Production of erythrocyte autoantibodies in NZB mice is inhibited by CD4 antibodies

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SUMMARY

NZB mice spontaneously develop haemolytic anaemia as the result of production of erythrocyte autoantibodies. The mechanisms leading to breakdown in tolerance to erythrocyte autoantigens are unknown. Antibodies to CD4 have been successfully used to treat several murine models of autoimmune disease. In this study we injected NZB mice with non-depleting CD4 antibodies and were able to prevent and abrogate erythrocyte autoantibody production in young (Coombs' negative) and old (Coombs' positive) mice, respectively. Our data indicate the dependency of autoantibody production on $CD4^+$ T cells. However, withdrawal of anti-CD4 antibodies resulted in the appearance of erythrocyte autoantibodies, showing that under these conditions we were unable to re-establish tolerance to autoantigens on erythrocytes using anti-CD4 treatment.

Keywords NZB mice   therapy of autoimmune disease   anti-CD4 antibodies

INTRODUCTION

New Zealand Black (NZB) mice spontaneously develop auto-immune haemolytic anaemia [1,2]. By 9 months of age, most of the mice have both IgM and IgG autoantibodies bound to their erythrocytes which can be detected by the Coombs' antiglobulin test [3,4]. These autoantibodies accelerate the removal of erythrocytes from the circulation, probably through destruction by phagocytic cells in the spleen and in the liver [5]. It is likely that when the erythropoiesis is unable to compensate for the loss of erythrocytes, the mice become anaemic. We have recently shown that the pathogenic autoantibodies are directed to band 3 on the erythrocytes [6].

The mechanisms of breakdown in tolerance to self band 3 on the erythrocytes are unclear, although a deficiency in regulatory cells has been suggested [7–9]. Previous studies by others have shown the requirement of T cells for the production of erythrocyte autoantibodies [10]. In a number of other experimental models of autoimmunity, antibodies to CD4 inhibit the development of the disease and/or even reverse ongoing disease [11–16]. Furthermore, treatment with the anti-CD4 MoAbs which do not deplete the CD4 population has also been shown to result in the induction of tolerance to soluble proteins [17,18] and to minor histocompatibility antigens when administered in combination with anti-CD8 [19]. Thus, anti-CD4 treatment may not only be able to treat autoimmune disease, but re-establish tolerance to the autoanti-

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gens. In fact, this is supported by experiments in the non-obese diabetic (NOD) mouse model for diabetes [20,21].

In this study we examined the effects of a putative non-depleting CD4 antibody on the development of spontaneous autoantibodies to erythrocytes and on autoantibodies in NZB mice with established disease. Our data indicate that CD4 antibodies can prevent the appearance of erythrocyte autoantibodies, but this does not induce 'tolerance' to the autoantigens. In addition, we show that anti-CD4 eliminates erythrocyte autoantibodies in NZB mice with ongoing disease.

MATERIALS AND METHODS

Animals
NZB mice were purchased from Harlan Olac Ltd. (Oxford, UK). BALB/c mice were obtained from Harlan Olac or A. Tuck and Son Ltd. (Battlesbridge, UK). The mice were maintained in the animal care facility of the Immunology Department of UCL Medical School.

Antibody used
Mice were treated with a putative non-depleting rat IgG2a MoAb to mouse CD4 (YTS 177.9; a kind gift from Professor Herman Waldmann, Cambridge, UK). The antibody was prepared from ascitic fluid by ammonium sulphate precipitation and dialysis against PBS.

'Prevention' protocol
Two groups of eight 12-week-old NZB female mice were used. One group received injections of 1 mg and the other 2 mg of

antibodies to CD4. The initial injection was intravenous (i.v.), and this was followed by three intraperitoneal (i.p.) injections weekly for 19 weeks. A group of eight age- and sex-matched NZB mice and BALB/c mice received injections of 0·2 ml saline as positive and negative controls, respectively. In a second experiment, only 14-week-old NZB female mice which were Coombs' negative were used. BALB/c age- and sex-matched controls also received 2 mg anti-CD4 or saline in the protocol used above.

'Treatment' protocol
Seven 39-week-old NZB female Coombs' positive mice received injections of 2 mg of anti-CD4. The treatment schedule was the same as for prevention of development of the disease, and mice were injected three times weekly for 14 weeks. Controls received 0·2 ml saline three times weekly.

Bleeding
Blood was collected from the retro-orbital plexus (approximately 100 µl) and EDTA added as anticoagulant.

Coombs' test
This was carried out as previously described [22]. Briefly, 25 µl of approximately 5% washed mouse erythrocytes were mixed on a glass slide with 25 µl of 1:40 heat-inactivated rabbit anti-mouse serum. The slides were incubated for 30 min in a humid chamber and then scored as follows both by naked eye and microscopy: 0, no agglutination; −/+ and +, slight or strong agglutination by microscopy; ++ and +++, agglutination and strong agglutination seen by naked eye.

Measurement of serum antibodies to ssDNA and dsDNA
Antibodies to DNA were measured by ELISA. Ninety-six-well microtitre plates (Nunc, Roskilde, Denmark) that had been precoated with poly-L-lysine (50 µg/ml), were coated overnight at 4°C with either calf thymus dsDNA, ssDNA (heat denatured) or distilled water. The wells were washed three times with PBS. The wells were charge neutralized with poly-L-glutamate (100 µg/ml), washed three times with PBS and blocked with 5% bovine serum albumin (BSA) (both steps at 37°C for 1 h). The wells were washed three times with PBS-0·1% Tween 20. The test sera (from seven mice per group) and the positive control pool of sera were incubated at 37°C for 1 h (in dilutions starting from 1:50 up to 1:12 800 in PBS-0·1% Tween 20). Pooled high-titre anti-DNA sera from 6 to 8 months old NZB/NZW F₁ served as positive control. The wells were washed three times with PBS-0·1% Tween 20. Goat anti-mouse IgA, IgM and IgG alkaline phosphatase conjugate (Sigma Chemical Co., Poole, UK) was then added (at 1:500 dilution) and incubated for a further 1 h at 37°C. The wells were washed six times with PBS-0·1% Tween 20 and plates incubated with *p*-nitrophenyl phosphate (Sigma) for a further 45 min. Plates were read using a Dynatech MR 4000 ELISA reader with 405 nm filter. Concentration of the sera giving an OD of 0·5 was expressed as a percentage of the positive control at the same OD value.

Lymphocyte counts
Blood films were air-dried onto slides, fixed with methanol and then stained with Wright–Giemsa (Sigma WG-16). At least 200 leucocytes were counted per slide. The data are expressed as the mean ± s.d. for four to eight mice.

Fluorescence analysis of lymphocyte subpopulations
Blood samples from four to eight mice per group were dispensed in 30-µl aliquots into tubes (LP3, Luckman) and washed once with PBS. Cells were incubated for 30 min with 10 µl anti-CD4-PE conjugate (L3/T4-RD1; Coulter 6604360), anti-Ly5-PE (Coulter 6604358) or a control rat MoAb IgG-2b-PE, of irrelevant specificity (R-PE ; Bradsure R2b04). They were then washed once with PBS and further incubated for 30 min with 10 µl of anti-Ly2-FITC-conjugated (Coulter 6604363), anti-CD3-FITC (Serotec MCA 500F) or control rat IgG-2b-FITC (of irrelevant specificity, Bradsure, R2b01), respectively. Becton Dickinson FACS lysing solution (92-0002) was added for 5 min to lyse the erythrocytes. The cells were washed, fixed with 1% formaldehyde in PBS and analysed for two-colour fluorescence using a Becton Dickinson FACScan 30. Lymphocytes were gated using forward angle and side scatter and at least 3000 cells analysed.

Statistical analysis
The non-parametric Fisher test was used to compare two independent groups of small sample size, and the non-parametric Mann–Whitney test was used to compare two small groups of ranked samples (i) for the lymphocyte subpopulations, and (ii) for the values of anti-DNA antibodies as a percentage of the positive controls.

RESULTS

Anti-CD4 prevents the development of erythrocyte autoantibodies in NZB mice
Treatment of NZB mice with either 1 or 2 mg of anti-CD4 prevented the development of Coombs' positive antibodies (Fig. 1). This was statistically significant at week 15 and week 21 compared with the saline-treated group. Similar results were obtained in a second experiment, but none of the mice after 13 weeks of anti-CD4 treatment was Coombs' positive (data not shown).

Tolerance to erythrocyte autoantigen(s) is not induced by anti-CD4 treatment
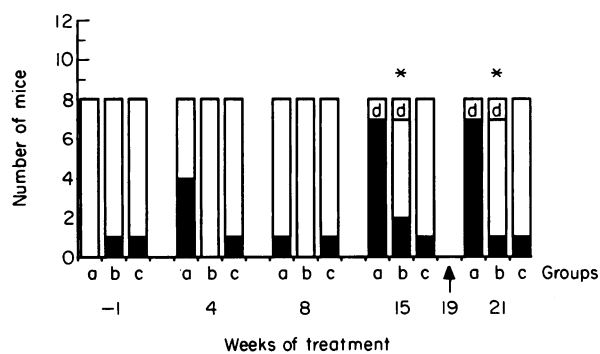The mice in the above experiment (see Fig. 1) were followed for

Fig. 1. Prevention of the development of autoantibodies in NZB by anti-CD4 treatment. The Coombs' test was carried out as described in Materials and Methods. □, Negative; ■, positive. Mice were treated with 2 mg anti-CD4 (group b), 1 mg anti-CD4 (group c) or saline (group a). Fisher test: *P < 0·05 (a *versus* b); P < 0·05 (a *versus* c). Arrow, treatment was stopped; d, died.
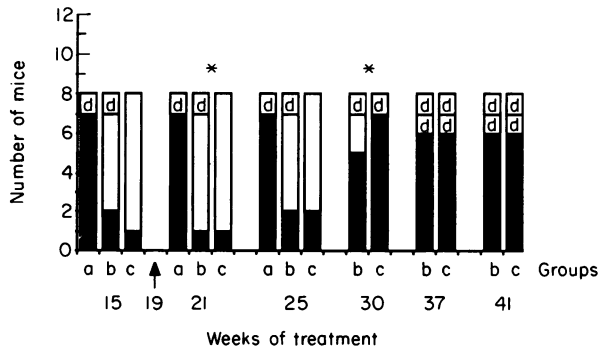
Fig. 2. Erythrocyte autoantibody production following the withdrawal of anti-CD4 treatment in NZB mice. The Coombs' test was carried out as described in Materials and Methods. □, Negative; ■, positive. Mice were treated with 2 mg anti-CD4 (group b), 1 mg anti-CD4 (group c) or saline (group a). Fisher test comparing groups at 21 and 30 weeks: *$P < 0.05$ (b *versus* b); $P < 0.05$ (c *versus* c). Arrow, treatment stopped; d, died.

several weeks after withdrawal of the anti-CD4 treatment. Most mice became Coombs' positive by 11 weeks after the last injection of anti-CD4 (Fig. 2). Thus, the anti-CD4 treatment failed to induce tolerance to erythrocyte autoantigens.

Anti-CD4 treatment abrogated erythrocyte autoantibody production in Coombs' positive mice
Anti-CD4 antibody treatment abrogated anti-erythrocyte production. Only two NZB mice out of seven and none of seven were Coombs' positive after 9 and 14 weeks of treatment, respectively. All mice receiving saline were still Coombs' test positive when examined at 9 and 14 weeks (Fig. 3).

Anti-CD4 suppressed the production of antibodies to DNA
Treatment with 2 mg anti-CD4 reduced the spontaneous production of both anti-ssDNA and anti-dsDNA antibodies (Fig. 4). Mean values of 29·4% and 29·7% for ssDNA and dsDNA antibodies were obtained for control mice expressed as a
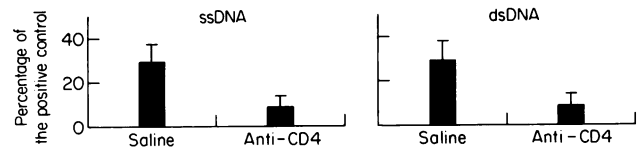
Fig. 4. Antibodies to ssDNA and dsDNA in serum of NZB mice treated with saline or anti-CD4. Serum from seven mice per group, bled at week 22 after starting the treatment with anti-CD4 or saline, were analysed. $P < 0.001$.
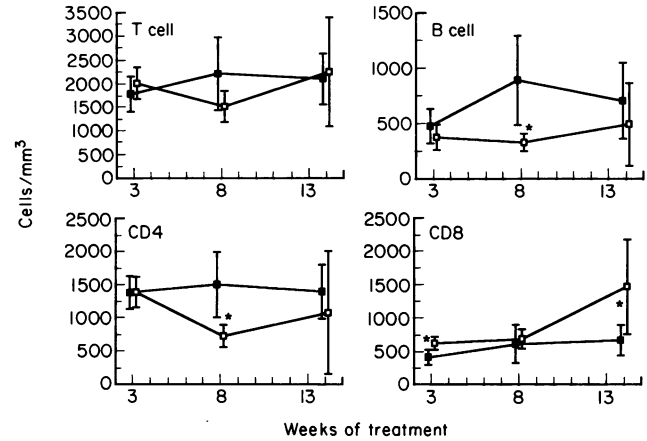
Fig. 5. Changes in circulating lymphocyte subpopulations of NZB mice during anti-CD4 treatment. FACScan analysis was carried out as described in Materials and Methods. Data are from mice treated with 2 mg anti-CD4 (□), and control saline-treated mice (■). Mann–Whitney test: *$P < 0.05$. Mean ± s.d.

percentage of positive pooled sera values (see Materials and Methods). A significant decrease in antibodies to ssDNA and dsDNA was seen in the anti-CD4-treated mice (to 9% and 8·8% of control values, respectively; $P < 0.001$).

The effects of anti-CD4 treatment on circulating lymphocyte subpopulations
There was a considerable heterogeneity in the absolute numbers of the different lymphocyte populations evaluated (Fig. 5). However, there was a significant increase in CD8+ T cells at 3 and 14 weeks, whereas CD4+ T cells and B cells were decreased at 8 weeks after anti-CD4 treatment compared with controls. In another experiment which was terminated at 5 weeks, only CD8+ T cells were increased in the anti-CD4-treated group at this time (data not shown).

DISCUSSION

In this study we show that antibodies to CD4 molecules *in vivo* both prevent and abrogate the spontaneous production of autoantibodies to erythrocytes in young and old NZB mice, respectively. These findings are consistent with several other studies in which anti-CD4 antibodies were successfully used either to retard, prevent or reverse the clinical and immunological manifestations of several murine models of autoimmune diseases such as diabetes in NOD mice [14,20,21], collagen-
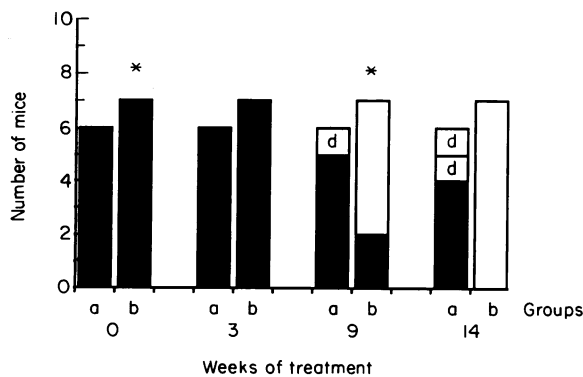

Fig. 3. Reversal of autoantibody production by anti-CD4 treatment in Coombs' positive mice. The Coombs' test was carried out as described in Materials and Methods. □, Negative; ■, positive. Mice were treated with 2 mg anti-CD4 (group b) or saline (group a). Fisher test comparing groups at 0 and 9 weeks: *$P < 0.05$ (b *versus* b). d, Died.

induced arthritis in DBA/1 mice [15], systemic lupus erythematosus (SLE) in NZB/NZW F_1 [11,12] and BXSB mice [13], and chronic relapsing encephalomyelitis in SJL/J [16].

In these long-term experiments we have used saline rather than an irrelevant rat MoAb since the experiments of Wofsy [23] with BXSB mice showed that mice receiving rat IgG over a long period died prematurely, presumably due to T-dependent anti-rat antibodies. This cannot occur when the T cells are compromised by the anti-CD4 specificity of the rat antibodies [13,18].

In NZB mice, the autoimmune response has previously been shown to be T cell-dependent *in vitro* [10]. The present studies confirm the role of T cells and show further that $CD4^+$ T cells are mandatory for the production of the erythrocyte autoantibodies. The non-T cell-dependent $CD5^+$ B cell population has been implicated in the production of erythrocyte autoantibodies in NZB mice [23], but our data would support a non-$CD5^+$ B cell origin for these antibodies unless an unusual switch of $CD5^+$ B cells to T-dependence was a feature of the disease process. Furthermore, sequencing studies of erythrocyte autoantibody-producing clones from NZB mice have suggested that they are derived from $CD5^-$ B cells [24]. Since some NZB mice spontaneously develop anti-DNA autoantibodies, it was important to determine whether or not anti-CD4 prevented the development of these autoantibodies. Our results showed that $CD4^+$ T cells were required for production of autoantibodies to DNA (Fig. 4). This is consistent with the data of Wofsy & Seaman, who found a decrease of DNA antibodies in NZB/NZW F_1 mice treated with anti-CD4 [11]. From cloning studies of NZB/NZW F_1 T cells it appeared that there were two kinds of T helper cells involved in the production of DNA autoantibodies. One helped via a cognate interaction and MHC class II, and the other through a bystander effect [25]. If such T cells are involved in the production of DNA autoantibodies in NZB mice it is possible that the anti-CD4 inhibits one or other of these T cells or both.

The mechanisms whereby anti-CD4 inhibited the production of erythrocyte autoantibodies are at present unclear. It is unlikely to be direct CD4 T cell lymphopenia, since we purposely used a putatively non-depleting antibody, and CD4 levels were only slightly decreased (Fig. 5). Although changes in lymphocyte numbers have not been seen previously following short-term treatment with a putatively non-depleting anti-CD4 antibody [18], it is possible that the minor fluctuations in absolute numbers of CD4 and $CD8^+$ T cells, and B cells seen here are the result of long-term therapy, which might modify homeostatic mechanisms required for maintenance of lymphocytes in the circulation. It cannot, however, be excluded that the increase in $CD8^+$ T cells seen after 14 weeks of treatment with anti-CD4 might be contributing to the suppression of erythrocyte autoantibodies (and possibly DNA autoantibodies) seen at this time. There are a number of possibilities which might explain the inhibitory effects of anti-CD4 on autoantibody production:

1 *Blindfolding or blockade.* Anti-CD4 antibodies could block the interaction between CD4 T cells and autoantigen(s) presented by MHC class II molecules on accessory cells in the same manner in which it has been shown that T cell responses can be blocked both *in vivo* and *in vitro* by antibodies to anti-MHC class II [26,27]. Furthermore,

murine experimental SLE, encephalomyelitis and myasthenia gravis have been treated successfully with anti-MHC class II antibodies [28–31].

2 *Down-regulation of the expression of CD4 molecules.* Continuous treatment with anti-CD4 reduced the levels of CD4 detectable on circulating T cells (data not shown). This would also result in reduced interaction between CD4 on T cells and MHC class II on accessory cells.

3 *Generation of a direct negative signal to the CD4 cells.* It has been shown that antibodies to CD4 can inhibit IL-2 production by T cell hybridomas stimulated with concanavalin A (Con A), a system that does not need accessory cells [32].

4 *Depletion of specific antigen-reactive CD4 T cells through engagement of CD4 and autoantigen.* Since the number of specific T helper cells for autoantigen are small, this would probably not have been detected in our analysis.

5 *Transient induction of active suppressors.* It cannot be excluded that there was a transient anti-CD4-induced generation of specific suppressor cells. Previous studies have indicated that mice treated with thyroglobulin and anti-CD4 generate transferable specific suppressor cells in antigen-naive animals [33]. Specific suppressor cells against transplantation antigens have also been demonstrated [34]. In the latter model these were shown to be long-lived.

In previous studies, tolerance to repeated injections of a soluble antigen was achieved when anti-CD4 treatment was discontinued [17,18,35]. With regard to tolerance induced to autoantigens, 17 out of 18 NOD diabetic mice given long-term anti-CD4 treatment remained normoglycaemic at least up to 7·5 months following withdrawal of treatment [21]. Interestingly, these same mice had insulitis, suggesting that additional factors are required for the clinical manifestation of the disease. In our experiments, however, we failed to induce tolerance to the erythrocyte autoantigens, even though the presence of self erythrocytes is tantamount to the repeated exposure to antigens, which was a feature of the experiments cited above [18,35]. This difference in the outcome may be due to: (i) the fact that tolerance using anti-CD4 might require a soluble form of an (auto)antigen, whereas in the case of erythrocytes the autoantigen is particulate. This seems unlikely, since tolerance to minor histocompatibility antigens has been achieved with anti-CD4, although anti-CD8 was also required to produce long-term survival of skingrafts [19]. Since we have recently shown that erythrocyte autoantibodies in NZB mice are directed to band 3 erythrocyte molecules [6], tolerance induction is currently being tested using anti-CD4 and a soluble form of this antigen; (ii) an inherent defect in T/B cell development in NZB mice [9,10,36] might make tolerance induction more difficult. In fact, high doses of bovine gammaglobulin or human gammaglobulin (HGG) given to 5–8-week-old NZB mice failed to induce tolerance [37]. In preliminary studies measuring antibodies to HGG, we have shown an incomplete tolerance following injection of 9–10-week-old NZB mice with HGG under the cover of anti-CD4. This same protocol resulted in complete tolerance in CBA/Ca mice ([18], unpublished observations); (iii) NZB B cells have been shown to be hyperresponsive [36] and may require only very small numbers of primed $CD4^+$ T cells to help autoantibody secretion by B cells. In this regard, it is interesting that one mouse in each of the groups receiving either 1 or 2 mg anti-CD4 was Coombs'

positive even after 21 weeks of treatment (see Fig. 1). We are currently analysing the requirement for band 3-specific CD4$^+$ T cells to help B cells secrete erythrocyte autoantibodies; (iv) it is envisaged that tolerance is only induced by anti-CD4 in T cells which are actively engaging the antigen. Possibly the relevant T helper cells are sequestered at a site not easily accessible to erythrocyte autoantigen(s), for example in the peritoneal cavity. Interestingly, mice made transgenic for NZB antibody genes coding for erythrocyte autoantibody became anaemic, even though one would expect the B cells to be tolerized in the excess of circulating erythrocytes. However, injection of erythrocytes into the peritoneal cavity resulted in inhibition of this autoantibody production [38], thus suggesting that autoreactive B cells remaining unexposed to autoantigen in the peritoneal environment are not made tolerant.

Even though the anti-CD4 treatment does not appear to induce tolerance in these experiments, the ability of these antibodies to reverse the manifestations of autoimmune disease is encouraging. Indeed, clinical trials with antibodies to CD4 carried out in patients with rheumatoid arthritis, psoriatic arthritis, and multiple sclerosis [39–42] hold out some promise for long-term therapy.

ACKNOWLEDGMENTS

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