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Circulating T-cell populations during mercuric chloride-induced nephritis in the Brown Norway rat

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SUMMARY

Fluorescence-activated cell sorter analysis was used to study the peripheral lymphocyte populations during mercuric chloride ($HgCl_2$)-induced autoimmune nephritis in the Brown Norway (BN) rat. Sequential studies showed a transient loss of T cells from peripheral blood attributable to decreases in the percentage of T-helper cells. In addition, there was a decrease in the percentage of T-cytotoxic/suppressor cells prior to the appearance of circulating anti-GBM antibodies, followed by elevated levels of T-suppressor cells during down-regulation of the response. This method may allow closer inspection of the events linking changes in T-cell populations and induction and termination of an autoimmune response.

INTRODUCTION

Abnormalities in the proportion of B and T lymphocytes and T-cell subsets have been noted in a number of autoimmune diseases, or diseases with a suspected autoimmune aetiology, in both peripheral blood and lymphoid organs such as the spleen (Chatenoud & Bach, 1981; Okita, Row & Volpe, 1981; Miller & Schwartz, 1982). Such changes, which include decrease in percentage and/or absolute numbers of T-cytotoxic/suppressor cells (Duke *et al.*, 1983; Craig *et al.*, 1985), and increase in T-helper cells or T-helper cell function (Sundeen *et al.*, 1979), have been thought to reflect alterations in the immune system that are important in the pathogenesis of the autoimmune response, either directly, where cell-mediated injury occurs, or indirectly, by indicating loss of regulation of autoantibody production.

Sequential studies of lymphocyte subsets in experimental models of autoimmunity may help in an elucidation of their relationship with disease pathogenesis, and in this way we have studied mercuric chloride ($HgCl_2$)-induced, anti-glomerular basement membrane (GBM) autoantibody-mediated nephritis in the Brown Norway (BN) rat. This is a genetically restricted autoimmune disease, which is biphasic, consisting initially (within 2 weeks) of production of anti-GBM antibodies, which fix in a linear pattern along the GBM, followed by a second phase, where granular deposits of immunoglobulin are apparent (Sapin, Druet & Druet, 1977; Druet *et al.*, 1978; Bowman *et al.*, 1984). The disease is self-limiting, and during down-regulation of anti-GBM antibody production it has been found that a population of T-cytotoxic/suppressor cells appears in the spleen, which can transfer suppression to normal syngeneic recipients (Bowman *et al.*, 1984).

The mechanism by which $HgCl_2$ exerts its effect in this model is not clear; some have suggested that it may act as a polyclonal activator of B cells (Hirsch *et al.*, 1982), and others that it may directly stimulate T-helper cells (Pelletier *et al.*, 1985a, b). Interestingly, in the PVG rat, where $HgCl_2$ produces an immune complex-mediated glomerulopathy, *in vitro* studies have shown that $HgCl_2$ affects T-suppressor cell function (Weening, Hoedemaeker & Bakker, 1981).

The aims of this study were to determine whether $HgCl_2$ produced changes in circulating lymphocyte populations similar to those that we had demonstrated in the spleen, and whether it was feasible to study these in serial blood samples. This was achieved using a method of labelling lymphocytes in small volumes of peripheral blood, which in turn circumvented two major problems; firstly, anaemia (likely to occur because of frequent blood sampling during a short-lived autoimmune response), and secondly, distortion of the lymphocyte populations, which can occur when lymphocytes are separated by methods such as Ficoll density gradient centrifugation (Pontesilli *et al.*, 1986).

This paper describes the whole blood-labelling technique, using indirect immunofluorescence and flow cytometry, and its use in analysing sequential changes in T-cell subsets in $HgCl_2$-

Abbreviations: BN, Brown Norway; EAE, experimental allergic encephalomyelitis; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; mAb, monoclonal antibody(ies); PBS, phosphate-buffered saline; RIA, radioimmunoassay; SD, standard deviation; Th, T-helper (cell); Tc/s, T-cytotoxic/suppressor (cell); WBC, white blood cell.

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induced nephritis in the BN rat. We have found transient decreases in total T cells, which are attributable to decreases in MRC W3/25+ cells (T helper). In addition, it was noted that decreases in T-cytotoxic/suppressor cells, which preceded the anti-GBM autoantibody response, were followed, in a proportion of rats, by abnormally high levels of T-cytotoxic/suppressor cells during down-regulation of anti-GBM antibody production.

MATERIALS AND METHODS

Animal protocol

BN rats were obtained from the Repgo Institute (Rijswick, The Netherlands) and a breeding colony established. Wistar rats were purchased from Charles River, U.K., Ltd, London. Animals were fed standard laboratory diet and water *ad libitum*. Female rats, aged 7–12 weeks, were injected three times a week for 2 weeks with 1 mg/kg $HgCl_2$, given subcutaneously as a 0·1% solution in distilled water. Control animals were injected with phosphate-buffered saline (PBS). Serial blood samples were taken by tail artery puncture under ether anaesthesia and collected into Heparin (Monoparin, Weddel Pharmaceuticals Ltd, Wrexham).

Antisera and proteins

Mouse monoclonal antibodies (mAb) MRC OX8, MRC W3/25, MRC OX19 and MRC OX12, which bind to rat T-cytotoxic/suppressor, T-helper (Brideau *et al.*, 1980), T (Dallman, Thomas & Green, 1984) and B cells (Hunt & Fowler, 1981) were purchased from Serotec Ltd (Bicester, Oxon). An irrelevant mouse monoclonal antibody to cytomegalovirus was kindly provided by Dr B. Rogers, RPMS, London. FITC goat anti-mouse Ig was a kind gift of D. Grennan, RPMS, London. For some experiments FITC goat anti-mouse Ig was bought from Coulter Ltd (Luton, Beds).

Anti-GBM radioimmunoassay (RIA)

Antibodies to GBM were assayed using a solid-phase RIA with a substrate of collagenase-digested rat GBM, as described in detail elsewhere (Bowman, Peters & Lockwood, 1983).

Total and differential white blood cells (WBC)

Total WBC counts were determined using a ZF model Coulter Counter on heparinized blood samples lysed with Zaponin (Coulter Ltd). Differential WBC counts were performed on ethanol-fixed blood smears using modified Giemsa stain (R. A. Lamb, RPMS, London) and standard techniques.

Flow cytometry cell analysis and lymphocyte subsets

Lymphocytes were labelled with the monoclonal antibodies MRC OX19, MRC OX8, MRC W3/25 and MRC OX12 using a whole-blood technique. From sequential samples of heparinized blood, 100-μl aliquots were washed three times in 10 ml PBS and incubated with 20 μl of 1/100 mAb in PBS 1% bovine serum albumin, or medium for 30 min at room temperature (RT). After further washing with PBS, labelled cells were detected by incubation with microfuged FITC goat anti-mouse Ig in 20% normal rat serum/PBS for 20 min at RT. Cells were then washed with PBS and erythrocytes lysed by suspending the pellet in 1 ml lysing buffer (0·15 M NH_4Cl, 10 mM $KHCO_3$, 0·5 mM EDTA, pH 7·4), incubating on ice for 5 min and washing with PBS. Cells were fixed in 1% paraformaldehyde in PBS, and kept in the dark until analysis, preferably within 48 hr.

Analyses were performed on a Coulter EPICS C fluorescence-activated cell sorter, using forward light scatter and 90° light scatter. A total of 10^5 cells from the lymphocyte-rich fraction of mononuclear cells were analysed for labelled cells, expressed as a percentage, and compared to the negative control (no first antibody), which was subtracted. Initially negative controls were labelled with an irrelevant mouse monoclonal antibody to cytomegalovirus, until this was found to give results not significantly different to controls incubated with medium. Absolute numbers of positive lymphocytes/ml peripheral blood could be derived using percentage positive cells and total and differential WBC counts. Percentages of T-cell subsets in spleens were determined in a similar method using 10^6 WBC/test but replacing the lysis step with distilled water shock lysis.

Statistical analysis

Statistical analysis was performed using the Wilcoxon rank sum method for non-parametric significance testing.

RESULTS

Anti-GBM antibodies and WBC counts

In $HgCl_2$-treated BN rats, anti-GBM antibodies were detectable in the circulation after Day 7, peaked between Days 10 and 14, and then fell spontaneously (Fig. 1). Similar kinetics of antibody

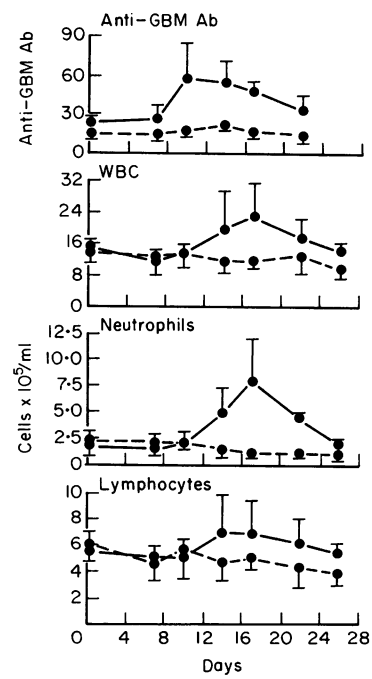

Figure 1. Time–course of anti-GBM antibody, total WBC, neutrophils and lymphocytes in $HgCl_2$-treated BN and Wistar rats (combined results from two experiments). Groups of female BN (●—●) or Wistar (●- - -●) rats ($n=6$) were subjected to the standard $HgCl_2$ injection protocol and sequential assays of anti-GBM antibody, and total and differential WBC counts performed, from which were also derived the number of neutrophils and lymphocytes/ml. Results shown represent the mean ± SD of each group, expressed as percentage positive binding (anti-GBM antibody) or cells/ml.

production were found if $HgCl_2$ was given continuously compared with $HgCl_2$ given for only 2 weeks (data not shown). Peripheral WBC counts increased after Day 12, peaked at Days 14–17, and then usually returned to pretreatment levels by Day 26 (Fig. 1). The increase in total circulating WBC coincided with an increase in neutrophils, peaking at Day 17 (neutrophils: Day 0 *vs* Day 17, $P<0{\cdot}02$). The number of circulating neutrophils returned to pretreatment levels by Day 26. This neutrophilia was reproducible in two separate experiments. Percentage as well as total numbers of neutrophils showed this transient increase. No significant change occurred in lymphocyte numbers, although great variability was present (Fig. 1). Less variation was seen in analysis of percentage lymphocytes.

Wistar rats, which are not susceptible to $HgCl_2$-induced nephritis, did not produce circulating anti-GBM antibodies and did not show this cellular proliferation (Fig. 1).

Lymphocyte subsets

Labelling of lymphocytes by the whole blood technique, using a monoclonal antibody such as MRC W3/25, followed by flow cytometry on a Coulter EPICS C cell sorter (analysis by 90° light scatter and forward light scatter) yields the distribution of cells shown in the contour map (Fig. 2). Red cell debris, polymorphs and, to a certain extent, monocytes may be effectively discriminated from lymphocytes, which may then be analysed for labelled cells.

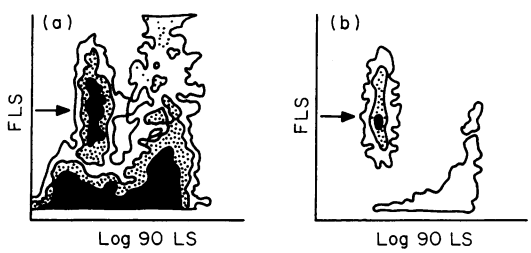

Figure 2. Contour map of MRC W3/25-labelled BN cells. Aliquots (100-μl) of heparinized normal BN peripheral blood were labelled with MRC W3/25 mAb and FITC goat anti-mouse Ig and analysed by flow cytometry, forward angle light scatter and 90° light scatter, ungated (a) and gated (b) for fluorescence. The lymphocyte-rich area is marked by the arrow.

The effect of $HgCl_2$ treatment in BN rats on lymphocyte subsets in peripheral blood was examined. In a pilot experiment T cells (MRC $OX19^+$) generally decreased while B cells (MRC $OX12^+$) increased in a reciprocal manner (Fig. 3). This reciprocity was especially marked early in the disease. In more detail, the percentage of T cells showed a decrease after Day 10, from Day 0 values of $36{\cdot}7 \pm 1{\cdot}8$ to $22{\cdot}3 \pm 4{\cdot}5$ by Day 18 ($P=0{\cdot}05$, Fig. 4). In some experiments an additional early transient decrease at Day 7 was also noted (Fig. 5, Day 0 *vs* Day 7, $P=0{\cdot}05$, see also Fig. 3). Levels rose to normal by Day 40 (Fig. 4). The decrease(s) in percentage T lymphocytes could be accounted for by the decrease in MRC $W3/25^+$ (T helper, Th) cells, which followed similar kinetics to the T cells (Figs 4 and 5). Non-susceptible Wistar rats did not have significant changes in MRC $OX19^+$ or $W3/25^+$ cells (Fig. 5, Day 0 *vs* Day 17: $Ox19^+$, not significant; $W3/25^+$, not significant).

MRC $OX8^+$ (T-cytotoxic/suppressor, Tc/s) cells were often found to have an early decrease at Days 5–10, from Day 0 values

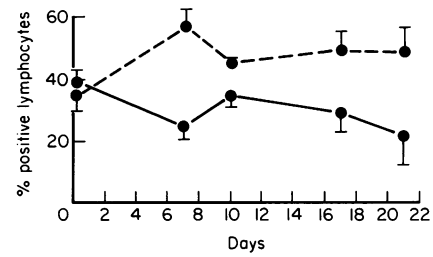

Figure 3. Reciprocal changes in percentage peripheral B and T lymphocytes in $HgCl_2$-treated BN rats. A group ($n=3$) of female BN rats were subjected to the standard $HgCl_2$ injection protocol and sequential B (MRC OX12, ●- - -●) and T (MRC OX19, ●—●) cells determined on a Coulter EPICS C cell analyser. A time-course of the mean ± SD of the percentage positive lymphocytes is shown.

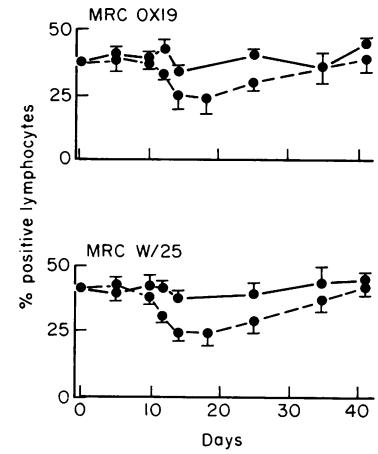

Figure 4. Sequential changes in percentage peripheral T and Th cells in $HgCl_2$-treated and control BN rats. Groups of BN rats ($n=4$) were subjected to the standard $HgCl_2$ injection protocol (●- - -●), or injected with PBS (●—●) and sequential MRC $OX19^+$ (T cells, upper graph) or MRC $W3/25^+$ (Th, lower graph) cells analysed by flow cytometry. Results show the mean ± SD, expressed as percentage positive lymphocytes. Similar transient decreases occurred in the percentage of MRC $OX19^+$ and MRC $W3/25^+$ cells in $HgCl_2$-treated but not control BN rats.

of $5{\cdot}0\% \pm 1{\cdot}3$ to $2{\cdot}49\% \pm 0{\cdot}75$ at Day 5 ($P=0{\cdot}02$, Fig. 6; also Day 0 *vs* Day 7, $P=0{\cdot}02$). This occurred in 12 out of 15 rats examined, and additionally some rats (7/15) showed a later rise above the normal range at Days 12–22 (Day 0 *vs* minimum, $P<0{\cdot}001$; minimum *vs* maximum, $P<0{\cdot}001$, Fig. 7). $HgCl_2$-treated Wistar rats, where the percentage of Tc/s cells is more than double that of BN rats, at Day 0, failed to show this early decrease (Day 0 *vs* minimum and minimum *vs* maximum: not significant) and late increase, but sometimes exhibited a decrease in percentage Ts cells after Day 12 (data not shown).

When the number of positive cells/ml peripheral blood was calculated instead of percentages, similar trends in MRC $OX19^+$, $W3/25^+$ and $OX8^+$ cells emerged, although greater variation was seen between rats due to the large variation in the number of lymphocytes/ml in the rats. In a small number of spleens examined the percentages of MRC $OX19^+$ and $W3/25^+$

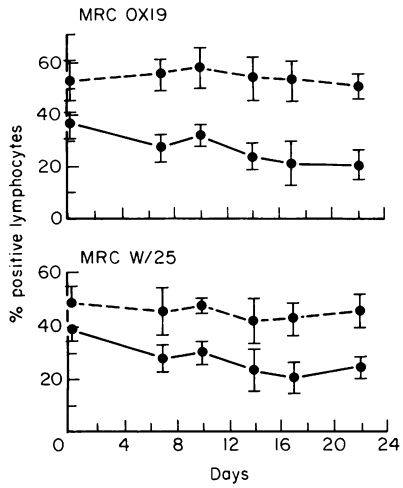

Figure 5. Sequential changes in the percentage peripheral T and Th cells in $HgCl_2$-treated BN and Wistar rats. Groups ($n=6$) of BN (●—●) or Wistar (●- - -●) rats were subjected to the standard $HgCl_2$ injection protocol and sequential MRC $OX19^+$ (T cells, upper graph) or MRC $W3/25^+$ (Th, lower graph) cells analysed by flow cytometry. Results show the mean ± SD, expressed as percentage positive lymphocytes. Decreases in MRC $OX19^+$ and MRC $W3/25^+$ cells occurred in $HgCl_2$-treated BN but not in Wistar rats.

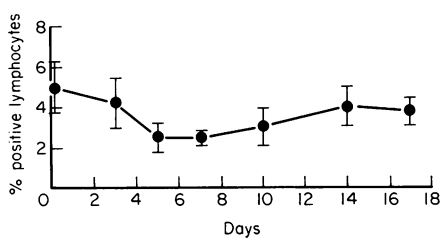

Figure 6. Sequential changes in percentage peripheral MRC $OX8^+$ (Tc/s) cells in $HgCl_2$-treated BN rats. Female BN rats ($n=6$) were subjected to the standard $HgCl_2$ injection protocol and MRC $OX8^+$ cells analysed by flow cytometry. Results show the mean ± SD, expressed as percentage positive lymphocytes.

lymphocytes also decreased during the disease, while the percentage of MRC $OX12^+$ cells showed a transient increase (data not shown).

DISCUSSION

The importance of changes in peripheral lymphocyte populations in the pathogenesis of human autoimmune diseases is not yet known. Studies have primarily been confined to multiple sclerosis and experimental allergic encephalomyelitis (EAE). In multiple sclerosis, studies equating Ts with a Tg cell (receptor for IgG) showed that such suppressor cells decreased or increased in peripheral blood in parallel with exacerbations and remissions (Huddlestone & Oldstone, 1979), while more recent work, using conventional T8 phenotype analysis of cells, has shown that patients with active disease have abnormally low Tc/s cells, while patients with inactive disease have normal Ts levels (Craig *et al.*, 1985). In EAE, Lyt 1^+ cells decrease in the peripheral blood and appear in the central nervous system

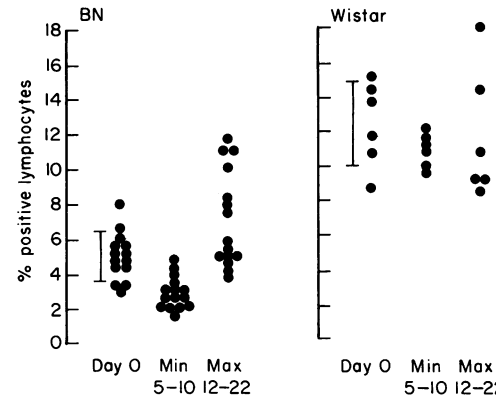

Figure 7. MRC $OX8^+$ cells in peripheral blood of individual $HgCl_2$-treated BN or Wistar rats (combined results of four separate experiments). BN or Wistar rats were subjected to the standard $HgCl_2$ protocol and MRC $OX8^+$ cells analysed sequentially by flow cytometry. Data shown represent the Day 0 levels, the minimum level at an early stage (Days 5–10) and the maximum level at a later stage (Days 12–22), expressed as percentage positive lymphocytes. The normal range (mean ± SD) of Day 0 values is shown by the bar for each strain. Note that an early decrease and late rebound in Tc/s cells occurred in $HgCl_2$-treated BN but not Wistar rats.

(Hauser *et al.*, 1984). However, EAE is a cell-mediated disease, and even less is known about the changes in peripheral T-cell subsets in autoantibody-mediated disease. Examination of any such sequential changes in experimental models of autoimmunity may aid in an understanding of their link with pathogenesis. However, most experimental models are in small laboratory animals, and this imposes limitations on the volume of blood that may be taken for sequential studies in individual animals. In addition, even when sufficient blood may be removed, the use of Ficoll to separate lymphocytes may itself affect the T-cell populations (Pontesilli *et al.*, 1986). We have developed a technique for labelling lymphocytes in small volumes of whole blood by indirect immunofluorescence followed by flow cytometry.

During $HgCl_2$ treatment of BN rats the total WBC count approximately doubled before returning to normal by Day 26, and this was mainly due to a short-lived neutrophilia. This response did not occur in the non-susceptible Wistar strain and so was not simply an inflammatory response. There was little change in the overall lymphocyte count and so the percentage of T and B cells derived from flow cytometry analysis gave an accurate estimate of their relative numbers. We found a transient reduction in the percentage and numbers of circulating T cells that did not occur in the non-susceptible Wistar rat. It occurred both slightly preceding (Day 7) and also at the time of the anti-GBM antibody response (Day 12 onwards). The B cells showed a corresponding increase, in both percentage and absolute numbers, as would be expected during polyclonal B-cell activation.

The decrease in T cells was found to be due in part to decreases in T-helper cells. Similar decreases in peripheral Th cells have been observed in the autoimmune responses studied in EAE (Hauser *et al.*, 1984) and in the diabetes-prone BB rat (Yale & Marliss, 1984), and in EAE this fall in circulating Th cells was accompanied by an influx of these cells into the central nervous system to cause autoimmune injury. However, in $HgCl_2$-treated

BN rats we have noted little or no mononuclear cell infiltrate into the kidney or thyroid (the polyclonal autoimmune response after $HgCl_2$ also includes antibodies to thyroglobulin) that could account for the loss of Th cells from the circulation, although other organs have not yet been examined (C. Bowman *et al.*, submitted).

Of interest was the early decrease in T-cytotoxic/suppressor cells at Days 5–10, which preceded the autoantibody response. If, for example, Tc/s cells from BN rats were more susceptible to the toxic effects of $HgCl_2$, their subsequent low numbers might result in a temporary loss of autoregulation and allow the B-cell production of autoantibodies to go unchecked. It is of note that mercury treatment of PVG rats, which results in a glomerulopathy, also causes a general reduction in T-cell reactivity to PHA- and Con A-induced suppressor function (Weening *et al.*, 1981). The onset of disease in spontaneously occurring systemic lupus erythematosus in NZB/NZW mice is also associated with loss in suppressor cell function (Krakauer, Waldmann & Strober, 1976), while a similar mechanism has been suggested for autoimmune thyroiditis, where a specific T-suppressor dysfunction has been postulated (Kidd *et al.*, 1980). In multiple sclerosis, loss in T-suppressor cells (as indexed by low T4/T8 ratio) occurs in active disease but not in remission (Craig *et al.*, 1985). One further factor that may be important is that BN rats have a particularly low percentage of Tc/s compared to other rat strains such as the Wistar. A low level of Tc/s and a high percentage of B cells in BN compared to Lewis rat spleens has previously been reported (Rosenberg & Feldman, 1982).

There was further evidence of a reciprocal relationship between the percentage of Tc/s cells and antibody levels at the time of down-regulation of the anti-GBM response when levels of Tc/s cells were seen to rise in many BN rats. However, no relationship was found between individual levels of anti-GBM antibody and the minimum numbers of Tc/s during the early phase, or the maximum numbers of Tc/s during the down-regulation phase.

The T-cytotoxic/suppressor cell decrease and recovery also precede the rise and fall in the number of circulating neutrophils, and it is interesting to speculate on the possible relationship between the two phenomena since, for other granulocytes, studies in thymectomized irradiated mice have shown that T cells may regulate parasite-stimulated eosinophilia (Walls *et al.*, 1971). Thus, the neutrophilia might represent loss of T-cell control of neutrophils, indicating a further, strain-related, action of $HgCl_2$ on T-cell function.

In studies on flow cytometry analysis of T-cell subsets, recognition must be given to the fact that differences in intensity of staining may occur during the disease, giving rise to weakly positive cells, such that the cells labelled with the mAb may not be representative of the normal distribution of cells of that particular phenotype. This has been found in the MRL-lpr/lpr mouse, where cells stained with Lyt 1 have an abnormally low intensity compared to other mouse strains (Lewis, Giorgi & Warner, 1981). In addition, care must be taken in studies involving the whole-blood technique of labelling to ensure that an efficient lysing method is used, as the presence of more than minimal amounts of red blood cells during flow cytometry analysis results in a falsely low percentage value for positive cells, as the unlabelled contaminating red cells move into the area sampled by the cell analyser as lymphocytes.

In conclusion, we have developed a method of labelling lymphocytes in whole blood in rats for the analysis of T-cell subsets by flow cytometry. This method uses minimal amounts of heparinized blood and is therefore suitable for sequential studies in individual animals. We have applied this technique to a study of T-cell subsets in $HgCl_2$-induced autoimmune nephritis in the BN rat and have found a transient loss in T cells from the peripheral blood, attributable to decreases in T-helper cells and associated with reciprocal increases in B cells. In addition, there is a decrease in T-cytotoxic/suppressor cells prior to the appearance of anti-GBM antibodies in the circulation, and abnormally high levels of Tc/s cells during down-regulation of this autoantibody. Analysis of such changes may help in an understanding of the induction and pathogenesis of autoimmunity.

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