complement to cell transfer experiments in elucidating the mechanisms whereby natural and acquired resistance to autoimmune disease is maintained.

ACKNOWLEDGMENTS

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