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Effects of cyclophosphamide on autoantibody synthesis in the Brown Norway rat

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SUMMARY

The effects of cyclophosphamide on autoantibody synthesis were studied in an experimental model of glomerulonephritis due to autoantibodies to the glomerular basement membrane (GBM). Brown Norway rats develop anti-GBM antibodies, as part of a polyclonal response, when repeatedly injected with mercuric chloride ($HgCl_2$). Anti-GBM antibody levels peak between days 11 and 14 and thereafter rapidly fall; convalescent animals show a time-dependent resistance to rechallenge with $HgCl_2$ which remains significant for up to 3 months. The administration of cyclophosphamide, as a single intramuscular injection at day 0, has three distinct dose-dependent effects on anti-GBM antibody production. Firstly, lower doses (2·5 mg/kg) increase antibody levels at the time of peak response; secondly, higher doses ($\geqslant$20 mg/kg) prevent antibody synthesis following $HgCl_2$; and thirdly, the higher doses also reduce the response to rechallenge with $HgCl_2$ 3–4 months later. These effects of cyclophosphamide also apply to the polyclonal response to $HgCl_2$, as judged by measurement of total IgG concentrations. Further investigation of the mechanisms of action of cyclophosphamide in this model should provide information relevant to the treatment of human autoimmune disease.

Keywords cyclophosphamide autoantibody synthesis glomerular basement membrane

INTRODUCTION

The recognition that autoantibodies are important in the pathogenesis of diseases such as Goodpasture's syndrome (Lerner, Glassock & Dixon, 1967) and myasthenia gravis (Appel, Almon & Levy, 1975), has led to the development of treatment regimens designed to reduce antibody levels. Until recently, the conventional approach was the administration of various immunosuppressive agents, although their effects were too slow to be of benefit in many cases of Goodpasture's syndrome. We introduced plasma exchange (PE) to remove circulating autoantibody, pending the action of steroids and cytotoxic drugs (Lockwood *et al.*, 1976). Our clinical studies indicate that PE and cyclophosphamide can bring about rapid control of autoantibody synthesis in nephritis due to antibodies to the glomerular basement membrane (GBM) (Peters *et al.*, 1982). In order to rationalize the management of autoimmune disease, it is necessary to understand the mechanisms by which these therapies act. We have therefore examined the effects of cyclophosphamide in an experimental model of autoantibody-mediated glomerulonephritis produced in the Brown Norway (BN) rat by injection of mercuric chloride ($HgCl_2$) (Sapin, Druet & Druet, 1977; Bowman *et al.*, 1981). In this study, we find that cyclophosphamide may have various immediate and long term effects depending upon the dosage used, and believe that these findings could have important therapeutic implications.

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MATERIALS AND METHODS

Animals. Brown Norway rats were obtained from the Repgo Institute, Rijswijk, Holland, and a breeding colony established. Male animals, aged between 8 and 24 weeks, were used in all experiments involving the effects of cyclophosphamide. Experimental groups of rats were matched for age and weight, housed in standard conditions and fed normal laboratory diet and water *ad libitum*.

Disease model. Anti-GBM antibodies were induced by four subcutaneous injections of $HgCl_2$, 1 mg/kg body wt, given on alternate days as a 0·1% solution in distilled water. Blood samples were taken by tail artery puncture under ether anaesthesia.

Cyclophosphamide administration. Freeze dried cyclophosphamide (Farmitalia, Carlo Erba) was reconstituted with distilled water to a concentration of 20 mg/ml. Freshly prepared cyclophosphamide was administered by intramuscular injection to experimental animals, whilst controls received an equal volume of 0·9% saline. Two regimens were used: in initial experiments involving total doses of 20–160 mg/kg body wt, cyclophosphamide was given as four divided doses at the time of each injection of $HgCl_2$; in subsequent experiments involving doses of 1·25–20 mg/kg body wt, it was given as a single dose at the time of the first injection of $HgCl_2$.

Assay for anti-GBM antibody. Anti-GBM antibody was measured by a solid phase, radioimmunoassay, as previously described (Bowman, Peters & Lockwood, 1983). A collagenase digest of normal rat GBM was coated onto microtitre plates overnight at 4°C, and test or control sera were applied for 1 h at 37°C. Binding of specific antibody was recognized by further incubation for 1 h at 37°C with affinity purified ^{125}I-labelled rabbit anti-rat IgG.

Assay for IgG. Total IgG concentrations were measured by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) into 1·5% agarose gel in complement fixation diluent (CFD, Oxoid) containing rabbit anti-rat IgG (Miles).

Statistical analysis. Differences between data were determined by Student's *t*-test.

RESULTS

Effect of $HgCl_2$

All control rats injected with $HgCl_2$ made detectable circulating anti-GBM antibody, the levels of which peaked between days 11 and 14 and thereafter fell. The kinetics of antibody production were the same in animals receiving four injections of $HgCl_2$, as in those given repeated injections (Bowman *et al.*, 1981).

Rechallenge with $HgCl_2$

Rats that have recovered from $HgCl_2$-induced nephritis show resistance to further injections of $HgCl_2$. To determine the duration of this resistance, groups of rats were rechallenged with $HgCl_2$ between 1 and 4 months after an initial course of four or five injections. Pooled results of these experiments are shown in Fig. 1. Significant resistance to rechallenge was found at 1 month and this effect was still present, although less marked, at 3 months.

Effect of cyclophosphamide

The effect on antibody synthesis of doses of cyclophosphamide from 1·25–160 mg/kg was examined. In each experiment four to six animals were studied at different doses. Anti-GBM antibodies were measured serially for 4 weeks, and when possible daily samples were taken during the time of peak response (days 10–14). Initial experiments, using four divided doses totalling 20–160 mg/kg, revealed that the surviving treated animals made no, or barely detectable, antibody. Animals receiving 160 mg/kg died within 2 weeks, whereas those receiving 80 mg/kg or less survived long term.

We subsequently studied the effect of 1·25–20 mg/kg given as a single injection at day 0. The kinetics of antibody synthesis in one of four experiments are shown in Fig. 2. It can be seen that

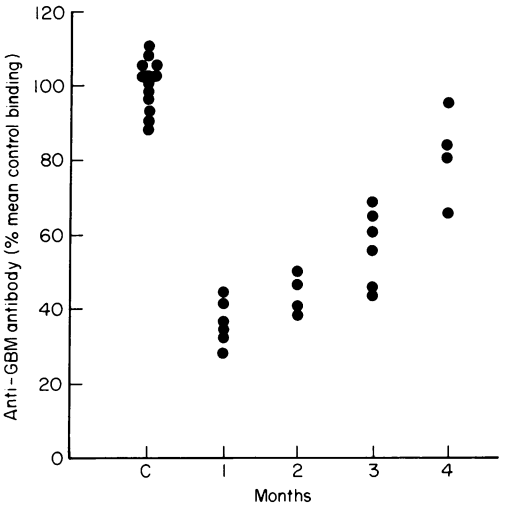

Fig. 1. Peak anti-GBM antibody levels following $HgCl_2$ administration to naive controls (C), and to animals being rechallenged after 1–4 months. Control results were significantly different to those of rechallenge at 1 month ($P < 0{\cdot}001$), and 3 months ($P < 0{\cdot}001$).

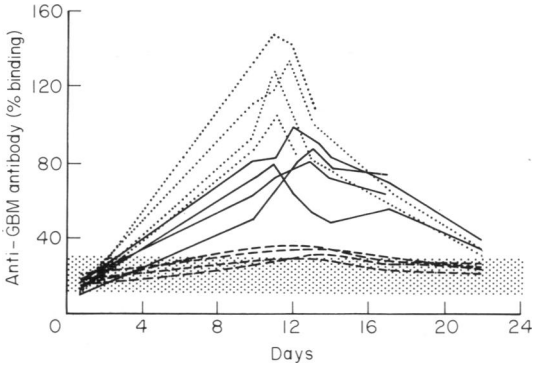

Fig. 2. Time course of anti-GBM antibody synthesis following $HgCl_2$ administration to controls (——), and to animals receiving cyclophosphamide 2·5 mg/kg (. . . .) and 20 mg/kg (- - - -). The normal range is shown by the stippled area.

animals given 2·5 mg/kg made higher peak levels than controls, but that subsequent control of antibody production was normal. Animals receiving 20 mg/kg made minimal amounts of antibody at the time of peak response. Pooled results of four experiments are shown in Fig. 3. An increase in peak antibody levels was seen in animals receiving 2·5 mg/kg, and a striking reduction in antibody synthesis observed in those given 20 mg/kg.

Rechallenge of cyclophosphamide treated rats

To determine whether the effect of higher doses of cyclophosphamide on antibody production was long lasting, animals which had been treated with cyclophosphamide were rechallenged at 3–4 months, by which time $HgCl_2$-induced resistance was diminishing. Animals which had received 20–80 mg/kg as divided doses in the initial experiments made a minimal response, as compared with their controls rechallenged after $HgCl_2$ alone (Fig. 4). Of the animals which had received 1·25–20 mg/kg as a single dose, those given 1·25–2·5 mg/kg produced similar peak antibody levels to controls, those given 5–10 mg/kg made less antibody, and those given 20 mg/kg made a greatly

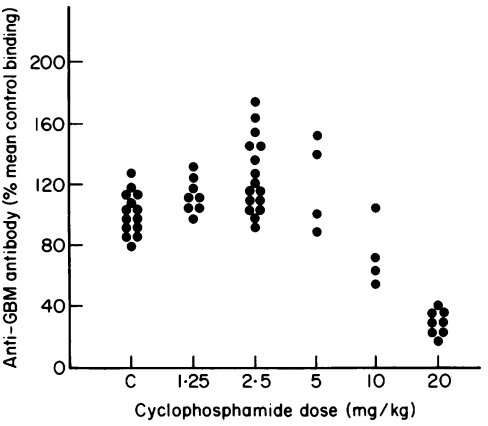

Fig. 3. Peak anti-GBM antibody levels following $HgCl_2$ administration to controls (C), and to animals receiving cyclophosphamide 1·25–20 mg/kg as a single dose. Control results were significantly different to those receiving 2·5 mg/kg ($P < 0{\cdot}01$) and 20 mg/kg ($P < 0{\cdot}001$).

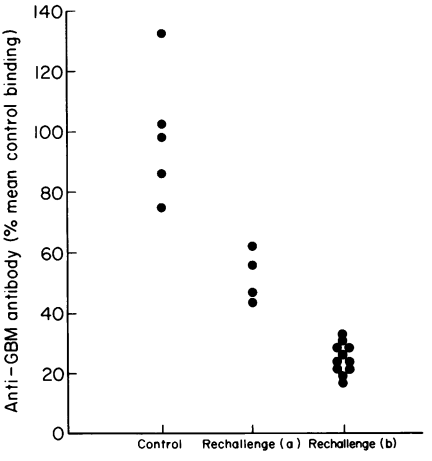

Fig. 4. Peak anti-GBM antibody levels following $HgCl_2$ administration to controls, to animals being rechallenged after 3 months (a) and to rechallenged animals that had also received cyclophosphamide 20–80 mg/kg in divided doses (b). Control results were significantly different to those of rechallenge after cyclophosphamide ($P < 0{\cdot}001$). Results of the rechallenged group were also different to those rechallenged after cyclophosphamide ($P < 0{\cdot}001$).

reduced response (Fig. 5). Although results from experiments using divided doses or a single higher dose were similar, they should be regarded separately as the effects of the drug could be different in each situation.

Pre-treatment with cyclophosphamide alone

To determine whether cyclophosphamide pre-treatment, without an initial $HgCl_2$ challenge, would have long term effects on the ability to respond to $HgCl_2$, groups of rats were given 5–40 mg/kg cyclophosphamide alone and challenged with $HgCl_2$ 3 months later. The pooled results are shown in Fig. 6. Cyclophosphamide given alone had no effect on the subsequent antibody response to $HgCl_2$.

Effects on total IgG concentration

Initial challenge. The effect of $HgCl_2$ on the polyclonal response, in certain of the experiments,

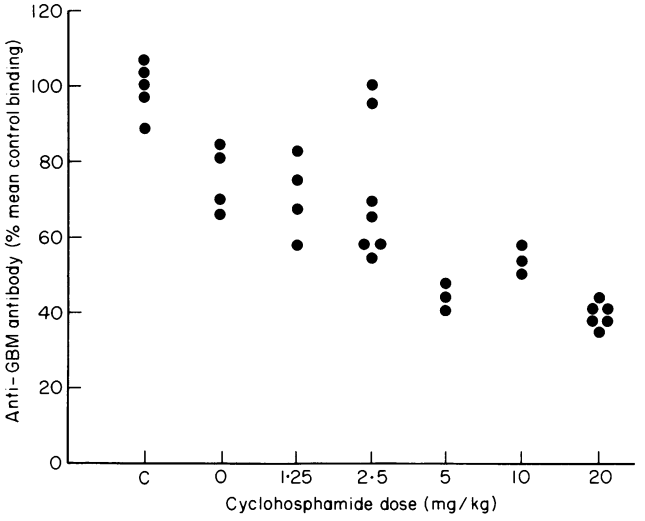

Fig. 5. Peak anti-GBM antibody levels following $HgCl_2$ administration to controls (C), to animals being rechallenged after 3–4 months (0), and to rechallenged animals that had also received cyclophosphamide 1·25–20 mg/kg as a single dose, 3–4 months previously. Control results were significantly different to those of rechallenge after cyclophosphamide 20 mg/kg ($P < 0{\cdot}001$). Results of the rechallenged group were also different to those rechallenged after cyclophosphamide 20 mg/kg ($P < 0{\cdot}001$).

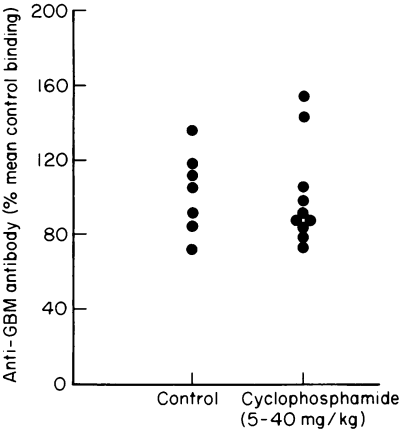

Fig. 6. Peak anti-GBM antibody levels following $HgCl_2$ administration to controls, and to animals that had received cyclophosphamide alone 3 months previously at doses of 5 mg/kg ($n = 3$), 20 mg/kg ($n = 3$) and 40 mg/kg ($n = 4$). There was no difference between the groups.

was assessed by comparing the ratio of peak to initial IgG levels in individual animals. This ratio was used because IgG concentrations rose with age in our rats. Pooled results are shown in Fig. 7a; a striking increase in total IgG followed challenge with $HgCl_2$ alone, and this response was reduced by cyclophosphamide 20 mg/kg.

Rechallenge. On rechallenge of animals which had received $HgCl_2$ alone 3–4 months previously there was a smaller relative rise in IgG. This response was reduced or absent in rats which had received cyclophosphamide 20 mg/kg at the time of the initial challenge (Fig. 7b).

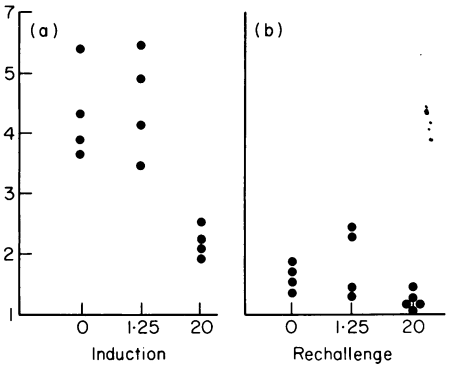

Fig. 7. Ratio of peak to initial IgG levels. (a) Following $HgCl_2$ administration to controls (0), and to animals receiving cyclophosphamide 1·25 mg/kg and 20 mg/kg. (b) Following $HgCl_2$ rechallenge of controls (0), and of animals that had also received cyclophosphamide 1·25 mg/kg, 5 mg/kg and 20 mg/kg, 3–4 months previously.

DISCUSSION

The development of autoantibodies to GBM in BN rats injected with $HgCl_2$ is a self-limiting, strain-dependent phenomenon, related to the major histocompatibility complex RT1 and to at least one other gene (Druet *et al.*, 1977). It has been demonstrated that $HgCl_2$ acts as a T-dependent polyclonal activator in the BN rat (Hirsch *et al.*, 1982) and our observations of a striking rise in total IgG concentration, and of the formation of a variety of other autoantibodies, provide further evidence for this effect. Anti-GBM nephritis in man is also genetically restricted (HLA DR2 in 32 out of 36 of our cases), and autoantibody formation is self-limiting, although this may take up to 2 years (Wilson & Dixon, 1981). These similarities, together with the consistency of the antibody response in $HgCl_2$ challenged animals, make the BN model suitable for investigating the effects of cyclophosphamide on autoantibody synthesis.

We observed three separate dose-dependent phenomena; firstly, lower doses of cyclophosphamide (2·5 mg/kg) increased antibody levels at the time of peak response but did not affect subsequent control; secondly, higher doses ($\geqslant$ 20 mg/kg) prevented antibody synthesis at the time of the initial response; and thirdly, these higher doses also reduced the response to rechallenge with $HgCl_2$ 3–4 months later. Although it is difficult to compare dosages between species, as there are differences in the rate of metabolism and sensitivity of target cells (Freireich *et al.*, 1966), the wide range of doses used in this study includes equivalent doses commonly used in man (20 mg/kg in rat compares with 3 mg/kg in man on a mg/m^2 basis).

The enhancement of antibody formation with 2·5 mg/kg of cyclophosphamide could be explained by the relatively greater susceptibility of T suppressor cells or their precursors. Although we have not demonstrated this in our present study, there is good evidence for such selectively in mouse (Schwartz, Askenase & Gershon, 1978; Diamentstein *et al.*, 1981) and man (Stevenson & Fauci, 1980; Ozer *et al.*, 1982). The target cell at our higher doses is unknown, and could be a T helper or B lymphocyte, as the response to $HgCl_2$ is T-dependent (Hirsch *et al.*, 1982). The B cell has been shown to be more susceptible to cyclophosphamide in other species (Schwartz *et al.*, 1978; Stevenson & Fauci, 1980), and is perhaps the more likely target, particularly as cyclophosphamide can suppress B cell function at non-cytotoxic doses (Shand & Howard, 1979; Cupps, Edgar & Fauci, 1982).

The mechanisms of long term resistance to rechallenge with $HgCl_2$ following high doses of cyclophosphamide may be of particular clinical relevance. The lack of effect of pre-treatment with cyclophosphamide alone indicates a selective action on those cells altered by $HgCl_2$. One possible explanation is functional deletion of the stimulated B cell clones; the alternative is that cyclophosphamide has induced a population of cells capable of long term suppression of the

autoantibody response to $HgCl_2$ (reviewed by Moreno, 1982). Spleen cell transfer experiments from rats with cyclophosphamide-induced resistance will help to resolve this question. We have performed such experiments to investigate the short term resistance following $HgCl_2$ alone, and have shown that this can be transferred by spleen cells, with the T suppressor population playing an important role (Bowman *et al.*, unpublished data). However, the duration and extent of resistance following cyclophosphamide treatment make it likely that different mechanisms are involved.

Clinical observations suggest that cyclophosphamide can prevent recurrence of antibody formation following PE in Goodpasture's syndrome (Peters *et al.*, 1982) and in systemic lupus erythematosus (SLE) (Verrier Jones *et al.*, 1981). One hypothesis to explain this effect is that PE may result in clonal stimulation of the cells involved in the antibody response, rendering them more susceptible to cylophosphamide. Such a mechanism would be compatible with the immediate and long term effects of higher doses of cyclophosphamide in the BN rat; these doses reduced the polyclonal response induced by $HgCl_2$ as well as specific anti-GBM antibody production. The therapeutic implications of our experimental findings may therefore extend from anti-GBM nephritis to diseases such as SLE, in which polyclonal activation occurs and relapses are characteristic.

Our study demonstrates the varied effects of different doses of cyclophosphamide on autoantibody synthesis in the rat. It is possible that the long term resistance to autoantibody formation seen after higher doses will be of particular clinical relevance. Further investigation of the cellular basis of these phenomena will clarify the mechanisms of action of cyclophosphamide, and should allow the introduction of more rational treatment regimens for human autoimmune disease.

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