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## The mouse immune response to carrier erythrocyte entrapped antigens

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**Abbreviated article title:**

Carrier erythrocyte entrapped antigens

## 1. Introduction

Increasing attention is being devoted to the development of novel delivery systems that enable sustained *in vivo* delivery and specific targeting of antigens to the professional antigen presenting cells. Targeted antigen delivery to specific cells of the immune system has the potential to maximise immunological responses to smaller doses of antigen, and overcome the disadvantage of conventional immunization schedules which often employ adjuvants. Despite the increasing interest in the development of vaccine adjuvants, many of the experimental adjuvants advancing as far as clinical trials have proved too toxic for routine clinical use. Even alum adjuvanted vaccines, which have a good safety record, have been documented to induce immunoglobulin E antibody responses and allergic reactions of varying severity [1-3]. Moreover, alum is a weak adjuvant for antibody induction to protein subunits and a poor adjuvant for cell-mediated immunity. The choice of adjuvants for vaccination is currently a compromise between a requirement for improving the immune response to vaccine antigens and an acceptable low level of side effects. Delivery systems employing carrier vehicles with an enhanced *in vivo* half life have the added advantage of providing a sustained or pulsed release of encapsulated antigen, thus eliminating the necessity for multiple immunizations for the induction of a protective immune response. Such delivery systems are of particular interest in the development of single dose vaccines.

Antigen delivery systems that offer potential alternatives to conventional vaccination protocols are liposomes, immunostimulatory complexes (ISCOMs), poly-lactide-co-glycolide microspheres and virosomes [4-8]. Significant technological and economic obstacles have, however, impeded their clinical application, such as antigen instability induced by the production process, low encapsulation efficiencies, significant production costs associated with aseptic processing, and the complexity of standardizing antigen entrapment under good manufacturing practice conditions.

Erythrocytes have been proposed as an antigen delivery system [9-12]. Senescent erythrocytes are naturally sequestered from the vascular compartment by macrophages of the monocyte-macrophage system based in the spleen, liver and bone marrow. Although among the antigen presenting cells, dendritic cells are thought to be the most efficient at initiating antigen-specific responses, macrophages are also able to facilitate the presentation of peptides to T lymphocytes [13, 14]. Therefore the association of antigens with erythrocytes offers an immunization strategy of delivering antigens directly to the immune system. Magnani and colleagues have shown that multiple administrations of antigens conjugated to the erythrocyte membrane via biotin-avidin-biotin bridges are able to elicit immune responses in mice, similar to or higher than those obtained using soluble antigen in Freund's adjuvant [9-11]. Other studies showed that multiple injections of bacterial antigens encapsulated within erythrocytes also resulted in the production of antigen specific antibodies [12]. The entrapment of substances within erythrocytes is readily achieved using a variety of techniques including electroporation, drug-induced endocytosis, osmotic pulsing and hypo-osmotic haemolysis (either by direct dilution or hypo-osmotic dialysis). We have previously shown that carrier erythrocytes prepared using the reversible hypo-osmotic dialysis technique have *in vivo* survival times comparable to those of native erythrocytes, and that entrapped agents have *in vivo* half-lives similar to the erythrocyte carrier [15-17]. Thus carrier erythrocytes have the potential to enhance antigen stability and in doing so have the potential to target antigen delivery to the antigen presenting cells in a sustained fashion.

We report here investigations on single administrations of carrier erythrocyte entrapped antigen to facilitate an antigen-specific humoral response in the Balb/c mouse. Humoral responses at three different time points to primary immunizations of erythrocyte encapsulated antigens were measured and compared with those observed using the conventional subcutaneous immunization route. Ig isotype responses to primary immunizations of carrier erythrocyte entrapped antigen and

subcutaneous antigen were compared to responses observed in mice that subsequently received a secondary booster immunization with the un-entrapped antigen.

## **2. Materials and Methods**

### **2.1. Mice**

Female Balb/c mice (Harlan UK Ltd.) of 8 to 10 weeks of age were used either as blood donors for carrier erythrocyte preparation or for immunization studies. Animals were fed a standard mouse diet and housed in a temperature- and light-cycle controlled facility.

### **2.2. Blood preparation**

Whole blood was collected from mice by intracardiac puncture; anaesthesia was induced with 2.5% halothane and maintained at 1% for bleeding out, and the blood collected into 2 ml syringes spiked with a low molecular weight heparin (Fragmin, 14U/ml blood). Blood was pooled and centrifuged at 1,100 x g for 10 minutes, and the plasma and buffy coat removed. Erythrocytes were washed twice in cold (4°C) supplemented phosphate buffered saline (SPBS: 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 136.89 mM NaCl, 8.10 mM Na<sub>2</sub>HPO<sub>4</sub>, 5mM MgCl<sub>2</sub>, 5mM adenosine, 3mM glutathione and 5mM glucose), pH 7.4.

### **2.3. Antigens**

Four antigens were studied; keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA), cholera toxin b subunit (CTB) and bovine adenosine deaminase (ADA). KLH, BSA and CTB were selected on the basis that they are established and available model antigens that are known to cause strong antibody responses in mice on subcutaneous administration. ADA was chosen on the basis of being an example of a weak antigen with which we had previous experience. Fatty acid free BSA, KLH and CTB were obtained from Sigma-Aldrich, UK. Therapeutic grade bovine ADA with an average specific activity of 252 IU/mg protein was supplied by Roche Pharmaceuticals, Germany.

#### **2.4. Carrier erythrocyte preparation**

Antigen-loaded and unloaded carrier erythrocytes were prepared under sterile conditions using a hypo-osmotic dialysis technique [16, 18]: 7 volumes of washed and packed erythrocytes were mixed with 3 volumes of cold SPBS, containing the antigen to be entrapped (in the case of unloaded carrier erythrocytes no antigen was added). A range of antigen concentrations was used. The cell suspension was placed in a dialysis bag with a molecular weight cut-off of 12,000 Da and then dialysed against hypo-osmotic buffer (5 mM  $\text{KH}_2\text{PO}_4$ , 5 mM  $\text{K}_2\text{HPO}_4$ , 5mM  $\text{MgCl}_2$ , 5mM adenosine, 3mM glutathione and 5mM glucose, osmolality of 53 mOsm/kg) at 4°C with rotation for 90 minutes. The lysed erythrocytes were resealed by dialysis against SPBS, pH 7.4 at 37°C with rotation for 60 minutes. The resulting carrier erythrocytes were then washed three times in SPBS, with centrifugation at 100 x g for 20 minutes and counted using a Coulter counter. The osmolality of the washed and packed cells ranged between 320-335 mOsm/kg. To determine whether antigen was binding to the exterior surface of the erythrocyte, control native erythrocytes were dialysed against SPBS for 90 minutes at 4°C (instead of hypo-osmotic buffer) with the same amount of antigen used for loading carrier erythrocytes. No binding was found with the four antigens used.

#### **2.5. Carrier erythrocyte entrapped antigen quantification**

Antigen entrapment studies were performed to determine the concentration of antigen required to achieve a specific antigen dose.

ADA activity was assayed by following the deamination of adenosine to inosine; diluted and haemolysed carrier erythrocytes were incubated with 3.75 mM adenosine in 100 mM phosphate buffer (pH 7.4) for 30 minutes. The reaction was stopped with 40% trichloroacetic acid and the protein precipitate removed by centrifugation for 1 minute at 12,000 x g. The supernatant was extracted with water-saturated diethyl ether to pH 5.0 to remove the trichloroacetic acid. The



reaction products were separated by HPLC using a Waters trimodular system with a 5 $\mu$ m Spherisorb ODS2 column (125mm x 4.9 mm ID, Hichrom, UK) and an isocratic solvent system consisting of 40 mM ammonium acetate buffer and 5mM tetrabutylammonium hydrogen sulphate (pH 2.75), with a run time of 8 minutes.

BSA was measured using a bovine albumin ELISA quantification kit (Bethyl Laboratories, Inc); bound BSA was detected by horseradish peroxidase-conjugated anti-BSA second antibody using OPD peroxidase (0.4 mg/ml 2-phenylendiamine dihydrochloride and 0.4 mg/ml urea hydrogen peroxide in 0.05 M phosphate-citrate buffer, pH 5.0) as a substrate, and measuring the absorbance at 450 nm using a microplate reader (Anthos). Carrier erythrocyte entrapped BSA was determined from a standard curve of known concentrations of BSA. The coefficients of intra- and inter-assay variation were between 1.8% and 4.9%, and 5.9% to 6.2%, respectively.

KLH was measured using a developed sandwich ELISA method: Microtitre plates (Costar) were coated for 18 hours at 4°C with 0.1  $\mu$ g/ml goat anti-KLH (Biogenesis) in 0.05 M sodium bicarbonate-carbonate buffer, pH 9.6. The plates were washed with phosphate-buffered saline, pH 7.0, containing 0.05% Tween 20 (PBS/Tween 20) and then blocked with 3% BSA in PBS/Tween 20 for 2 hours at room temperature. The washed plates were incubated for 60 minutes at room temperature with 100  $\mu$ l/well of lysed KLH-loaded carrier erythrocytes diluted in blocking buffer, washed with PBS/Tween 20 and incubated for 60 minutes at room temperature with 100  $\mu$ l/well of rabbit anti-hemocyanin second antibody (Sigma-Aldrich, UK) diluted 1:1000 in blocking buffer. Plates were then washed with PBS/Tween 20, incubated for 60 minutes at room temperature with 100  $\mu$ l/well of alkaline phosphatase conjugated anti-rabbit IgG (Sigma-Aldrich, UK), washed with PBS/Tween 20 and then incubated with 100  $\mu$ l of 1.0 mg/ml p-nitrophenyl phosphate in 0.2 M Tris buffer (Sigma-Aldrich, UK) for 30 minutes at room temperature. The absorbance was measured at 405 nm and entrapped KLH concentrations were determined from a standard curve of known

concentrations of KLH. The coefficients of intra- and inter-assay variation were from 0.7% to 1.4% and from 4.9% to 6.8%, respectively.

Carrier erythrocyte entrapped CTB concentrations were determined using an ELISA protocol: microtitre plates (NUNC) were coated with 50µg/ml gangliosides (type III, Sigma-Aldrich, UK) in PBS, pH 7.4 for 18 hours at room temperature. The plates were washed with PBS/Tween 20, blocked with 1% BSA in PBS/Tween 20 for 2 hours at room temperature, and incubated for 60 minutes at 37°C with 100µl lysed CTB-loaded carrier erythrocytes diluted in blocking buffer. Plates were washed with PBS/Tween 20 prior to the addition of 100 µl 1µg/ml rabbit anti-CTB IgG (Sigma-Aldrich, UK) diluted in blocking buffer and incubated for 60 minutes at 37°C. After further washing, antigen detection was performed as described above for KLH using alkaline phosphatase conjugated anti-rabbit IgG and p-nitrophenyl phosphate as the substrate. Entrapped antigen concentrations were determined from a standard curve of known concentrations of CTB. Intra- and inter-assay variation ranged between 1.9% and 5.3%, and 5.7% and 6.3%, respectively.

## **2.6. Measurement of carrier erythrocyte survival *in vivo***

*In vivo* survival studies of antigen-loaded carrier erythrocytes were performed to determine the optimal timings for immunization test bleeds.

Using a sterile technique, 2 ml of washed and packed antigen-loaded carrier erythrocytes were gently mixed with 7.4 MBq sodium [<sup>51</sup>Cr] chromate BP (Amersham Biosciences, UK) and allowed to stand at room temperature for 30 minutes. Unbound chromium was removed by incubating in 20 mg ascorbic acid BP (100 mg/ml, Evans Medical, Leatherhead, UK) for 5 minutes, followed by a single wash in SPBS, with centrifugation at 100 x g for 20 minutes. Following re-suspension in an equal volume of plasma, 200 µl of cell suspension (final haematocrit of 17 to 20%) were injected via the tail vein into each of 7 randomly selected mice. Each syringe was weighed before and after

injection to determine the exact amount of cell suspension infused into each mouse. *In vivo* survival was assessed by monitoring the disappearance of label from the circulation; one mouse was bled by intracardiac puncture under terminal anaesthesia at each time point, at days 1, 3, 7, 14, 21, 28, and 35. Mice were weighed immediately after carrier erythrocyte infusion, and then again immediately prior to bleeding out. The percentage haematocrit of the blood samples was determined using a microhaematocrit centrifuge (Hawksley, West Sussex) and a microhaematocrit reading device, and aliquots of blood were assayed for radioactivity. Counts per minute (cpm) were corrected for vascular expansion (due to weight gain) by using the accepted assumption that the total blood volume of mice is 7% of its body weight [19] and then expressed as cpm/ml of packed cells. The percentage of  $^{51}\text{Cr}$  labelled erythrocytes remaining in the circulation was determined from the amount of radiolabel injected into each the mouse. Percentage raw cell survival was calculated by expressing cpm/ml packed cells as a percentage of the calculated zero time value (determined from the amount of radiolabel injected into each the mouse), after correction for natural decay. The data were then plotted as a function of time on a semi-logarithmic scale and a best-fit straight line drawn. The half-life ( $t_{1/2}$ ) was taken as the time for the concentration of  $^{51}\text{Cr}$  in the circulating blood to fall to 50% of its initial value. For the determination of mean cell life (MCL), cpm/ml packed cells (corrected for both natural decay and chromium elution from the red cells) were expressed as a percentage of the calculated zero time value. Cell survival data were plotted against time on a linear scale and the MCL span derived from the intercept obtained by extrapolating the line to zero activity [16]. Plasma was also measured for  $^{51}\text{Cr}$  activity to check for intra-vascular haemolysis, and thus antigen release into the circulation. Three time courses per antigen were performed.

## **2.7. Immunization protocols**

For each antigen, mice were randomly assigned to five groups of 9 animals; Group 1 were immunized subcutaneously with antigen suspended in 100  $\mu\text{l}$  of TiterMax Gold<sup>TM</sup> adjuvant (Sigma-Aldrich, U.K.), Group 2 were immunized intravenously via the tail vein with antigen entrapped

within 100 µl carrier erythrocytes suspended in 100 µl plasma (total infusion volume of 200 µl), Group 3 were injected intravenously with 100 µl of unloaded carrier erythrocytes suspended in 100 µl of plasma, Group 4 were injected intravenously with free antigen mixed with 100 µl of unloaded carrier erythrocytes suspended in 100 µl of plasma, and Group 5 were injected intravenously with 100 µl lysed antigen-loaded carrier erythrocytes suspended in 100 µl plasma. Antigen-loaded carrier erythrocytes were lysed by freezing and then thawing. These studies were designed to administer an antigen dose of approximately 20 µg; however, due to the absence of detectable immune responses using 20 µg ADA via both the subcutaneous and carrier erythrocyte immunization routes, ADA immunization doses of approximately 40µg were also investigated.

In a second set of immunization experiments, the effect of a secondary subcutaneous booster immunization on the Ig isotype profile was investigated. Mice were randomly assigned to two groups of 9 animals for each antigen, and immunized as described in Groups 1 and 2 above. Secondary immunizations containing 20µg of KLH, BSA or CTB, or 40µg ADA, suspended in 100µl phosphate-buffered saline (without adjuvant) were administered on the day corresponding to the half-life day for each antigen to obtain a theoretical equivalence in residual antigen levels; 8 days post-primary immunization for ADA, 11 days for BSA, 10 days for CTB and 9 days for KLH.

## **2.8. Detection of antigen specific IgG**

Mice were bled prior to immunization and at 14, 35 (both via the tail vein), and 40 days (via intracardiac puncture) post primary immunization. Sera were prepared by centrifugation of clotted blood at 2,000 x g for 5 minutes and then stored at -80°C. Antigen-specific immunoglobulins (IgG) were detected using direct ELISAs; microtitre plates (Costar) were coated with 10 µg/ml antigen in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6 for 18 hours at 4°C. Plates were washed with PBS/Tween 20 between incubations. With the exception of the anti-BSA immunoglobulin assay, the plates were blocked with 1% BSA in PBS/Tween 20 for 2 hours at room temperature. Sera

diluted in PBS/Tween were added to the wells and incubated at room temperature for 2 hours. Antigen-specific antibodies were detected by incubation for 1 hour at room temperature with 100 $\mu$ l/well of a 1:1000 dilution of goat anti-mouse IgG whole molecule conjugated to alkaline phosphatase (Sigma-Aldrich, UK) followed by a 30 minute incubation at room temperature with 4-nitrophenyl phosphate. The absorbance was measured at 405 nm. The overall intra- and inter-assay coefficients of variation were: anti-BSA, 3.0% and 4.9%; anti-KLH, 5.2% and 6.8%; anti-CTB, 4.8% and 5.3%; anti-ADA, 1.9% and 6.5%.

### **2.9. Characterization of antigen specific antibody isotypes**

Antigen specific antibodies were isotyped using a mouse monoclonal antibody isotyping kit (Sigma ISO-2). Sera diluted in PBS/Tween 20 were added to wells coated with the relevant antigen and incubated for 2 hours at room temperature. Diluted (1:1000) goat anti-mouse isotypes (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) were added to the wells and incubated for 30 minutes at room temperature. Antigen-specific antibody isotypes were detected using alkaline phosphatase conjugated anti-goat IgG whole molecule and 4-nitrophenyl phosphate substrate. The absorbance was measured at 405nm.

### **2.10. Data analysis**

Results are expressed as mean  $\pm$  SEM. Data from the *in vivo* survival studies were analysed using a one-way analysis of variance (ANOVA) test, followed by an unplanned multiple comparison using Tukey's HSD test where significant differences were found. Homogeneity of variance was tested using Levene's Test and was met prior to analysis. A mixed ANOVA, with one between subjects factor and one within subjects factor was used to analyse differences in immune IgG responses between the two immunization routes. The between subjects factor was administration route with two levels (subcutaneous and carrier erythrocyte), and the within subjects factor was immune response day with three levels (14, 35 and 40 days). Data which showed significant differences in

the within subjects factor were further analysed using the Bonferroni pairwise comparison test to aid identification of main effects. Homogeneity of covariance was tested using the Mauchly's Test of Sphericity and was met prior to each test. A two factor ANOVA was also used to analyse differences in specific isotype profiles. The two between subjects factors were administration route, with levels subcutaneous and carrier erythrocyte, and immunization schedule, with levels primary immunization, and primary immunization followed by a subcutaneous booster immunization. Differences were considered significant when  $P < 0.05$ . SPSS 12.0 for Windows was used to analyse the data. Within and between assay variation was determined for each ELISA; the intra-assay of six samples were calculated from six replicate determinations in a single assay and the inter-assay variation was calculated from the results of four separate assays using six replicates of six samples.

### **3. Results**

#### **3.1. Carrier erythrocyte antigen entrapment**

Figure 1 shows the entrapment of ADA, BSA, CTB and KLH as a function of antigen concentration added to the dialysis bag; in each case the amount of antigen entrapped increased linearly with increasing dialysis antigen concentration. The mean entrapment efficiencies expressed as a percentage of antigen added to the dialysis bag were  $65.5 \pm 1.3 \%$  (n=11) for ADA,  $41.7 \pm 1.0 \%$  (n=9) for BSA,  $59.8 \pm 2.8 \%$  (n=13) for CTB and  $35.1 \pm 2.2 \%$  (n=9) for KLH. Using this entrapment efficiency data, the dialysis antigen concentrations required to obtain approximate entrapped antigen doses of  $20\mu\text{g}/100\mu\text{l}$  carrier erythrocytes ( $40\mu\text{g}/100\mu\text{l}$  in the case of ADA) were calculated to be 2.8 mg, 2.2 mg, 1.5 mg and 2.6 mg per 5ml dialysis volume for ADA, BSA, CTB and KLH, respectively.

The haematological and biochemical parameters of the carrier erythrocytes were closely similar to those of the unmodified erythrocyte (data not shown), as previously reported [17, 18].

### **3.2. *In vivo* survival of antigen-loaded carrier erythrocytes**

Optimal timings for test bleeds and secondary subcutaneous booster immunizations were determined from *in vivo* survival studies of antigen-loaded carrier erythrocytes. Table 1 shows the *in vivo* survival parameters of carrier erythrocytes loaded with the four different antigens. MCL ranged between 30.0 and 38.6 days, and cell half life ranged between 8.1 and 10.6 days. The mean cell lives of four different antigen-loaded carrier erythrocytes differed significantly beyond the 0.01 level:  $F(3, 8) = 9.17$ ;  $P = 0.006$ . An unplanned multiple comparison using Tukey's HSD test showed that ADA-loaded carrier erythrocytes had a significantly lower MCL compared to BSA-loaded ( $P = 0.008$ ) and CTB-loaded ( $P = 0.01$ ) carrier erythrocytes, although there was no significant effect of antigen on the mean cell half life:  $F(3, 8) = 1.30$ ;  $P = 0.34$ . There was no detectable label in the plasma indicating that there was no intravascular haemolysis of the antigen-loaded carrier erythrocytes, and thus indicating that there was no release of antigen into the circulation.

### **3.3. IgG antibody responses to administered antigens**

Figure 2 shows the IgG antibody responses to subcutaneous immunizations of 20 $\mu$ g of BSA, CTB and KLH, and 40 $\mu$ g of ADA mixed with TiterMax Gold<sup>TM</sup> and intravenous immunizations of carrier erythrocyte entrapped antigen. Antigen doses administered via the carrier erythrocyte route were  $43.7 \pm 1.3\mu$ g (ADA),  $22.9 \pm 1.2\mu$ g (BSA),  $19.7 \pm 0.7\mu$ g (CTB) and  $17.8 \pm 0.5\mu$ g (KLH). IgG antibody responses to carrier erythrocyte entrapped BSA and CTB at 14, 35 and 40 days post immunization were not significantly different from those observed in mice immunized subcutaneously,  $F(1, 16) = 3.340$ ;  $P = 0.086$  for BSA and  $F(1, 16) = 0.303$ ;  $P = 0.589$  for CTB. Mice immunized with carrier erythrocyte entrapped KLH elicited significantly higher IgG antibody

responses compared to mice immunized with subcutaneous KLH,  $F(1, 16) = 6.12$ ;  $P = 0.025$ . An ADA dose of  $40\mu\text{g}$  resulted in IgG antibody responses that were similar in both groups:  $F(1, 16) = 1.847$ ;  $P = 0.193$ . The mean antibody titre at the three sampling times differed significantly:  $F(2, 32) = 7.433$ ;  $P = 0.002$ ; subsequent analysis using the Bonferroni pairwise comparison test showed that this difference was due to significantly lower IgG responses at day 14 post immunization compared to the responses observed at days 35 ( $P = 0.049$ ) and 40 ( $P = 0.016$ ). Mice injected with unloaded carrier erythrocytes, lysed antigen-loaded carrier erythrocytes, and unloaded carrier erythrocytes mixed with antigen demonstrated no measurable IgG antibody responses.

### **3.4. Ig isotype profile and IgG1:IgG2a ratio**

Figure 3 shows the antibody isotype profile responses at 40 days post primary immunization in mice which received either a single primary immunization of antigen via the carrier erythrocyte or subcutaneous route (open bars), or a primary immunization via the carrier erythrocyte or subcutaneous route followed by a secondary subcutaneous booster immunization (hatched bars). With the exception of anti-CTB IgG2b, significant differences in the percentage isotype titres were observed in all cases for administration route (carrier erythrocyte or subcutaneous) and/or for immunization schedule (primary or secondary). Figure 4 shows the IgG1: IgG2a ratios. This ratio ranged between 1.0 and 1.1 in mice receiving only primary immunizations of carrier erythrocyte entrapped antigens, signifying that both Th1 and Th2 types of immune responses were induced. However, when a secondary booster immunization of subcutaneous ADA or BSA was administered, ratios of 6.7 and 4.5, respectively, were obtained indicating that the immune response had shifted to towards a Th2 profile. Primary immunization with ADA, CTB and KLH via the subcutaneous route resulted in IgG1/IgG2 ratios of 1.0, 1.1 and 1.0 respectively and therefore also demonstrated equal Th1 and Th2 responses. A secondary booster immunization with KLH resulted in an IgG1/IgG2a ratio of 3.5, indicating a shift towards a Th2 phenotype. Secondary booster immunizations with ADA and CTB did not change the IgG1/IgG2a ratio. Primary and secondary



booster subcutaneous immunizations with BSA produced IgG1/IgG2a ratios of 3.2 and 3.0 respectively, suggesting that this antigen induced a predominantly humoral immune response by this vaccination route.

#### **4. Discussion**

Carrier erythrocytes have been investigated as carriers of therapeutic agents for clinical applications for more than two decades [17, 18, 20-24]. The present work investigated the potential of Balb/c carrier erythrocytes to entrap model antigens and deliver them in a sustained manner to the monocyte-macrophage system for the purpose of eliciting a humoral response.

The molecular weight of the substance to be entrapped is a major factor in determining the efficiency of entrapment, although other factors such as diffusion coefficient, surface charge, polarity, molecular shape and hypo-osmotic dialysis time are also thought to be involved [17, 25-27]. The higher entrapment efficiencies observed for CTB and ADA would be predicted based on their relatively low molecular weights of 12,000 and 33,000 Da. The entrapment efficiency of ADA observed here is comparable to that reported in our previous *in vitro* studies on human carrier erythrocytes [17, 26, 27]. The percentage entrapment of BSA observed is similar to that reported in previous studies investigating the encapsulation of human serum albumin by human carrier erythrocytes [28, 29]. KLH consists of two different polypeptides of molecular weights 449,000 and 392,000 Da, existing in aggregation states ranging between  $4.5 \times 10^5$  and  $1.3 \times 10^7$  Da depending on pH and divalent ion concentration [30, 31]. Based on the observations of Deloach *et al* [32] who reported an entrapment efficiency of 39% for  $\beta$ -galactosidase (molecular weight 595,000 Da), an entrapment efficiency of 35% would suggest that KLH existed in a low state of aggregation during the hypo-osmotic dialysis procedure. The hypo-osmotic dialysis process permits the reproducible entrapment of macromolecules; from initial experiments where the entrapment efficiencies of a

range of antigen concentrations were measured (Figure 1), we were able to accurately calculate the amount of antigen to be added to the dialysis tubing to obtain the desired dose of carrier erythrocyte entrapped antigen.

The *in vivo* survival parameters of BSA-loaded, CTB-loaded, and KLH-loaded carrier erythrocytes are comparable to those of the unloaded carrier erythrocyte [33, 34]. The decreased MCL and mean cell half-life of the ADA-loaded carrier erythrocytes is likely to be a consequence of elevated activities of entrapped adenosine deaminase altering the balance of the erythrocyte adenine nucleotide pool. Hereditary hyperactivities of erythrocyte ADA have been reported to cause depletion in erythrocyte ATP and non-spherocytic haemolytic anaemia [35]. The survival of carrier erythrocytes is highly dependent on intracellular ATP levels; dog and human carrier erythrocytes with depleted cellular ATP concentrations have reduced *in vivo* survival rates, but if the resealing buffer is supplemented with glucose, magnesium chloride and adenosine, carrier erythrocyte energy metabolism is preserved and *in vivo* survival times are comparable to the unmodified native erythrocyte [15, 16, 34, 36, 37]. The *in vivo* survival results reported here, together with the efficient antigen loading of these cells, demonstrate the potential of the carrier erythrocyte to behave as a slow or sustained antigen release system by delivering antigens to the antigen presenting cells over a period of 30 to 38 days, mimicking the delivery of small antigen boosters.

The results of the immunization studies demonstrate that a single administration of carrier erythrocyte entrapped antigen is capable of eliciting significant humoral responses which are comparable or in the case of KLH, superior to those obtained using the potent adjuvant TiterMax Gold™ via the subcutaneous vaccination route. It may be hypothesised that the greater humoral immune response to KLH via the carrier erythrocyte route is due to a combination of antigen size and immunization route. Indeed, studies in the Sprague Dawley rat have shown that a single subcutaneous immunization with KLH resulted in a much weaker primary antibody response

compared to the responses obtained by either the footpad or intravenous route [38]. Esposito *et al* reported that a primary immunization and two booster immunizations of erythrocyte encapsulated synthetic peptide (NANP)<sub>40</sub> were required to produce a low antibody response [39]. The release of 65-75% of the internalised peptide and the parallel release of haemoglobin after a 24 hour *in vitro* incubation suggests that these loaded cells may have had poor *in vivo* survival characteristics. Higher antibody responses to erythrocyte entrapped diphtheria and tetanus toxoids compared to free antigen administered intraperitoneally required the use of an immunization schedule with two to four booster injections [12]. The requirement for boosters in this latter study may be related to the initial immunization doses administered (21 to 24µg), since the toxoid-loaded carrier erythrocytes exhibited normal *in vivo* half-lives of 10 days. In the current studies, primary immunizations with 20µg ADA also failed to elicit an IgG antibody response via both the subcutaneous and carrier erythrocyte routes - unlike with BSA, CTB and KLH. However, increasing the dose of ADA to 40µg produced IgG antibody responses that were similar in both groups, though the immune responses at 35 and 40 days were significantly higher than observed at 14 days for both immunization routes, and was not observed with the other three antigens. Further studies are required to determine whether these significant differences are specific to ADA, or due to the administration of a higher antigen dose. The entrapment within carrier erythrocytes is able to replicate the potency of TiterMax Gold™ as an adjuvant for weaker antigens such as ADA which required doubling the dose to elicit immunogenicity. The immune response to ADA-loaded carrier erythrocytes is of significant interest with regard to our clinical application of ADA-loaded carrier erythrocytes in adenosine deaminase deficiency; despite raising an anti-ADA antibody response, the carrier erythrocyte simultaneously protects entrapped ADA from circulating antibodies [17, 21].

The differentiation of naive T cells into Th1 or Th2 cells determines the type of immune response generated; Th1 responses are characterized by the generation of cell-mediated immune responses against intracellular invaders by activating macrophages, natural killer cells and CD8<sup>+</sup> cytotoxic T

lymphocytes, and Th2 responses are characterized by the generation of B-cell-dependent humoral immune responses against antigens. The subclass of IgG induced after immunization is an indirect indicator of the relative contribution of Th2-type and Th1-type cytokines; in particular, the production of IgG1 and IgG2a are primarily induced by Th2 and Th1 responses respectively, and thus the ratio of IgG1:IgG2a can define the T-cell phenotype induced by immunization [40, 41]. A primary administration of all four antigens via the carrier erythrocyte route resulted in IgG1: IgG2a ratios which ranged between 1.0 and 1.1, indicating that both Th1 and Th2 responses were induced. However, a subsequent secondary subcutaneous booster immunization with ADA and BSA switched this response to one that was predominantly Th2. More detailed studies are required to determine the effect of secondary immunizations of carrier erythrocyte-entrapped antigens on the type of immune response induced. Both Th1 and Th2 responses were also induced in mice vaccinated with primary subcutaneous administration of ADA, CTB and KLH. Whereas a secondary subcutaneous immunization with KLH induced a switch to a predominant Th2 response, secondary immunizations with ADA and CTB produced no change in the relative titres of IgG1 and IgG2a isotypes. Dominici *et al* demonstrated that mice which received a primary immunization of HIV-1 Tat protein conjugated to erythrocytes, followed by three boosters, all via the intra-peritoneal route, produced both type Th1 and Th2 immune responses [11]. A number of factors are thought to be involved in the polarization towards a Th1 or Th2 response, including the route of immunization, antigen dose, the number of doses administered, duration of T-cell receptor ligation and type of antigen presenting cell involved [42, 43]; the results presented here suggest that the type and balance of response induced after a secondary subcutaneous immunization is antigen-specific to some degree. Antigen targeting via the carrier erythrocyte should allow further exploration of the molecular mechanisms leading to Th1/Th2 polarisation following an antigenic challenge and thus enhance the design of vaccination protocols.

A majority of the currently available vaccinations stimulate potent humoral responses capable of mediating long-term protection. There is growing evidence, however to suggest that a protective immunity against viruses and tumour cells requires the induction of both Th1 helper-mediated and cytotoxic T cell-mediated responses. Current studies are exploring the potential of carrier erythrocyte entrapped antigens to stimulate both CD4<sup>+</sup>- and CTL-mediated responses. Of particular interest is whether entrapped antigen can be presented to CD8<sup>+</sup> T lymphocytes on MHC class I molecules by a process of cross presentation [44-46], or whether manipulation of the carrier erythrocyte is required for antigen delivery to the cytosolic compartment for MHC I presentation. As dendritic cells are currently believed to be the most potent T-cell activators, carrier erythrocyte-mediated antigen targeting to this type of antigen presenting cell is also being investigated [47].

In conclusion, this study demonstrates that antigen-loaded carrier erythrocytes are able to elicit humoral immune responses without the need to use adjuvants, and with further study may prove to have the potential to provide the equivalent of priming and boosting immunizations with a single administration. In current immunization protocols a large proportion of administered antigen is catabolised and cleared before it reaches the antigen presenting cells; entrapment within the carrier erythrocyte protects the antigen from degradation by proteolytic enzymes, therefore having the potential to improve vaccine stability *in vivo* and reduce the dose of antigen required for inducing protective immunity. This immunization strategy may have development potential for antigens such as recombinant proteins and peptides, or for synthetic peptides to which a weak, equivocal, or nil response is obtained by current immunization approaches, and may be particularly pertinent to some tumours. Unlike other developing biological carriers, including viral vectors and bacterial ghosts, there are no safety issues associated with the administration of erythrocyte carriers. Carrier erythrocyte administration is simple, especially with the small volumes of erythrocytes that would be required for antigen delivery. Autologous carrier erythrocytes are safe and practical in biweekly

clinical practise [48] and may be prepared using a semi-automated red cell loader within 2 hours at room temperature under blood banking conditions [49].

### **Dedication and acknowledgement**

This paper is dedicated to the memory of our second author, Ian Pearson (1952- 2005).

The authors acknowledge the technical expertise of Will Murphy and Robert Hall. These studies work were supported by the Biotechnology and Biological Sciences Research Council.

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Table 1.

*In vivo* survival parameters of antigen-loaded carrier erythrocytes in mice

Entrapped antigen	MCL (days) <sup>a</sup>	T <sub>1/2</sub>
ADA	30.0 ± 1.6	8.1 ± 0.6
BSA	38.6 ± 1.9	10.6 ± 1.5
CTB	38.2 ± 0.1	10.1 ± 1.0
KLH	34.1 ± 0.9	9.4 ± 0.6

Results represent the mean ± SEM of three time courses

<sup>a</sup>  $P < 0.01$ , using one-way ANOVA

MCL = mean cell life

## Captions to illustrations

**Fig 1.** The entrapment of ADA, BSA, CTB and KLH by mouse carrier erythrocytes. Each point represents one dialysis experiment.

**Fig 2.** IgG antibody responses to intravenous carrier erythrocyte entrapped antigen (open bars) and subcutaneous antigen mixed with TiterMax Gold™ (hatched bars) at 14, 35 and 40 days post immunization. Doses administered were approximately 20µg for BSA, CTB and KLH, and 40µg for ADA. Results are expressed as mean ± SEM for 9 mice.

**Fig 3.** Ig isotype profile of responses in mice at 40 days after primary immunization with antigen (open bars), or primary immunization followed by a secondary subcutaneous booster of antigen (hatched bars). Primary immunizations were either carrier erythrocyte entrapped antigen (CE) or subcutaneous antigen mixed with TiterMax™ (Sub). Results are the mean ± SEM for 9 mice. Statistically significant differences for route of administration (R) and/or immunization schedule (S) are \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ), \*\*\* ( $P < 0.001$ ).

**Fig 4.** Mean IgG1:IgG2a isotype ratios in mice at 40 days after primary immunization with antigen (open bars), or primary immunization followed by a secondary subcutaneous booster of antigen (hatched bars). Primary immunizations were either carrier erythrocyte entrapped antigen (CE) or subcutaneous antigen mixed with TiterMax™ (Sub).

Figure 1

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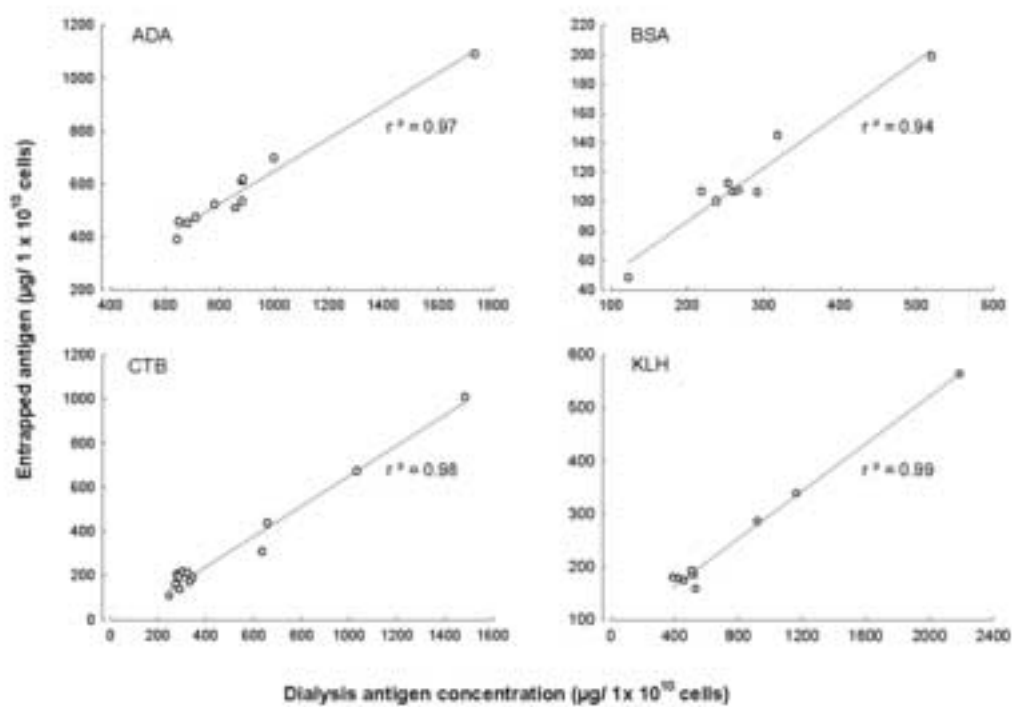


Fig 1. The mouse immune response to carrier erythrocyte entrapped antigens. Bax et al.

Figure 2

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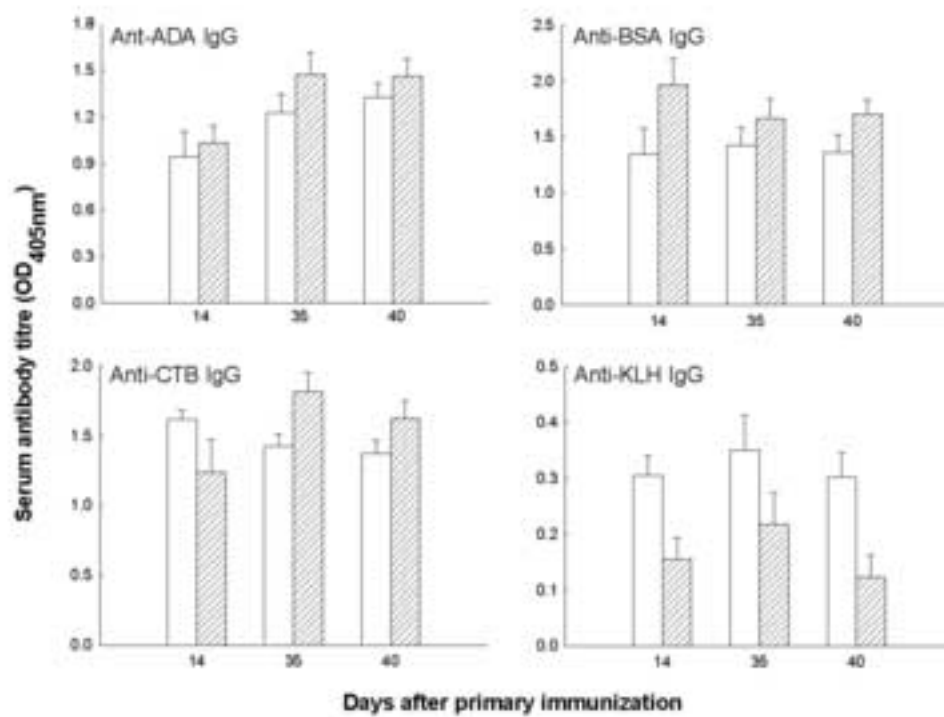
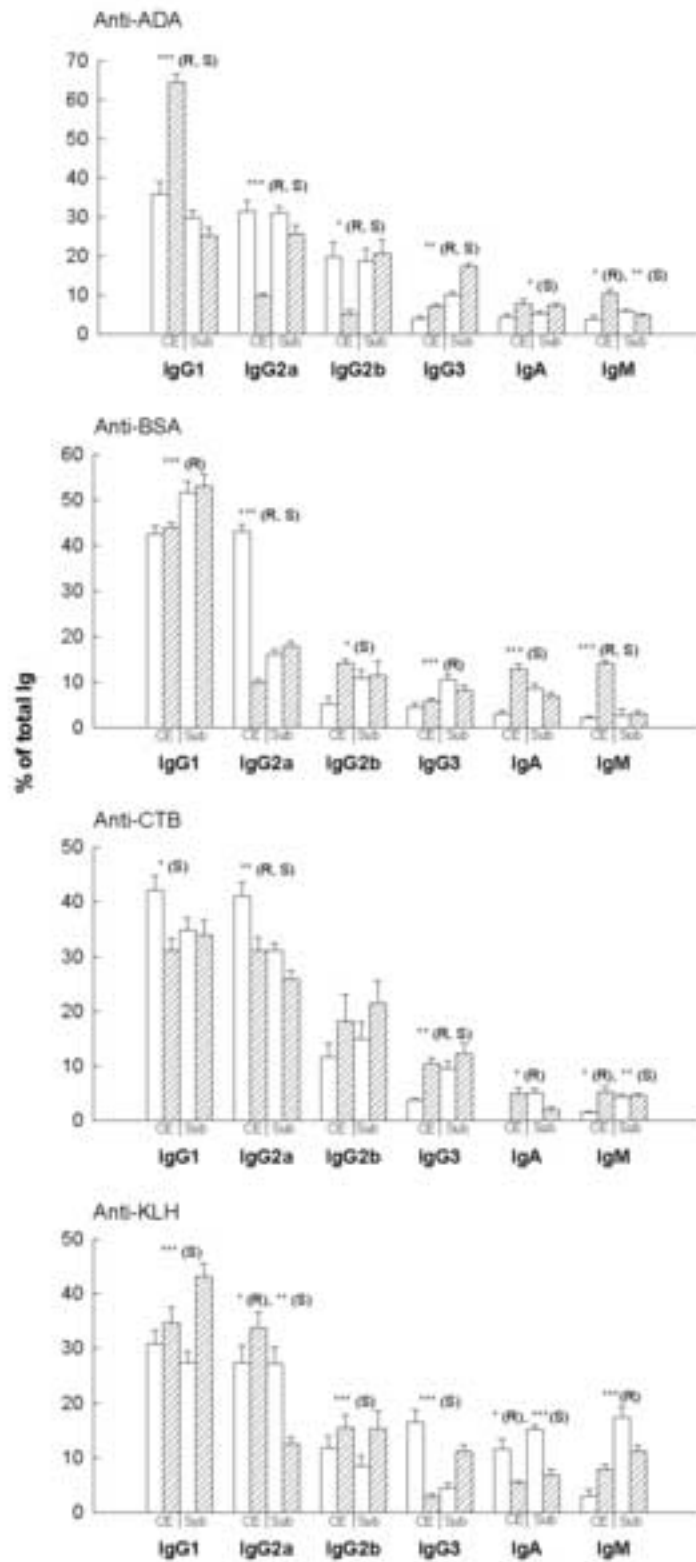


Fig 2. The mouse immune response to carrier erythrocyte entrapped antigens. Bax et al.

**Figure 3**  
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**Fig. 3** The mouse immune response to carrier erythrocyte entrapped antigens. Bax et al.



Figure 4  
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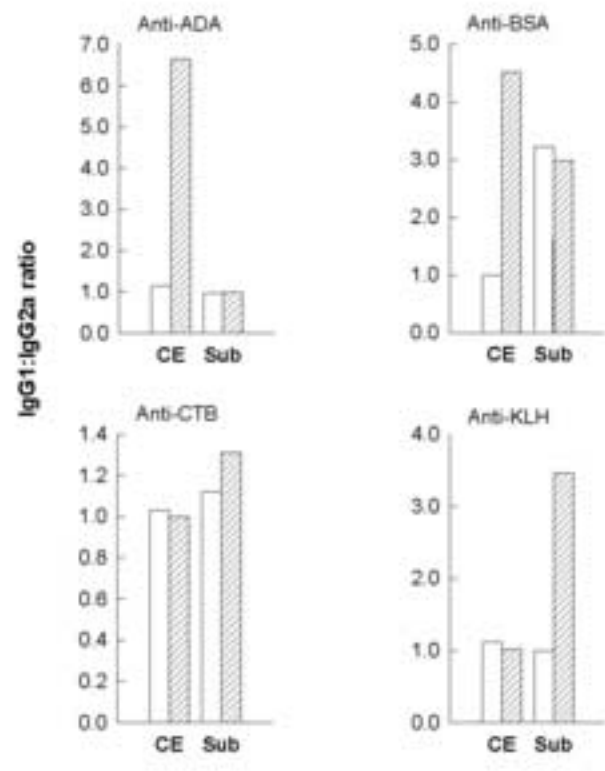


Fig 4. The mouse immune response to carrier erythrocyte entrapped antigens. Bax et al.



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4<sup>th</sup> May 2006

A D.M.E. Osterhaus  
Regional Editor  
Vaccine

Dear Professor Osterhaus,        **Re: revised manuscript JVAC-D-06-00081**

Thank you for sending us the reviewers' constructive comments regarding our manuscript titled "The mouse immune response to carrier erythrocyte entrapped antigens" authored by Anne M. Murray, Ian F.S. Pearson, Lynette D. Fairbanks, Ronald A. Chalmers, Murray D. Bain, Bridget E. Bax.

We have attached a detailed point-by-point reply to the Reviewers' comments together with a revised version; the manuscript has been revised precisely in accord with the reviewers' comments.

We hope that the revised reversion is now acceptable for publication in Vaccine. Thank you for your time.

Yours sincerely,

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## Comments Reviewer 1

Comment	Reply
The introduction should be shortened. The last paragraph of the introduction should be included in the Materials and Methods section.	We have reduced the length of the last paragraph and moved the text referring to the entrapment studies and <i>in vivo</i> survival studies to the Materials and Methods section.
There's some information about HPLC conditions missing. Additional statistical information about the analytical methods employed for the determination of the different antigens in loaded erythrocytes such as the variation coefficient or limit of quantification should be included.	We have included additional information about the HPLC conditions used and have included the coefficients of intra-and inter-assay variation for each ELISA.
The count cell is corrected for natural decay and blood haematocrit, but how this correction is performed it isn't explained.	We have now included in section 2.6 how this correction was performed.
The reason for using different post-primary immunization times for secondary booster depending on the antigen used must be mentioned.	We have re-written this sentence to clarify this (Line 16, Page 10).
Why isn't the same number of replicates for all the antigens used? More information about antigen carrier erythrocytes obtained as cell recovery or main haematological parameters of loaded erythrocytes should be included. It would have been interesting to include in the paper results about the <i>in vitro</i> release of the antigens from the loaded erythrocytes, which would facilitate the analysis and interpretation of the <i>in vivo</i> results.	<p>The number of entrapment experiments performed depended on the volume blood obtained from donor mice; this varied with mouse weight.</p> <p>The haematological parameters of loaded carrier erythrocytes are reported in the literature. We have included an additional sentence stating that the haematological and biochemical parameters of carrier erythrocytes are closely similar to those of the unmodified erythrocyte (Line 1, Page 13).</p> <p>We did not perform <i>in vitro</i> antigen release experiments. Our previous studies have shown that <i>in vitro</i> release of entrapped substances mirrors the release of haemoglobin, and this release is therefore consistent with cell lysis. In the current studies we measured plasma associated <sup>51</sup>Cr as a marker of intravascular haemolysis and thus antigen release. We found no detectable label. We have expanded sentence 13 on Page 13 to indicate our findings.</p>
Figure 1.- Linear correlation and the corresponding statistics should be included in the figure.	We have included the regression lines and coefficients of determination in Figure 1.
Figure 2.- The results about the immunization with the different antigens by intravenous (carrier erythrocytes) and subcutaneous (free antigen) routes of administration should be standardized with the exact dose of antigen administered, and the statistics need to be repeated.	We are not able to standardize the results in Figure 2 as we did not perform dose- response experiments; we thus can not make the assumption that the immune responses are dose dependent.
The discussion should be shortened. The first paragraph repeats some of the information given in the Introduction.	We have reduced the length of the first paragraph (Page 15).
For the majority of the antigens studied (ADA, BSA and CTB), the Ig antibody response is quite similar when intravenous (loaded erythrocytes) and subcutaneous (free antigen plus TiterMax Gold) are used. However, the immune response by intravenous route using KLH carrier erythrocytes is higher. These differences are not sufficiently explained in the Discussion.	We have expanded the discussion to include our hypothesised explanation for the larger humoral response to KLH. We have included a reference to support this hypothesis (Line 23, Page 16).

<p>It would be interesting to discuss the potential explanation for the higher immune response observed in 35 and 40 days in comparison to 14 days when using 40 <math>\mu</math>g, since there isn't such difference in other antigens results.</p>	<p>We have inserted a sentence stating that further studies are required to determine whether these differences are specific to ADA, or due to antigen dose (Line 15, Page 17).</p>
<p>The advantages of the erythrocytes like antigen biological carriers versus other promising antigen biological carriers like viral vectors or bacterial ghosts should be commented on.</p>	<p>We have inserted a sentence addressing the safety concerns of these other biological carriers (Line 22, Page 19).</p>

Comments Reviewer 2

<b>Comment</b>	<b>Reply</b>
<p>The choice of the 4 antigens needs to be more motivated in detail.</p> <p>Please insert Osmolarity of hypo-osmotic buffer and the osmolarity of erythrocytes after dialysis procedure.</p>	<p>We have clarified this sentence further (Line 21, Page 5).</p> <p>We have now included the osmolalities of the hypo-osmotic buffer and carrier erythrocytes (Page 6, Lines 9 and 13).</p>
<p>The injected suspension of loaded RBC should have theoretically an Ht of 15-20%. Please insert the real HT value of loaded RBC injected.</p>	<p>We have now inserted the final haematocrit of the infused carrier erythrocyte suspensions (Line 25, Page 8).</p>
<p>Insert shortly the calculation of MCL</p>	<p>We have inserted the method used to determine MCL (Page 9)</p>
<p>The scheme of immunization is not clear. Motivate the choice of T1/2 as second booster time.</p> <p>Insert in Table 1 the molecular characteristics of the 4 antigens, (ie. MW), the entrapment efficiency, and remove this information from discussion section.</p>	<p>We have re-written this sentence to clarify this (Line 14, Page 10).</p> <p>We have removed from the discussion the entrapment efficiencies of CTB and ADA. We have not included the molecular characteristics of the four antigens in table 1 because of the unknown aggregation state of KLH. We hope this is acceptable to the reviewer.</p>
<p>Lane 7 pag 18 " the subclass ...." paragraph should be moved to discussion.</p>	<p>We have moved this section to the discussion (Line 2, Page 18).</p>
<p>Pag 21 at the end. Naïve T cells instead of naïve CD4+</p>	<p>This error has been corrected</p>
<p>Pag 22 lane2 insert NK cells in the list.</p>	<p>We have included NK cells in the list</p>
<p>Pag 22 lane 3 antigens instead of extracellular organisms.</p>	<p>We have changed this.</p>
<p>Discuss the role of antibody anti ADA in circulation in case of enzyme replacement therapy by ADA loaded erythrocytes for the cure of ADA deficiency.</p>	<p>We have included a sentence to address this point (Line 19, Page 17).</p>
<p>Remove from results the explanation of the need to increase ADA amount used for the study otherwise it's redundant.</p>	<p>We have removed this explanation.</p>
<p>References:            Insert: Corinti et al J Leukoc Biol. 2002 Apr;71(4):652-8 to validate your idea that RBC antigen delivery can interact with dendritic cells.            Insert a more recent paper: Annese v. Et al Eur Rev Med Pharmacol Sci. 2006 Jan-Feb;10(1):27-31. or ross i l Blood Cells Mol Dis. 2004 Jul-Aug;33(1):57-63.</p>	<p>We have included all three references.</p>
<p>Figure 3 It's not easy to understand try to divide the figure in 2 part because the histogram are too little moreover using the same colours for CE and SUB is too confusing.</p>	<p>We agree with this comment and have split Figure 3 into two Figures (Figure 3 and Figure 4).</p>

**Suggested Reviewers:**

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