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# Anti-CD4 treatment of NZB mice prevents the development of erythrocyte autoantibodies but hastens the appearance of anaemia

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## 1. Summary

New Zealand Black (NZB) mice spontaneously develop autoimmune haemolytic anaemia as the result of production of autoantibodies to erythrocytes. We have recently shown that antibodies to CD4 prevent the development of erythrocyte autoantibodies in young mice (Coombs' negative). In spite of this inhibition of erythrocyte autoantibody production, the anti-CD4-treated mice show a precocious and severe anaemia. Balb/c mice treated with the same protocol do not develop anaemia. Our results suggest that erythropoiesis in NZB mice is particularly sensitive to depletion of CD4<sup>+</sup> T cells.

## 2. Introduction

Monoclonal antibodies (mAb) to CD4 have been used successfully to treat several different murine autoimmune models of human diseases. These include diabetes, arthritis, encephalomyelitis and systemic lupus erythematosus [1–7]. Furthermore, clinical trials with anti-CD4 mAbs in patients with rheumatoid arthritis, psoriatic ar-

thritis, and multiple sclerosis [8–11] have shown beneficial effects with the likelihood that such treatment could become standard for a number of human autoimmune diseases.

Autoimmune haemolytic anaemia develops spontaneously in New Zealand Black (NZB) mice following the appearance of erythrocyte autoantibodies [12] detectable by the Coombs' test [13]. Using this model we have recently shown that anti-CD4 treatment abolishes the erythrocyte autoantibodies in Coombs' positive mice and prevents the development of such autoantibodies in young mice [14].

In this paper we show that although erythrocyte autoantibodies do not develop in anti-CD4-treated mice, anaemia as detected by reduced haematocrit values occurs earlier than in control NZB mice treated with saline.

## 3. Materials and Methods

### 3.1. Mice

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

### 3.2. Antibody used

Mice were treated with a putative non-deplet-

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ing rat IgG2a mAb to mouse CD4 (YTS 177; a kind gift from Prof. Hermann Waldmann, Cambridge). The antibody was prepared from ascitic fluid by ammonium sulphate precipitation and dialysis against phosphate-buffered saline.

### 3.3. Injection protocol

Two groups of eight 12-week-old NZB female mice were initially used for these experiments. One group received injections of 1 mg and the other 2 mg of antibodies to CD4 per injection. The initial injection was intravenous (i.v.) and this was followed by 3 intraperitoneal (i.p.) injections weekly for 19 weeks. In a second confirmatory experiment, 14-week-old Coombs' test negative female NZB mice and Balb:c mice (both 8 per group) received 2 mg of antibodies to CD4 or saline using the same protocol.

### 3.4. Coombs' test

This was carried out as previously described [14,15]. Briefly, 25  $\mu$ l of approximately 5% washed mouse erythrocytes were mixed on a glass slide with 25  $\mu$ l of 1:40 heat inactivated rabbit anti-mouse serum. The slides were incubated for 30 min in a humid chamber and then scored 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).

### 3.5. Haematocrit

Anticoagulated blood (50  $\mu$ l) was introduced into microtubes and the ends were sealed. Tubes were then microcentrifuged for 5 min and the haematocrits were measured [16].

### 3.6. Statistical analysis

The non-parametric Fisher test was used to compare two independent groups of small sample size and Mann-Whitney test was used to compare two independent small groups of data.

## 4. Results

### 4.1. Anti-CD4 prevents the development of erythrocyte autoantibodies in NZB mice

Treatment with both 1 and 2 mg of anti-CD4 prevented the development of Coombs' positivity compared with the saline controls (experiment 1). The average Coombs titres are shown in Fig. 1. Comparing the positive and negative Coombs' values for each group at a given time point, this was statistically significant by the 15th week of treatment (Fisher test). Similar results were obtained in the second experiment (data not shown).

### 4.2. Anti-CD4 treatment hastens the development of anaemia in NZB mice

Significantly lower haematocrit levels were detected in both NZB groups treated with anti-CD4 in the first experiment (data not shown). These findings were confirmed in the second experiment where by the 17th and 22nd weeks after starting treatment the anti-CD4-treated group showed a decrease in haematocrit levels as compared with the saline control group (Fig. 2a). No such changes were seen in Balb:c mice treated with anti-CD4 (Fig. 2b).

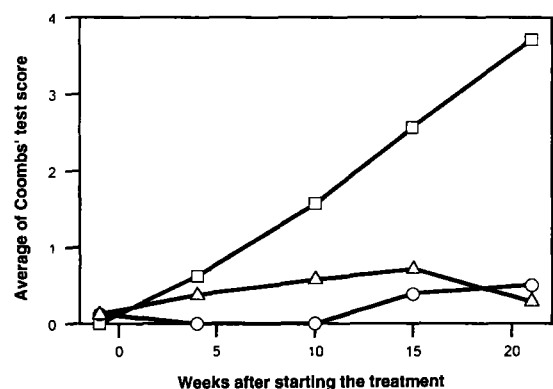


Fig. 1. Anti-CD4 prevents the development of autoantibodies in NZB mice. This graphic shows the average Coombs' test scores of (□) saline control group, (△) 1 mg and (○) 2 mg anti-CD4-treated groups.

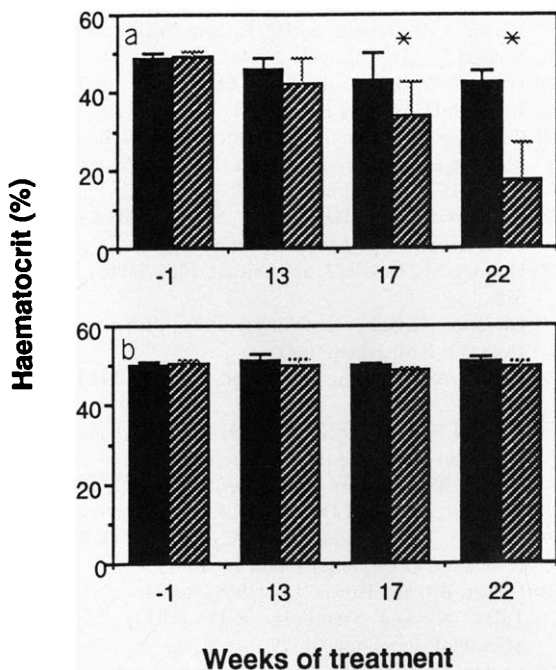


Fig. 2. Anti-CD4 treatment hastens the development of anaemia in NZB mice. Haematocrit values of blood from NZB (a) and Balb/c mice (b) treated with saline (■) or anti-CD4 (▨). Mean  $\pm$  SD. Mann-Whitney  $^*P < 0.01$

## 5. Discussion

In this study we confirm that antibodies to CD4 molecules *in vivo* prevent the development of erythrocyte autoantibodies in NZB mice (Fig. 1) [14] but show that they hasten the development of anaemia (Fig. 2). This was shown in two different experiments for 2 mg doses, but mice receiving 1 mg of anti-CD4 also showed anaemia; however, too few mice were used to show statistical differences from the 2 mg dose (data not shown).

It was interesting and unexpected that anaemia developed in the NZB mice in the absence of erythrocyte autoantibodies as detected by the Coombs' test. There are a number of possibilities which might account for this observation.

(1) T-cell-derived cytokines may play a critical role in erythropoiesis in NZB mice, and some strains of mice seem to be specially sensitive to T-cell depletion. In fact, following neonatal thymectomy or treatment with anti-lymphocyte anti-

bodies NZB mice have previously been shown to develop a wasting disease and anaemia [17,18]. In the present studies, Balb/c mice given comparable treatment with anti-CD4 did not develop anaemia.

(2) TNF has been shown to induce anaemia in mice [19]. In preliminary studies we have found just detectable serum levels of TNF- $\alpha$  in the NZB anti-CD4-treated group compared with NZB controls (Oliveira and Taverne, unpublished observations). Although requiring further study, we feel that this is unlikely to be due to contamination of our anti-CD4 with endotoxin since 100  $\mu$ g/ml of this preparation *in vitro* (equivalent to a maximum *in vivo* concentration) failed to stimulate peritoneal macrophages to produce detectable levels of TNF. This was tested under conditions where 16 ng/ml of endotoxin (LPS) produced 14 ng/ml TNF (Oliveira and Taverne, unpublished observations).

In preliminary experiments, following 22 weeks of anti-CD4 treatment we found that reticulocyte numbers were lower in the anaemic anti-CD4-treated NZB mice ( $11\% \pm 10$ ) than the non-anaemic saline control group ( $26\% \pm 17$ ,  $P < 0.05$ ), suggesting that long-term treatment with anti-CD4 can influence the early stages of erythropoiesis. Possibilities 1 and 2 both might explain these findings.

(3) NZB mice develop autoantibodies to erythrocytes several months before they become anaemic [20]. This is probably due to the fact that immune complexes spontaneously produced by ageing NZB mice saturate the Fc receptors on phagocytic cells, diminishing the efficiency of antibody-coated erythrocyte removal from the circulation [21]. Recovery of phagocytic activity has been associated with removal of immune complexes from the circulation [22]. Anti-CD4 treatment might well prevent the formation of the majority but not all autoantibodies which include anti-DNA antibodies and anti-thymocyte antibodies [21]. Although unlikely, it is possible that some erythrocyte autoantibodies (insufficient to be picked up by the Coombs' test) might remain which would then allow clearance of antibody coated erythrocytes on recovery of phagocytic cell Fc receptor mediated function.

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