Detecting and predicting neutralization of alemtuzumab responses in multiple sclerosis

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Glossary: Anti-drug antibodies (ADA); Disease modifying treatment (DMT); Expanded Disability Status Scale (EDSS); Multiple sclerosis (MS); People with MS (PwMS); Welsh Neuroscience Research Tissue Bank (WNRTB)
ABSTRACT

Objective: To test the hypothesis that anti-drug antibodies against alemtuzumab could become relevant after repeated treatments for some individuals, possibly explaining occasional treatment resistance.

Methods: Recombinant alemtuzumab single-chain variable fragment antibody with a dual tandem nanoluciferase reporter linker was made and used to detect binding anti-drug antibodies. Alemtuzumab IgG Alexa-Fluor 488 conjugate was used in a competitive-binding cell based assay to detect neutralizing anti-drug antibodies. The assays were used to retrospectively screen, blinded, banked-serum samples from people with multiple sclerosis (n=32) who had received three or more cycles of alemtuzumab. Lymphocyte depletion was measured between baseline and about 1 month post-infusion.

Results: The number of individuals showing limited depletion of lymphocytes increased with the number of treatment cycles. Lack of depletion was also a poor prognostic feature for future disease activity. Anti-drug antibody responses were detected in 29/32 (90.6%) individuals. Neutralizing antibodies occurred prior to the development of limited depletion in 6/7 individuals (18.8% of the whole sample). Pre-infusion, anti-drug antibody levels predicted limited, post-infusion lymphocyte depletion.

Conclusions: Although anti-drug antibodies to alemtuzumab have been portrayed as being of no clinical significance, alemtuzumab-specific antibodies appear to be clinically relevant for some individuals, although causation remains to be established. Monitoring of, lymphocyte depletion and the anti-drug response may be of practical value in patients requiring additional cycles of alemtuzumab. Anti-drug antibody detection may help to inform on re-treatment or switching to another treatment.
INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated, demyelinating disease of the central nervous system. Memory T and B lymphocytes are key in the pathophysiology of MS, and these cell types are targeted by an increasing number of disease modifying treatments (DMTs) capable of inhibiting relapsing MS. These DMTs are administered continuously or given as a pulsed immune-reconstitution therapy to produce long-term disease inhibition[1]. Alemtuzumab was the first biological immune-reconstitution therapy licensed for the treatment of people with MS (PwMS)[1, 2]. This depletes lymphocytes in vivo and in vitro by a number of mechanisms, including complement fixation and antibody-dependent cellular cytotoxicity[1, 2], and has been shown to be highly efficacious in suppressing relapses in PwMS (CARE MS I and II)[3, 4]. However, a small number of people appear to be unresponsive to alemtuzumab and continue to experience clinical relapses despite treatment[5].

Although alemtuzumab was the first humanized monoclonal antibody engineered with the aim of reducing immunogenicity to the founding rodent molecule[6], surprisingly, it is one of the most immunogenic therapeutic proteins[7, 8] generating anti-drug antibodies (ADA) which may be either binding or neutralizing[8]. Yet, these have been reported to be of minimal clinical significance[9-11]. Indeed, the dosing schedule of alemtuzumab[9-12] avoids issues of ADA, which occur with high frequency (~85%) within 2 years of treatment[13-15]. Using the currently recommended treatment schedule, the infusion cycle ends before primary and secondary antibody responses will be generated, and the recommended interval of at least 12 months between treatment cycles allows ADA levels to subside[7, 13]. Neutralizing ADA, were not mentioned in the pivotal trial reports[10-12]. These only occurred in 0.6% of PwMS prior to the second infusion cycle[7], so would be infrequently problematic within the original two-year treatment cohort[1, 10, 11]. However, as additional treatment cycles were shown to be efficacious in people not adequately responding to two cycles[3, 4, 15], the European Medicines Agency supported the use of third and fourth treatment cycles in 2017. However, pre-dose binding and neutralizing ADA become far more prevalent following the second treatment cycle (75% and 31% at 24 months respectively), a factor which may limit the biological and clinical efficacy of the subsequent treatment cycles[7, 8]. In this study, we investigated the hypothesis that ADA become increasingly problematic after successive cycles of alemtuzumab treatment and that the ADA levels may be associated with diminished treatment effectiveness.
METHODS

**Anti-drug antibody assays:** In order to monitor PwMS in our care, we developed an in-house luminescence based, anti-globulin detection assays for binding anti-alemtuzumab-specific antibodies[16]. In brief, a recombinant single chain variable fragment based on alemtuzumab variable heavy and light chains was engineered as a fusion protein with two nanoluciferase reporter domains (GloBody). In the presence of ADA, GloBody-ADA complexes form which are captured on immobilised protein-G and the retained luciferase activity measured[16]. Additionally, competitive binding of Alexa Fluor 488-labelled alemtuzumab to adherent human CD52-expressing Chinese hamster ovary cells, coupled with serial dilutions of serum, was used as a neutralizing assay[17]. Detailed methodology has been reported previously[16, 17].

**Ethical approval:** Samples were collected with informed consent and ethical approval (Research Ethics Committee approval references: 19/WA/0058 and 05/WSE01/11).

**Alemtuzumab treatment.** People received five daily 12mg infusions at baseline and three daily 12mg infusions where administered twelve months later[11]. Following disease activity (typically ≥1 relapse and/or ≥2 unique lesions defined as either new/enlarging T2 hyper-intense and/or gadolinium-enhancing brain and/or spinal cord lesions via magnetic resonance imaging), additional cycles of three daily 12 mg infusions could be administered at least 12 months apart[3, 4].

**Samples:** The assays were applied to bioarchived serum samples from 32 PwMS who had all received three or more cycles of alemtuzumab[5]. Samples were obtained from the Welsh Neuroscience Research Tissue Bank (WNRTB) and had been donated as part of a long-running population study of MS, which has previously been described[18]. Analysis of ADA was performed blinded to clinical and laboratory data from the WNRTB. Individuals had received either three (n=24), four (n=3), five (n=4) or six (n=1) cycles of alemtuzumab. Absolute lymphocyte counts, taken from routine laboratory reports where available, from time points immediately before, and 1-2 month after each infusion were used to calculate relative depletion rates. Apparent lymphocyte depletion was defined as ≥ 35% reduction in absolute lymphocyte count pre- to post infusion and/or depletion below the lower limit of normal.

**Statistical analysis:** Statistical analysis was performed using t test/Mann Whitney U tests using Sigmaplot Software (Systat. San Jose, California, USA).

**Data sharing statement:** Anonymous data is available on request.
RESULTS

Cohort characteristics. At the time of sample requisition from the WNRTB, 137 people had received alemtuzumab within the South Wales cohort. In total, 40 people had received three or more treatment cycles of alemtuzumab. The rates of receiving a third (or subsequent treatment) in this cohort was 49% (39/80) of people followed up to 5 years and 50% (15/30) of people followed up to 10 years. Archived blood samples were available from 32 out of the total 40 individuals who had ever received a third cycle of treatment. The 32 people who had donated blood samples were 28 female (88%), and had a mean age at MS onset of 29 years (range 10 – 44). All PwMS received alemtuzumab as first-line treatment; the first cycle was administered after a mean duration of 4.5 years (range 0.6 – 17) from symptom onset.

Individuals do not deplete lymphocytes on repeated infusion of alemtuzumab. Lymphocyte depletion data was available for 32 out of 32 (cycle 1), 32 out 32 (cycle 2), and 31 out of 32 (cycle 3). At the population level, lymphocyte depletion was marked and significant (P<0.001) compared to pre-cycle treatment levels for cycle 1 to cycle 3, (Figure 1). However, at an individual level 31 out of 32 people (97%) showed apparent lymphocyte depletion following cycle 1, 30 out of 32 (94%) after cycle 2, 27/31 (87%) after cycle 3 in PwMS (Figure 1). Of the 4 people who did not show apparent lymphocyte depletion after cycle 3, two had previously shown limited depletion after cycle 2. Lymphocyte subset analysis was not available. Whilst it is recognized that these counts will reflect a composite of depleted and repopulating cells[7], whose composition may change due to the different reconstitution kinetics of distinct lymphocyte subsets[7], relative lack of depletion may be an indicator for the lack of efficacy of alemtuzumab, due to antibody neutralization.

Anti-drug antibody responses occur with high frequency. Baseline serum samples were available for 17/32 PwMS. Using a binding ADA assay[16], low titres 751 ±1,399 Lux were evident in subjects prior to first infusion (Figure 2). However, 29/32 (90.6%) people generated binding ADA with a titre > two standard deviations above the baseline mean (> 3,549 Lux) during their subsequent follow up (Figure 2). Some people demonstrated very high ADA titres (mean 1,288,805 ± 2,773,146 Lux with a range 1,296-9,965,386 Lux) following alemtuzumab treatment (Figure 2, Figure 4). A standard monoclonal anti-alemtuzumab IgG (Bio-Rad HCA-199) spiked into controls serum at ~50 μg/mL generated a signal of 21,159 ± 6,468 Lux. The maximum blood concentration range of alemtuzumab immediately after the third infusion is 2.3 ± 0.8 μg/mL[14], ADA values in the range of the spiked standard would be theoretically at least 16-fold molar excess of the circulating levels of the drug.
Relationship between lymphocyte depletion, ADA and treatment response. ADA were clearly present in 6/7 individuals before poor depletion occurred. Binding ADA titres were boosted with each cycle of treatment and the binding ADA became persistent in some individuals (Figure 3a-d). This was associated with increasing levels of antibody neutralization (Figure 3a-d). One individual appeared to demonstrate limited lymphocyte depletion in spite of an absence of neutralizing ADA (Figure 3e). However, in this case month-1 post infusion lymphocyte data was unavailable. Lymphocyte levels were first measured more than 2.5 months post-infusion, so we cannot exclude the possibility that depletion, followed by rapid repletion, occurred within this time window. Six of seven individuals who depleted poorly, exhibited disease activity within 2 years of infusion. In contrast, it was evident that only 13/28 PwMS with apparent lymphocyte depletion demonstrated disease activity after 3 cycles, with 14/16 people without disease activity having >5 years follow-up. People receiving 4 cycles (Figure 3. n=7/7) of alemtuzumab subsequently exhibited disease activity in this cohort. This study was not designed to monitor the clinical significance of antibody neutralization. However, when comparing baseline and 5 year EDSS, those who neutralised the alemtuzumab response after 3 cycles of treatment, showed a median 1.0 point EDSS worsening (Range 0.5-2.0. n=5), while those who did not develop neutralizing antibodies showed a median improvement of 0.5 EDSS points (range -2.0-2.5. n=26). This may suggest that antibody neutralization has clinical significance, but further studies are warranted.

Pre-dose ADA responses may detect subsequent lymphocyte depletion responses. As it is known that most people generate ADA that wanes over time (Figure 3B-D)[7, 8, 14], pre-cycle ADA levels and the subsequent lymphocyte depletion level will be most informative on the significance of ADA[8]. However, only 23 serum samples from 19 different individuals with lymphocyte depletion data were taken less than 2 months before infusion. This included five pre-dose samples from poor-depleting individuals whose ADA titres were: 2.7 x 10^4, 2.8 x 10^4, 8.9 x10^4, 1.2 x 10^5 and 1.0 x10^6 Lux units (Figure 3). Many people who depleted lymphocytes exhibited low titre (Below 1.5 x 10^3 Lux units) antibody responses (Figure 4A), however there were individuals that had relatively high titres (5.8 x 10^4 and 1.1 x 10^5 Lux units) of binding ADA but exhibited notable depletion (1.4 to 0.2, 1.8 to 0.4 cells 10^9/L. Figure 4A) and did not relapse for at least 4 and 7 years after infusion. However, these individuals did not exhibit neutralizing potential (Figure 4B, 4C). Therefore, binding (<15,000 Lux) and neutralizing (≥10 titre for virtually complete neutralization) titres perhaps could be adopted to suggest poor-depleters and depleters (Figure 4A). It remains to be established if this can be applied prospectively, but the data suggests that it is possible that pre-dose ADA titre limits may be set that can exclude potential futile treatments.
DISCUSSION

Alemtuzumab is a potent immune reconstitution therapy however, up to 40-50% of people receiving alemtuzumab for MS require three or more cycles of treatment[5, 15]. Third and subsequent cycles are often followed by a sustained remission of disease activity[4, 15], but cases are recognised to occur where people are unresponsive to alemtuzumab and continue to experience clinical relapses despite treatment. During the clinical development of alemtuzumab the impact of neutralizing antibodies was not mentioned and ADA have been repeatedly portrayed as being clinically insignificant[11, 12, 19].

However, our study suggests that some people develop persistent neutralizing antibody responses to alemtuzumab, and that this is associated with limited lymphocyte depletion and disease activity. Although, it is not possible to prove cause and effect, it seems likely that high-titre neutralizing responses would blunt or inhibit the lymphocyte depletion response. In this study 6/32 (18.8%) developed high titres of both binding and neutralizing ADA and showed subsequent evidence of relative lack of lymphocyte depletion. This is aligned with other reports in the literature of disease activity requiring further infusions associated with lack of lymphocyte depletion, which prompts switching to another DMT[20]. However, there are alternative explanations to explain poor depletion and disease breakthrough, such as alemtuzumab treatment leading to the emergence of CD52-negative lymphocytes[21, 22]. This may have contributed to poor depletion despite limited ADA response in one individual. Furthermore, pharmacogenomics, such as Fc receptor genetic variants, linking to antibody-dependent cellular cytotoxicity, may also be relevant, particularly if antibody titres become limiting[1, 23-25].

Although the precise molecular mechanisms that cause the formation of ADA remain to be defined, it is evident that the majority of people produce CD52-specific ADA following alemtuzumab infusion[7, 13]. This probably relates to a number of factors that include the: dose, dosing schedule, biology of alemtuzumab, CD52 expression profile and the repopulation kinetics of ADA forming cells and CD52-expressing regulatory subsets[26]. These ADA are boosted with an increasing number of infusions, such that they become relatively persistent in some individuals[7, 13]. Further prospectively collected data may help to validate and extend our findings and identify the important perturbations of the immune networks that facilitate ADA formation[7]. Importantly, re-evaluation of samples and data from CARE-MS and their extension studies[7, 8, 19], which contains immunophenotyping and ADA data[7], may provide further valuable insights into the relationship between both binding and neutralizing (inhibitory) antibody titres and the associated laboratory/clinical data. This may be particularly enhanced if events within lymphoid tissues can be also monitored, as they are the probably the source of ADA forming cells and are likely to deplete and repopulate differently to the peripheral blood[27, 28]. Previous reports of a
lack of association between ADA and lymphocyte depletion at the population level[10, 12, 15] is misleading, since population analyses are insensitive to low frequency events, as demonstrated here. The influence of neutralizing ADA alone has yet to be reported from the pivotal trial extension data, but people with the highest quartile of pre-dose ADA producers, deplete their lymphocytes post-dose, less efficiently than people in the lower 75% percentile[19], and suggests that analysis of the neutralizing response may be informative for treatment response in some individuals. Therefore, the neutralizing ADA data from the pivotal trial extension studies, should be reported. Future work may also further define levels of ADA that are predictive of disease activity.

The lack of general awareness of the potential of alemtuzumab to induce neutralizing antibodies, coupled with the suggestion that disease activity is not related to lymphocyte levels[29] may contribute as evidenced by disease activity to viewing lymphocyte depletion data as being unimportant. However, a failure of lymphocyte depletion is likely to imply alemtuzumab is less effective. Once an individual failed to deplete, this was often seen in subsequent treatment cycles. Whilst it will be the case that only a small subset of lymphocytes will be clinically important and these will not be accurately monitored by simply measuring lymphocyte numbers, a lack of absolute lymphocyte depletion should prompt extra vigilance.

Our study provides important preliminary data that pre-infusion binding ADA titres >15,000 and/or complete neutralization at >10 fold dilution, may be associated with poor post-infusion lymphocyte depletion. Furthermore, this appears to be a predictor for future disease activity and the need for further alemtuzumab infusions. Therefore, monitoring ADA before the second or subsequent infusion cycles may be valuable in guiding treatment decisions. The ADA detection technology used here could also be applied to other monoclonal antibody treatments, to inform whether to re-treat or to switch therapy. Identifying people who are at risk of ineffective treatment responses might save them from undergoing an expensive and futile treatment, and ensure cost-effective use of alemtuzumab.
### Appendix 1

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<tr>
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REFERENCES


Figure Legends

**Figure 1.** *Lymphocyte depletion following alemtuzumab treatment.* People with MS received three cycles (n=31). Few of these individuals received four cycles (n=7) cycles of alemtuzumab. The figures represent the mean and standard deviation of absolute peripheral blood lymphocyte count and the individual levels before and after treatment for each treatment cycle. Those with depletion that perhaps suggests atypical/insufficient (low change or absolute depletion level above the lower limit of normal) depletion (red), compared to those depleting (grey) are indicated. In some individuals (*) post-dose blood counts were more than 2 months from infusion. Statistical analysis was performed using t test/Mann Whitney U tests.

**Figure 2.** High titre ADA develop in most people following alemtuzumab treatment. Alemtuzumab globody was used to detect binding ADA in sera from PwMS who had received alemtuzumab. The results show individual baseline responses prior to the initial infusion (n=17), the response to a 50μg/mL alemtuzumab ADA monoclonal antibody standard (Bio-Rad HCA-199, n=32) and the highest titre for each individual (n=32), in any of 4-6 samples/individual banked during treatment. The mean ± standard deviation group scores are shown.

**Figure 3.** *Alemtuzumab neutralizing responses appear before PwMS apparently fail to deplete lymphocytes.* The (A-E) binding and (a-e) neutralizing responses of five individuals treated with alemtuzumab. (A-E) The results show the time of the beginning of each alemtuzumab infusion cycle, the absolute lymphocyte numbers and the binding ADA titre or the neutralizing responses. A-E Lower limit of normal of lymphocyte number (dashed line). In a-e, samples 1-6 or 1-4 are from the same individual at sequential time points during follow-up. Year 0 indicates 1 January of the year of the initial dosing and the time of treatments and sampling during the year are indicated.

**Figure 4.** *Alemtuzumab binding and neutralizing responses in people treated with alemtuzumab to predict treatment response.* Alemtuzumab globody and alemtuzumab Alexa Fluor 488 conjugates were used to detect (A) a combination of binding and neutralizing ADA or (B-D) neutralizing ADA in sera from PwMS who had received alemtuzumab. (A) The binding ADA response in individual samples taken within 2 months before infusion and the level of lymphocyte depletion within 2 months post-infusion compared to the pre-infusion level, from cycles 1-4. The neutralizing response was tested in individuals with a binding ADA level >15,000 lux (red and blue). Those with a titre above a 1:10 serum dilution that essentially completely neutralised the alemtuzumab Alexa Fluor 488 binding response are indicated (red). Poor depleters were classified, prior to analysis, as those exhibiting less than a 0.4 x 10⁹ cells/L depletion. Individuals with high titre binding ADA who depleted peripheral blood lymphocytes (Blue), were found (B, C) to lack notable neutralizing ADA responses, seen in individuals exhibiting poor depletion (Red. 4D).