ESTIMATION OF THE LYTIC CAPACITY OF HUMAN SERUM BY EXPERIMENTAL AND MATHEMATICAL ANALYSIS.

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Summary. *The dynamics of human complement consumption by immune complexes (ICs) was studied. ICs were formed between blood group A and B erythrocytes (solid phase) or synthetic blood group A and B active trisaccharides (fluid phase) respectively, and anti-A/anti-B monoclonal (IgM) blood group antibodies (mAb). We found that the lytic capacity of the human serum was already reduced when the estimated molecular concentration of complement components per erythrocytes were in large excess suggesting that the lytic capacity could be significantly smaller than that one would expect according to the one hit theory of complement-mediated lysis (Mayer M.M., 1961). A new mathematical model of complement consumption was successfully fitted to the entire experimental database containing 4672 measurements. It was used to estimate reactions in undiluted serum containing a physiological erythrocyte concentration, which cannot be measured directly. The model predicted that: (i) up to 40% of all the erythrocytes could be lysed; (ii) the lytic capacity can be significantly reduced by preincubation with 10 to 100 mg/ml ICs. The clinical implications of these findings concerning the reduction of harm caused by unintended transfusion of ABO incompatible blood, and the usefulness of synthetic oligosaccharides to overcome hyperacute rejection (HAR) of xenotransplants are discussed.*

Key words: human complement-mediated lysis, synthetic blood group active trisaccharides, immune complexes, mathematical analysis, ABO incompatibility, HAR.

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INTRODUCTION

The complement system has important functions *in vivo*: it promotes opsonization, mediates direct lysis, solubilizes immune complexes, and generates inflammation regulating chemotactic peptides (Frank M.M., 1992). Recognition of microbes by the innate immune system activates the alternative pathway of complement, which results in the covalent binding of C3 to the microbes (Muller-Eberhard H.J., 1988). The lectin pathway of complement activation, via the mannose-binding protein, may have an auxiliary role during the period of immunological vulnerability, which occurs after the decay of maternal IgG (Turner M.W., 1996) until the appearance of the infant's own natural antibody (Ab) repertoire. This provides a mechanism for activation of the classical complement pathway (Carroll M.C. & Fischer M.B., 1997). Decomplemented animals and guinea pigs deficient in C2, C3 or C4 showed that complement is also important for the generation of adaptive Ab response and immunological memory (Pepys M.B., 1974;Papamichail M. *et al*, 1975;Ochs H.D. *et al*, 1986;Bottger E.C. & Bitter-Suermann D., 1987). Furthermore, the classical pathway of the complement system prevents the formation of large immune complex (IC) lattices, whereas the alternative pathway is able to solubilize precipitated complexes (Miller G.W. & Nussenzweig V., 1975). Complement also promotes the clearance of immune complexes from the host via the mononuclear phagocytic system (Cornacoff J.B. *et al*, 1983; Cornacoff J.B. *et al*, 1984; Veerhuis R. *et al*, 1986; Schifferli J.A. *et al*, 1988;Davies K.A. *et al*, 1992; Walport M.J. & Davies K.A., 1996).

Fortunately, the mechanisms of IC clearance are abundant and only under very specific conditions does complement become "the limiting factor" when pathological consequences may occur, either due to that Ag/Ab complexes are formed in excess or the clearance mechanism is inefficient (Schifferli J.A., 1996). ICs, which form in the absence of complement, might escape elimination by the phagocytic system and deposit in tissues instead, which could result in inflammatory tissue injury (Davies K.A. *et al*, 1990). By causing inflammatory tissue injury, ICs may also release autoantigens, which in turn could stimulate autoimmune diseases. Consistent with this, most patients with hereditary homozygous C1q deficiency have severe autoimmune disease, primarily systemic lupus erythematosus (SLE) (Bowness P. *et al*, 1994). The depletion of complement is caused by the formation of large circulating IC in the simultaneous presence of hepatitis C virus antigen, Ab and rheumatoid factor (Schifferli J.A. *et al*, 1995). For example, in hypocomplementaemic urtical vasculitis, high affinity anti-C1q autoantibodies react with C1q and it is the anti-C1qAb/C1q complexes, which are probably responsible for complement depletion. In IgA vasculitis and nephritis, excessive production of IgA Ab and inefficient clearance of IgA IC together result in renal pathology. To prevent these processes, it is important to improve the understanding of the interaction of ICs with complement in new animal models and in more sensitive *in vitro* systems. As the transit time of ICs is extremely short (Davies K.A. *et al*, 1992) it is very difficult to study complement activation by pathologically relevant ICs *in vivo*.

We have demonstrated that sensitized human blood group A and B erythrocytes are convenient targets for studies of the classical pathway of the human complement cascade *in vitro* (Bakács T. *et al*, 1989; Bakács T. *et al*, 1990; Mushens R.E. and Bakács T., 1992). The numbers of A and B Ab-binding sites on erythrocytes are known and the biochemical structure of A and B antigens on erythrocytes have been defined (Howard J. & Hughes-Jones N., 1988; Mollison P.L., 1972). Although the difference between the immunodominant sugars of the A and B antigens is a single *N*-acetyl group, anti-A and anti-B mAbs do not cross react.

Therefore, these antigens can be used to study the influence of a single mAb, even if both anti-A and anti-B mAbs are simultaneously present in a mixture (Tusnády G. *et al*, 1992; Bakács T *et al*, 1993a; Bakács T *et al*, 1993b; Bakács T. *et al*, 1994). With the availability of synthetic blood group A- and/or B-oligosaccharides, it became possible to extend the scope of our human erythrocyte model system and study the complement activation by soluble ICs consisting of synthetic oligosaccharides and anti-blood group A and B mAbs.

In the present work, the dynamics of complement consumption by sensitized blood group A and B erythrocytes and well-characterized soluble ICs were studied *in vitro*. A general mathematical model of complement consumption was used to analyse experiments and the total lytic capacity of the human serum was extrapolated. The potential clinical implications of the findings concerning the transfusion-associated haemolysis and hyperacute rejection (HAR) of xenotransplants are discussed.

METHODS AND MATERIALS

Erythrocytes. Human blood group A and B erythrocytes were drawn from two normal donors into sodium citrate and stored (in glycerol) at -30°C until use (Krijnen H.W. *et al*, 1968). Immediately prior to assay the erythrocytes were thawed, washed in PBS and incubated with 1% papain for 10 min at 20 °C prior to labelling with 200 μCi of sodium ^{51}chromate (^{51}Cr) (Amersham International, U.K.), for 3 hr at 37°C. Following incubation, erythrocytes were washed extensively in RPMI-1640 supplemented with 10% heat inactivated foetal bovine serum (Sebak Biologische Forschungs GmbH, Germany), RPMI-FBS, and the cell concentration adjusted accordingly.

Synthetic blood group-related substances. Polyacrylamide-bound blood group A and B active trisaccharides without label (Atri-PAA and Btri-PAA respectively) and biotinylated polymeric probes (Atri-PAA-biot and Btri-PAA-biot respectively) with exactly defined molar content of the ligand (20 mol.% of ligands) were purchased from SYNTESOME (München, Germany).

Anti-A and anti-B reagents. Supernatants of cultured hybridomas were the sources of mouse monoclonal IgM antibodies (mAbs). BRIC.131 (anti-A) and BRIC.250 (anti-B) were generous gifts of R. Mushens (International Blood Group Reference Laboratory, Bristol, U.K.). The immunoglobulin concentration of the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) (see reference (Mushens R.E. *et al*, 1993)).

Immune complexes. Two types of ICs - formed either between IgM anti-A mAb and Atri-PAA or anti-B mAb and Btri-PAA - were made *in vitro*; equal volumes of various dilutions of mAbs and trisaccharides (50 μl respectively) were mixed in flat-bottomed 96-well microtest plates (Nunc, Roskilde, Denmark), which were sealed and incubated first for 1 hr at 37°C in a shaking incubator (Denley, Billingshurst, UK), then at 4°C overnight. Next morning following incubation, the microtest plates were used in the complement consumption assay. The concentrations of free reagents were determined by radioimmunoassay. 50 μl of biotinylated Atri-PAA and Btri-PAA trisaccharides (concentrations: 12, 6, 3, and 1.5 μg/ml), 50 μl of IgM anti-A mAb and anti-B mAb (concentrations: 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 μg/ml) and 100 μl of RPMI-foetal bovine serum (FBS) were pipetted into tubes and incubated for 24 hours at 4°C. Each determination was performed in triplicate. Following incubation, 500 μl of avidin coated magnetic immunosorbent (MIS) was added to each tube, vortex-mixed and incubated for 60 min at room temperature. The MIS particles were settled 5 min using a magnetic separator and 100 μl of the supernatant, IgM mAb standards (at concentrations of: 156, 78, 38, 19, 9.8, 4.9, 2.4, 1.2 and 0.6 ng/ml) and 200 μl biotinylated Atri-PAA and Btri-PAA trisaccharides (at a concentration of: 6.3 μg/ml) were pipetted into streptavidin coated polystyrene tubes. The tubes were incubated for 24 hours at room temperature and washed twice. Next 200 μl ^{125}I labelled anti-mouse IgM rabbit IgG was pipetted into all tubes and they were incubated for 4 hours at room temperature while shaking; then the tubes were washed twice. Each tube was counted for at least 60 seconds in a gamma-counter and the free IgM concentrations of the samples were calculated.

Complement. Serum from a blood group AB donor was the source of complement. Fresh serum was aliquoted and stored at -30°C until use.

Direct complement mediated lysis (DCL). Complement mediated lysis was measured according to Bakács *et al*. (Bakács T. *et al*, 1994). Equal volumes (50 μl) of various dilutions

of complement, mAbs, and ^{51}Cr-labelled blood group A or B erythrocytes were mixed in flat-bottomed 96-well microtest plates (Nunc), then the volumes were made up to 200 µl with RPMI-FBS. Spontaneous release of label was estimated from wells containing target cells and the mAb without complement and wells containing cells suspended in distilled water gave values for the maximum release. After varying incubation times of between 2 to 60 minutes at 37^{o}C in a shaking incubator (Denley), the microtest plates were cooled on ice and then centrifuged for 10 min at 447 g (Hettich, Tuttlingen, Germany). 100 µl of supernatants were removed and the radioactivity was measured in a gamma counter (Beckman, Palo Alto, CA). The spontaneous release varied between 2% and 3% and the maximum release between 97% and 100%. The percent specific lysis was calculated according to the formula: (^{51}Cr release in test - spontaneous release) / (maximum ^{51}Cr release - spontaneous release) x 100. The coefficient of variation was less than 2% (they are not shown).

Complement consumption assay (CCA). Various dilutions of AB serum (50 µl) were mixed with preformed ICs (100 µl; in flat-bottomed 96-well microtest plates; Nunc) and the mixture was preincubated at 37^{o}C in a shaking incubator (Denley) from 10 to 80 min. As complement consumption cannot be directly measured, the preincubation phase was followed by a DCL assay to determine the remaining lytic activity. Thus, equal volumes (50 µl) of constant concentrations of ^{51}Cr-labelled erythrocytes (96 x 10^{6}/ml containing 1 µg/ml sensitising mAb) were added to the microtest plates and the incubation was continued for a further 40 min at 37^{o}C in a shaking incubator (Denley). To avoid interference of components used during the first and second incubations, blood group B erythrocytes sensitized with anti-B mAb were employed if complement consumption of [Atri-PAA+anti-A] complexes was measured and vice versa. The second incubation was stopped, the radioactivity was measured and percent specific lysis was calculated as described for DCL.

Mathematical analysis. The interaction of different factors was investigated by BMDP program package (Dixon W.J., 1981). The differential equations were solved by Runge-Kutta numerical integration and the model parameters were fitted by the conjugate gradient method. These calculations were carried out on IBM PC by Pascal programs written in our laboratory.

RESULTS

Direct complement mediated lysis of sensitised erythrocytes. Experimental measurements of dynamics

In conventional *in vitro* assays, complement mediated lysis is usually studied as a function of a single component, which cannot properly describe the dynamics of a reaction influenced by more than one participant. In a DCL assay, there are four *input* parameters: 1) the amount of erythrocytes (*target*), 2) the dilution of serum (*effector*), 3) the amount of the sensitising mAbs (*antibody*), and 4) the duration of the experiment (*time*); whereas there is a single *output* parameter: the amount of *lysed* target cells. Lysis of sensitised blood group A and B erythrocytes was therefore measured as a function of all four parameters. The influence of relative sensitisation was determined since absolute mAb concentration was kept constant (1 μg/ml), while target cell number was varied (a total of 11 different concentrations between 96 to 768 x 10^6 cells/ml were used). Fifteen different dilutions (between 1/8 to 1/72) of allogeneic whole serum were used during eleven different incubation times (from 2 to 60 minutes) in eleven DCL experiments. From these experiments, a total of 1704 individual measurements were made (the complete experimental data base is available at http://www.renyi.hu/~mathimm). To ensure the accuracy of these experiments, conditions were chosen such that the vast majority of the lytic values were between 5% and 95%.

Four typical DCL experiments employing A or B erythrocytes (192x10^6 target cells/ml) in the presence of various serum dilutions are presented in Fig. 1 a, b, c, d. A general characteristic of measurements was that lysis increased during the first 20 to 30 minute incubation, but lysis reached a plateau up to 60 minutes and could not be increased by further incubation. It is important to note that increasing the dilution by a single step (from 1/16 to 1/32) resulted in a significant reduction of the lytic ability of the serum. Fig. 1 also demonstrates that predicted lyses are well fitted to the measurements of complement consumption by the mathematical model described below.

Next the influence of serum dilution was compared with the effect of increasing the number of erythrocytes. The starting assay conditions (96 x 10^6 target cells/ml, 1/16 serum dilution and 1 μg/ml mAb concentration) were chosen such to ensure lysis of all target cells. Two typical DCL experiments using A and B target cells respectively are presented in Fig. 2. a, b, c, d. A four-fold increase of serum dilution reduced lysis of A and B target cells from being practically complete to less than 20% and 40% respectively (Fig. 2. a, c). Whereas a four-fold increase of target cell number in the presence of a constant serum dilution, had almost the same effect (Fig. 2. b, d). The reduction of lysis was slightly smaller with B than with A erythrocytes.

IC formation between blood group active trisaccharides and anti-A or anti-B mAbs

The binding affinity between blood group active trisaccharides and anti-A and anti-B mAbs was measured by biotinylated polymeric Atri-PAA and Btri-PAA molecular probes (12.5 μg/ml respectively) and radiolabelled anti-(mouse) IgM reagents in a radioimmunoassay (Fig. 3. a). The binding affinity of the anti-B IgM mAb was slightly higher than that of the anti-A. The maximum difference was measured at 10 ng/ml mAb concentration, where 2.0 times more anti-B than anti-A mAb was complexed. The efficiency of IC formation was measured *in vitro*, using fourteen different concentrations of anti-A and anti-B mAbs (from 0.60 ng/ml to 5 μg/ml) as well as eight different concentrations of Atri-PAA and Btri-PAA trisaccharides (from 400 ng/ml to 50 μg/ml). As expected, when the trisaccharide concentrations were

increased, the amount of free mAb decreased. At higher reactant concentrations practically all anti-B mAb and nearly all anti-A mAb were complexed (Fig. 3. b, c).

Complement consumption by trisaccharide containing ICs. Experimental measurements of dynamics

Preformed ICs, containing either Atri-PAA or Btri-PAA, were incubated in fifteen different dilutions of allogeneic whole serum (between 1/12-1/72) during six different incubation times (from 10 to 80 minutes). The remaining lytic capacity was measured thereafter by adding a constant concentration of sensitized A or B erythrocytes (96×10^6 target cells/ml at 1 μg/ml mAb concentration) during a second incubation of 40 minutes. Thus, in a CCA experiment there are seven *input* parameters: 1) the concentration of ICs (*target 1*), 2) the concentration of erythrocytes (*target 2*), 3) the dilution of serum (*effector*), 4) the concentration of the mAbs complexed to trisaccharides (*antibody 1*), 5) the concentration of the mAbs used for the sensitization of erythrocytes (*antibody 2*), and 6) the duration of the CCA (*duration 1*), and 7) the duration of the DCL (*duration 2*). Twelve CCA experiments were carried out and under the conditions used, the majority of the measured values were between 5% and 95% lysis. From these experiments, a total of 2968 individual measurements were made (the complete experimental data base is available at http://www.renyi.hu/~mathimm).

The influence of trisaccharide and mAb concentrations on IC complement consumption is presented in a typical experiment in Fig. 4. a, b. Atri-PAA trisaccharide concentrations were varied from 1.6 to 25 μg/ml, while mAb concentration and serum dilution were kept constant (2.5 μg/ml and 1/16 respectively). The remaining lysis was plotted as a function of time. Surprisingly, increasing the trisaccharide concentration had little influence on complement consumption (Fig. 4. a). In contrast, if the mAb concentration was increased from 0.63 to 2.5 μg/ml, at constant trisaccharide concentration and serum dilution (6.25 μg/ml and 1/16 respectively), consumption efficiency was significantly increased (Fig. 4. b). These results were consistent with the DCL assay, when a 2.75-fold increase of serum dilution (from 1/16 to 1/44) resulted in a complete consumption of the lytic complement by both A and B trisaccharide containing ICs (Fig. 5. a, b). However, unlike the saturation type curves of the DCL assay, IC complement consumption increased continuously during the 60 minutes incubation. The agreement between the experimentally observed consumption and the predicted lytic values of the model was good (see section next).

A general mathematical model of complement consumption

The main purpose of the extended experimental measurements was to provide a sufficiently large database, from which all possible conditions could be obtained for a *mathematical analysis* of both DCL and CCA assays. A general model of complement consumption was generated by ordinary differential equations. The model was fitted to all of the 4672 measurements of the 23 experiments. The error of fit was compared with the error of repeated measurements between different experiments by selecting measurements with identical parameters (i.e. lytic values that were obtained using identical target, mAb, effector concentrations and incubation time) from the 23 experiments that were performed over a period of 2 years. The relative error of the repeated measurements of the DCL and CCA experiments were 6.5% and 10.2% respectively, whereas that of the model were 5.5% and 8.65% in the DCL and CCA assays respectively.

Dynamics of DCL and CCA processes are modelled by the same equations. The elements of the model are the functions *b*, *e* and *t* respectively, where *b* represents the relative amount of

sensitized target forming complex with the effector (i.e., complement), *e* describes the amount of effector and *t* is the quantity of free target, as functions of time. Then the model is

b' = e(B + b)(A-b),
e' = -tb', (*)
t' = -tb',

where prime denotes differentiation with respect to time. The initial conditions are: *b*(0) = 0, *e*(0) = starting effector concentration; *t*(0) = starting target concentration; *A* = starting mAb concentration, *B* = the initial concentration of the bound target (this is the shape parameter). The model equation system (*) contains one unknown parameter, which is B. There are three other numerical constants in (*): *A*, *e*(0) and *t*(0). In the course of the statistical parameter estimation procedure, an interface was incorporated between the system (*) and the data of the actual measurement. For each set of measurements from the same experiment, a specified collection of scaling factors for mAb, effector and target was used. The reason for this is that the system (*) is valid for quantities measured in arbitrary units. This is a compromise for a standardized system of equations containing only one unknown parameter. Scaling factors of different experiments are therefore different (Table 1.).

The system (*) of equations is called a *bond* model and refers to the binding of sensitised targets with effector. The first equation of (*) gives the dynamics of binding of effector molecules to units of sensitised targets. The speed of the process is proportional with the amount of remaining effector and both with the amount of bound and unbound targets. The second and third equations of (*) give the dynamics of the effector and target consumption. The binding process results in a logarithmic loss of target while the consumed effectors equal that of the consumed targets. The three equations of (*) show that binding alone does not immediately result in consumption of target and effector. The system of equations (*) was used for both DCL of sensitised erythrocytes and CCA of ICs. In CCA, the first consumption phase cannot be directly measured. However, measurement of the target cells that are left intact after the completition of second, lytic phase of the experiment can be connected with the first phase by the equations. The mathematical connection between the first and second phases is ensured by the equation

$$e_{CCA}(D) = e_{DCL}(D),$$

where e (d) and e (d) stand for the amount of effector at time d in phase CCA and DCL respectively, and D is the duration of the CCA phase. This assumes that the effector population remaining after CCA phase is left intact when entering the DCL phase of the experiment. Scaling parameters with shape parameter B are shown in Table 1. In the DCL assays, the system constants d could be grouped into two clusters; Version L and Version R respectively label the shape clusters.

Estimation of the total haemolytic capacity of serum

Using the scaling parameters from two different DCL experiments, the total haemolytic capacity of undiluted serum was extrapolated using the mathematical model; typical results are presented in Fig. 6. a, b, c, d. Predicted lysis of 100 x, 1000 x and 5000 x 10^6 target cells/ml, was similar with A and B erythrocytes (a, c and b, d respectively). A tenfold increase of the erythrocyte concentration (from 100 x to 1000 x 10^6 target cells/ml) prevented 100% lysis. The buffering effect of large erythrocyte concentrations against complement-mediated

lysis is more clearly demonstrated at physiological erythrocyte concentration (5000 x 10^6 target cells/ml); in this case, the predicted lysis was below 40%.

Estimation of the potential complement consumption capacity of ICs

The potential complement consumption capacity of ICs in undiluted serum was extrapolated from the CCA measurements by the mathematical model; typical results are presented in Fig. 7. a, b, c. The effect of increasing the mAb concentration is demonstrated at a constant trisaccharide concentration (10 μg/ml). No consumption was detectable at an mAb concentration of 1 μg/ml. At an mAb concentration of 10 μg/ml, consumption started after a lag phase of between 20-30 minutes and complete inhibition of lysis was achieved by 80 minutes. However, the haemolytic capacity was exhausted within a few minutes at an mAb concentration of 100 μg/ml (Fig. 7. a). The model predicted that there was an inverse relationship between the trisaccharide concentration and the consumption efficiency i.e. below 50 μg/ml trisaccharide concentration, the consumption increased whereas at higher concentration of trisaccharide (between 50 to 100 μg/ml) consumption decreased (Fig. 7. b). Furthermore, the model predicted that a lag phase, which precedes complement consumption, disappears at 1/16 serum dilution (Fig. 7. c).

DISCUSSION

We have measured the dynamics of complement consumption by ICs formed between blood group active immunodominant sugars and anti-blood group mAbs at the erythrocyte surface (DCL) and in the fluid phase (CCA). Since the relevant Ab binding epitopes in the two reactions phases were the same, the results of the DCL and CCA assays are comparable. Both solid and fluid phase reactions, using up to a 100-fold range of reactant concentrations, were analysed by a mathematical model (*bond* model). Initial DCL assay conditions were adjusted such that lysis was not limited by either the starting serum dilution (1/16), target cell number (96×10^6 cells/ml) or by the exogenous sensitising mAb concentration (1 μg/ml), i.e. all target cells were lysed. This suggested that all erythrocytes carried at least one effective membrane attack complex (MAC) of the complement cascade. Somewhat unexpectedly, however, we found that a fourfold further dilution of serum resulted in a strong reduction of lysis. Similarly, a fourfold increase of the starting target cell concentration also resulted in a strong reduction of lysis (see Fig. 2).

By multiplying the erythrocyte count by the number of A or B antigen sites per cell, the total number of available sensitisation sites can be calculated. Thus, using 100×10^6 A erythrocytes/ml and 1 μg/ml mAb concentration (and assuming that K_{aff} is 10^6 M^{-1}) there are 1.77×10^{-10} mol/L mAb-antigen complexes on all the erythrocytes (which is more than a 1000-fold excess per erythrocytes). By calculating an average concentration of the complement components and assuming that their average K_{aff} is 10^4 M^{-1} there are approximately 142 mAb-antigen-complement complexes on each erythrocyte. As one MAC binding to a single IgM mAb should be sufficient for the lysis of an erythrocyte (Mayer M.M. 1961), our finding was unexpected. In spite of the moderate dilution of the serum (up to 1/44) or the four-fold increase of the starting erythrocyte concentration (96×10^6 cells/ml), there was still a calculated excess of MACs over the erythrocytes. The results of CCA measurements were consistent with that of the DCL experiments with regards to a short preincubation of serum with ICs had a strong inhibitory effect in both systems on subsequent complement-mediated lysis (see Fig. 4, 5). The CCA results suggested that the lytic capacity of the human serum could be smaller than one would expect according to the one hit theory of complement mediated lysis (Mayer M.M., 1961).

The discrepancy between the calculated abundance of available mAbs, complement components and limited lytic capacity of serum may be explained by the uneven antigen expression of erythrocytes, which can differ up to 100-fold (Bakács T *et al*, 1993a). Complement distribution has been shown to be very sensitive to the relative concentrations of sensitising mAbs on competing targets. Even a slight difference in mAb concentration causes complement distribution to become amplified, because the complement distribution is proportional to the 5^{th} power of the ratios of competing mAb concentrations (Tusnády G. *et al*, 1992; Bakács T. *et al*, 1994). Thus, when mAbs become limiting as the erythrocyte concentration is increased, the erythrocytes that have the highest antigen expression will be preferentially sensitized. As a result, uneven sensitization would cause a greater asymmetrical complement distribution and consequently complement would accumulate on the erythrocytes with the highest number of antigen sites, whereas the rest of the erythrocytes would not be lysed. This phenomenon becomes more marked as the complement components become limiting factors as the serum is diluted.

Mathematical modelling has been found useful to predict consequences of interactions, which were not predictable by biological experimentation alone (Segel L.A., 1980). To this aim, we

have formulated a general mathematical model of complement consumption. Since the model was successfully fitted to a large experimental database, it can be used to estimate values, which cannot be measured directly; e.g., the lytic capacity of human serum in the presence of high (near to physiological) erythrocyte number, or the concentration of trisaccharides capable of inhibiting complement activity in undiluted serum. Although such predictions may in the future prove to be inaccurate, we believe that the *dynamics* of the process is correctly interpreted by the differential equations. The model suggests that: (*i*) in undiluted serum, at physiological erythrocyte and Ab concentrations, approximately 40% of the sensitized cells are lysed; (*ii*) the lytic capacity of the undiluted serum can be significantly decreased or even exhausted by 10 to 100 μg/ml IC concentrations. Although the experiments were carried out with papainized erythrocytes, which are known to be more sensitive to lysis than untreated erythrocytes, these predictions may have clinical implications.

When group-specific platelets are not available, it has become a common practice to transfuse ABO incompatible platelets. Since a small volume of plasma and erythrocytes may be present in most platelet concentrates, haemolysis due to ABO incompatibility continues to be a problem in transfusion of unmatched platelet concentrates. The potential severity of the administration of ABO-incompatible plasma in platelet concentrates is demonstrated in several case reports; e.g., it has been reported that haemolysis of an estimated 40% of the erythrocyte mass can be tolerated, but in other cases, the haemolytic reaction was fatal (Pierce R.N. *et al*, 1985; Conway L.T. & Scott E.P., 1984).

We showed recently that a_2M carrying A antigens formed complement activating ICs with anti-A mAbs in serum, and inhibited A erythrocyte lysis *in vitro*. Purified a_2M from pooled blood and endogenous a_2M in AB serum had the same effect (Bakács T. *et al*, 2000). Indeed, we speculated that increasing the concentration of a_2M could serve as a scavenger for anti-A Abs. It would prevent an immediate, severe transfusion reaction involving the ABO system by forming IC with anti-A Abs, which upon being opsonized, would be inactivated to iC3b- and C3dg-containing IC and cleared. The selective clearance of these Ab-complexes would steadily lower the total concentration of anti-A in blood. The data presented in this paper suggest that Atri-PAA and Btri-PAA trisaccharides might also serve as scavengers for anti-A and anti-B Abs, thus reducing the harm caused by unintended transfusion of incompatible blood and prevent an immediate, severe transfusion reaction by forming immune complexes with Abs, which upon being opsonized, would be inactivated and cleared. Synthetically produced trisacharides have, however, several advantages over purified a_2M from pooled blood. No side effects of i.v. trisaccharide therapy was observed in the baboons, they are rapidly excreted in the urine and bile (with half-life of <2 hr) and therefore long-term cumulative effects are negligible. Furthermore, because the trisaccharide is of low Mwt, its small structure and hapten-like behaviour does not lead to sensitization (Cooper D.K.C. & et al, 1993). Importantly, unlike with blood products, there is no danger of infection by transmissible agents.

Organ transplantation between distantly related species results in hyper acute rejection (HAR) of the transplant. This violent immune response involves the complement system, which is activated by human anti-pig Abs being directed against galactose alpha 1-3galactose (α Gal) epitopes on pig vascular endothelium. To overcome HAR either recipient Abs or complement are depleted or inhibited. Furthermore, immunological tolerance to pig organs is induced in the recipient by using transgenic pigs that do not express the α-Gal epitope and/or express a human complement inhibiting protein (i.e., DAF) (Brenner P. *et al*, 1999; Lavitrano M., 1999;

Buhler L. *et al*, 1999). The continuous i.v. infusion of synthetic A or B trisaccharides (300-500 mg/hr) in Baboons, beginning immediately pre-transplant and continued post-transplant for several days, prolonged allograft survival and prevented Ab-mediated rejection. It was therefore suggested that specific i.v. carbohydrate therapy should allow organ allografting to be performed across ABO blood group barrier in humans (Cooper D.K.C. & et al, 1993). Indeed, antigen-binding and cytotoxicity of anti-α Gal Abs against pig cells was prevented by synthetic oligosaccharides with terminal Galα1-->3Gal, most effectively as polyacrylamide-bound conjugates, *in vitro* (Rieben R. *et al*, 2000). We believe that our model might also be helpful for *in vitro* evaluation of synthetic oligosaccharides with terminal Galα1-->3Gal to overcome the increasing shortage of human donor organs.

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Table 1. Model parameters.

	DCL*		**CCA**	**DCL**
	L	R		
Shape				
Average	1,15E-01	4,85E+08	1,63E-02	6,79E-01
Min			1,40E-02	1,00E+00
Max			2,37E-02	1,02E-01
Target				
Average	1,36E-04	1,23E-04	4,54E+00	1,28E-04
Min			2,90E+00	1,24E-04
Max			7,68E+00	1,35E-04
Antibody				
Average	2,26E+03	5,14E+02	1,07E-01	1,14E+03
Min			1,52E-01	2,27E+03
Max			1,64E-02	5,17E+02
Effector				
Average	2,51E-01	3,93E-01		2,54E-01
Min	1,34E-01	2,58E-01		1,34E-01
Max	3,81E-01	7,67E-01		3,73E-01

*In the single-phase DCL experiments only two versions (L or R) of shape, target and antibody parameters, but individual effector parameters were used, whereas in each two-phase experiment individual parameters were used.

Figure legend

Fig. 1. a, b, c, d Direct complement-mediated lysis (DCL) of ^{51}Cr-labelled group A (a, b) and B (c, d) erythrocytes by IgM mAbs (BRIC 131, anti-A and BRIC 250, anti-B). Erythrocyte concentration: 192 x 10^6 cells/ml; mAb concentration: 1 μg/ml; AB serum (source of complement) dilutions: 1/16 (?), 1/24 (¦), 1/32 (?) respectively. The extent of lysis is given as the fraction of non-lysed erythrocytes. Continuous lines are predicted lytic curves of the model.

Fig. 2. a, b, c, d Direct complement-mediated lysis (DCL) of ^{51}Cr-labelled group A (a, b) and B (c, d) erythrocytes by anti-A and anti-B IgM mAbs. Erythrocyte concentration (a, c): 96 x 10^6 cells/ml; mAb concentration: 1 μg/ml; AB serum (source of complement) dilutions: 1/16 (?), 1/32 (¦), 1/64 (?) respectively. Erythrocyte concentration (b, d): 96x (?), 192x (¦), 384x (?) 10^6 cells/ml respectively; mAb concentration: 1 μg/ml; AB serum dilution: 1/16. The extent of lysis is given as the fraction of non-lysed erythrocytes. Continuous lines are predicted lytic curves of the model.

Fig. 3 a, b, c Measurement of bound and free IgM mAb concentration after complex formation with trisacharides. (a) Bound anti-A (?) / anti-B IgM (?) mAbs concentrations as a function of the starting IgM concentration by radioimmuno assay as described in Materials and Methods; Atri-PAA / Btri-PAA trisacharide concentrations were 12 μg/ml respectively. Free anti-A (b) and free anti-B IgM (c) mAbs concentrations as a function of the starting IgM concentration; concentrations of both trisacharides: 12 μg/ml; 6 μg/ml; 3 μg/ml; 1.5 μg/ml.

Fig. 4. a, b Measurement of complement consumption by soluble immune complexes formed between Atri-PAA trisacharides and anti-A mAbs in a CCA as a function of incubation time during the first phase of the assay (see in Materials and methods). (a) Concentration of trisacharides: 25 μg/ml (?); 6.25 μg/ml (¦); 1.6 μg/ml (?); mAb: 2.5 μg/ml; AB serum (source of complement) dilution:1/16. (b) Concentration of mAb: 2.5 μg/ml (?); 1.25 μg/ml (¦); 0.625 μg/ml (?); trisacharide: 6.25 μg/ml; AB serum dilution: 1/16. Remaining complement activity was measured by the extent of lysis of ^{51}Cr-labelled, sensitized group B erythrocytes during the second phase of the assay (40 minutes incubation). Erythrocyte concentrations: 96 x 10^6 cells/ml; sensitizing mAb concentrations: 1 μg/ml. The extent of lysis is given as the fraction of lysed erythrocytes. Continuous lines are predicted consumption curves by the model.

Fig. 5. a, b Measurement of complement consumption by soluble immune complexes formed between Atri-PAA trisacharides and anti-A mAbs (a) and Btri-PAA trisacharides and anti-B mAbs as a function of incubation time during the first phase of the assay; concentration of trisacharides: 12.5 μg/ml; mAbs: 2.5 μg/ml; AB serum dilutions: 1/16 (?), 1/32 (?), 1/44(?). Remaining complement activity was measured by the extent of lysis of ^{51}Cr-labelled, sensitized group B (a) and A (b) erythrocytes respectively during the second phase of the assay (40 minutes incubation). Erythrocyte concentrations: 96 x 10^6 cells/ml; sensitizing mAb concentrations: 1 μg/ml. The extent of lysis is given as the fraction of lysed erythrocytes. Continuous lines are predicted consumption curves by the model.

Fig. 6. a, b, c, d Virtual complement-mediated lysis of sensitized group A (a, c) and B (b, d) erythrocytes as predicted by the model using the scaling parameters of two different experiments (exp. No. 557 a, b and exp. No. 560 c, d respectively). Virtual erythrocyte concentration: 100 x 10^6 cells/ml (x); 1000 x 10^6 cells/ml (?); 5000 x 10^6 cells/ml (?); virtual

mAb concentration: 1 μg/ml; virtual AB serum dilutions: 1/1. The extent of lysis is given as the fraction of non-lysed erythrocytes.

Fig. 7. a, b, c Virtual complement consumption by immune complexes as predicted by the model. (a) Virtual mAb concentrations: 1 μg/ml (?); 10 μg/ml (?); 100 μg/ml (¸); virtual trisaccharide concentration: 10 μg/ml; virtual AB serum dilution: 1/1. (b) Virtual trisaccharide concentrations: 10 μg/ml (?); 50 μg/ml (?); 100 μg/ml (¦); virtual mAb concentration: 10 μg/ml; virtual AB serum dilution: 1/1. (c) Virtual AB serum dilutions: 1/1 (¦), 1/4 (?), 1/16 (?); virtual trisaccharide and mAb concentrations 10 μg/ml respectively. The extent of consumption is given as the fraction of lysed erythrocytes.

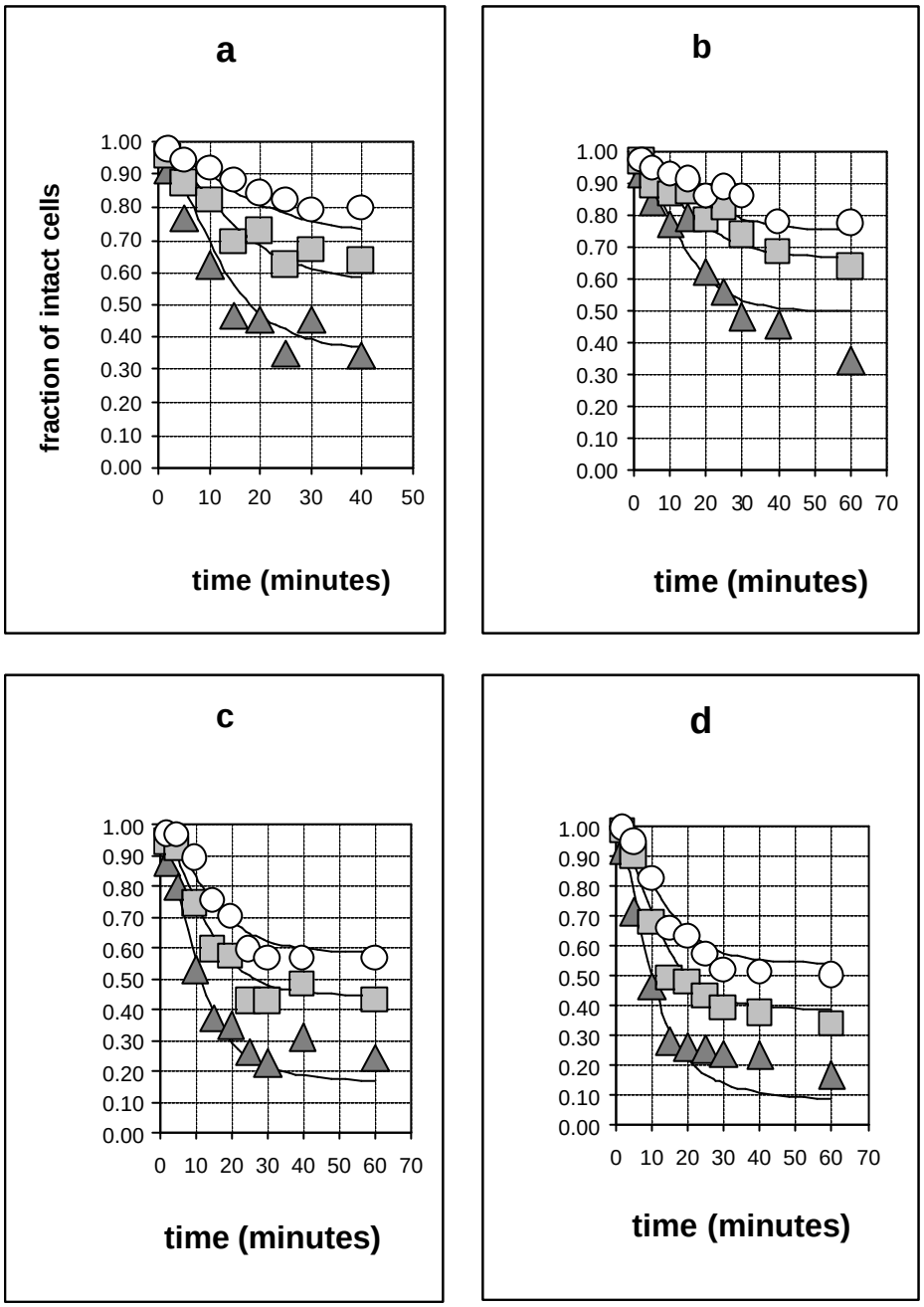
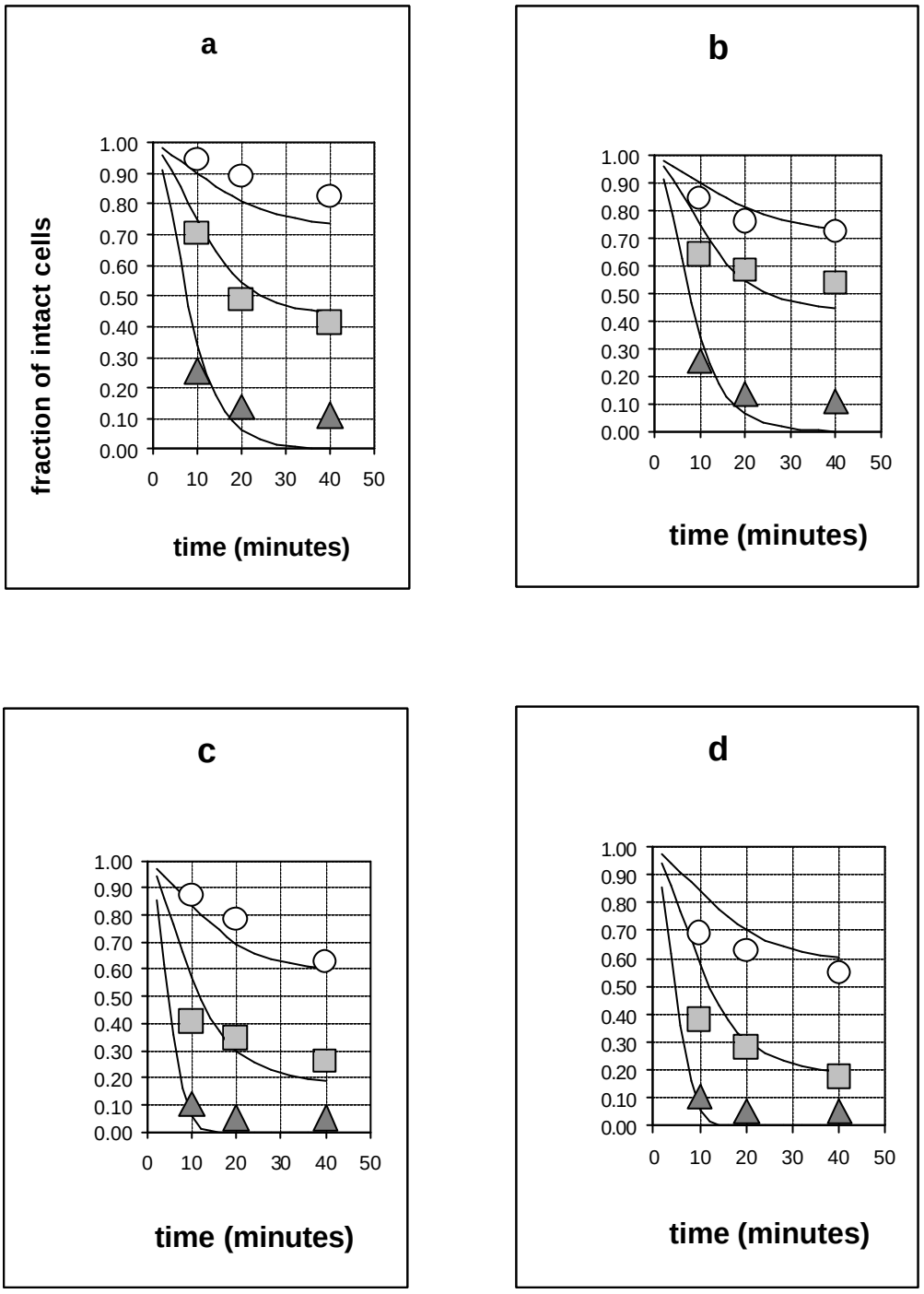

a
b
c
d
fraction of intact cells
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50
0 10 20 30 40 50 60 70
time (minutes)

Figure 1.


a
b
c
d
fraction of intact cells
1.00
0.90
0.80
0.70
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0.50
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0.20
0.10
0.00
0
10
20
30
40
50
time (minutes)

Figure 2.

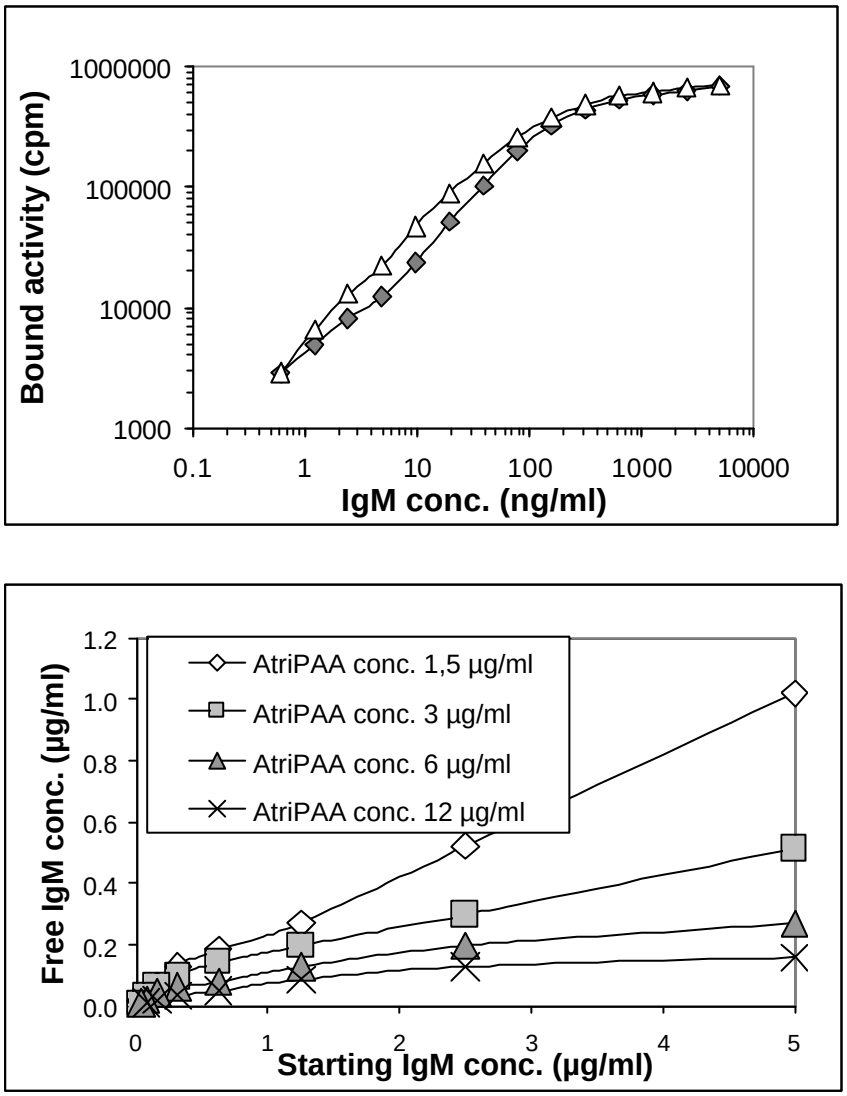

Bound activity (cpm)
1000000
100000
10000
1000
0.1
1
10
100
1000
10000
IgM conc. (ng/ml)
Free IgM conc. (µg/ml)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Starting IgM conc. (µg/ml)
AtriPAA conc. 1,5 µg/ml
AtriPAA conc. 3 µg/ml
AtriPAA conc. 6 µg/ml
AtriPAA conc. 12 µg/ml

Figure 3.

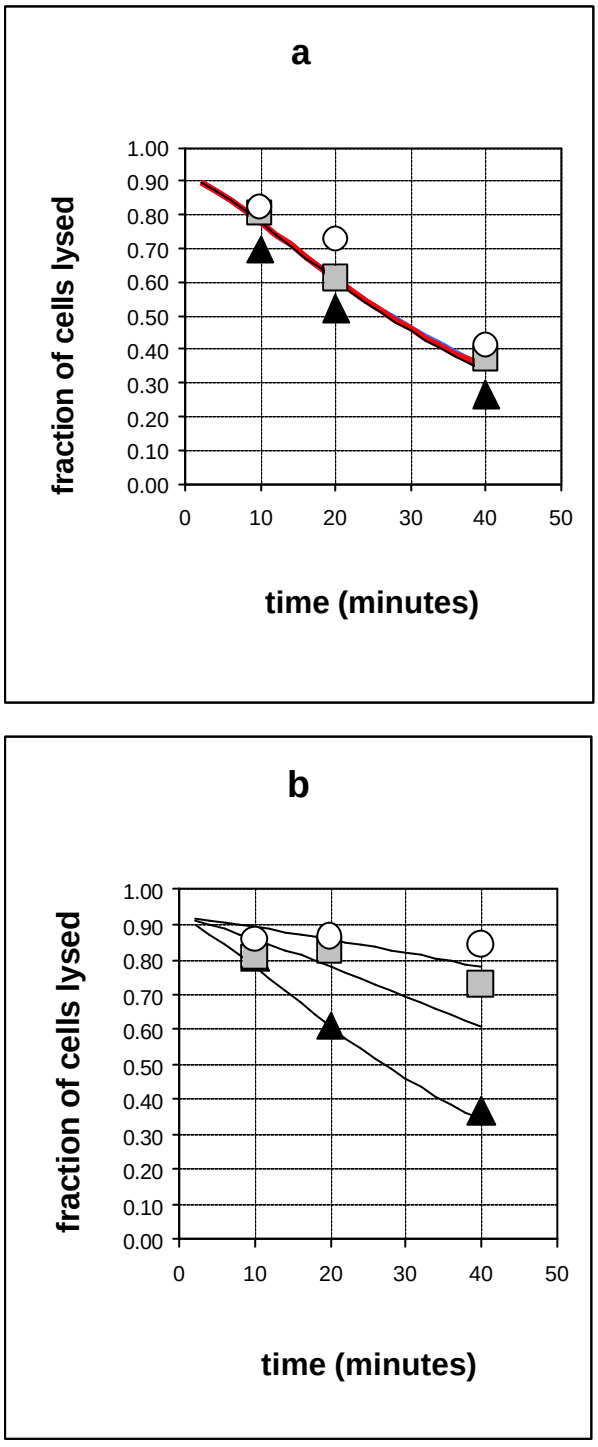
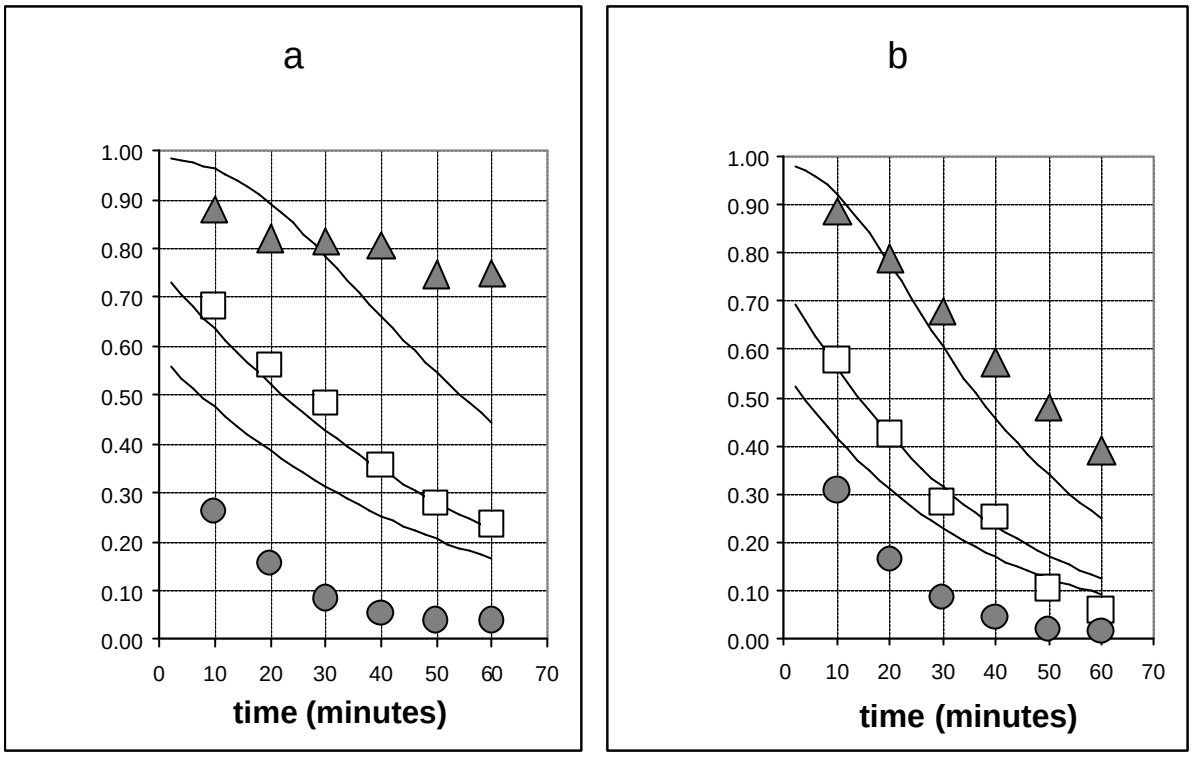

a
fraction of cells lysed
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
time (minutes)
b
fraction of cells lysed
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
time (minutes)

Figure 4.


a
1.00
0.90
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0.40
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0.20
0.10
0.00
0 10 20 30 40 50 60 70
time (minutes)
b
1.00
0.90
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0.10
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0 10 20 30 40 50 60 70
time (minutes)

Figure 5.

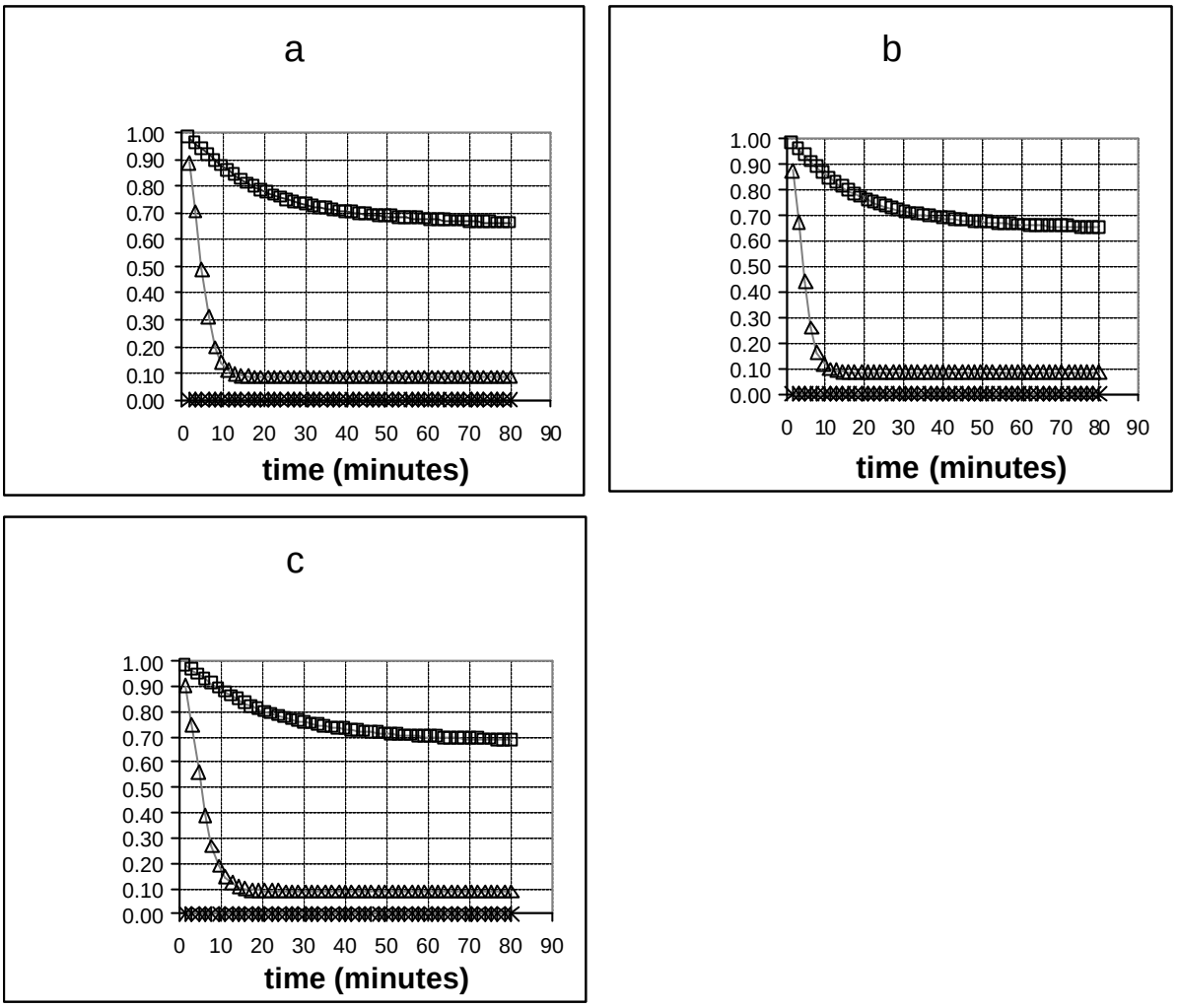
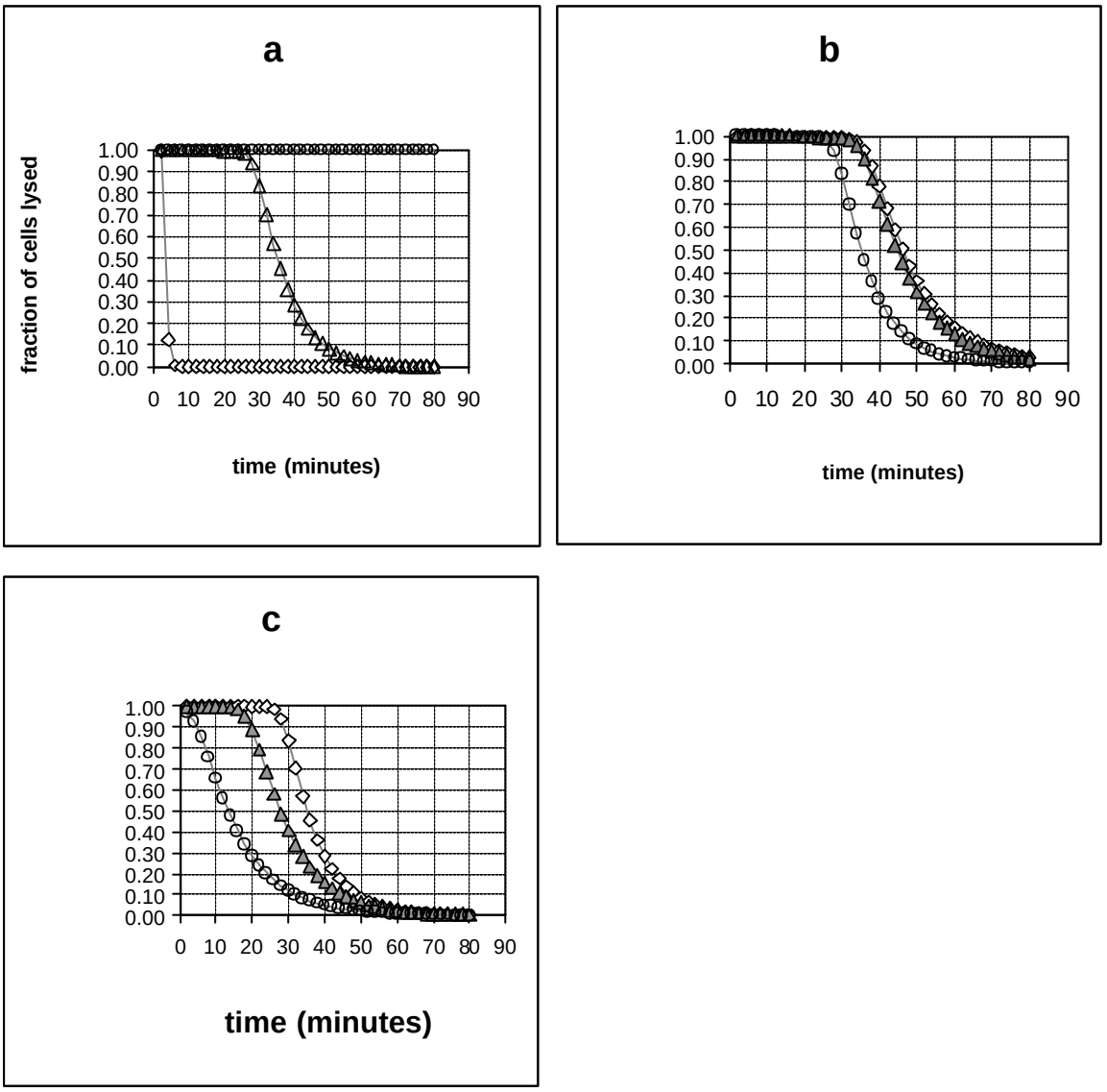

a
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90
time (minutes)
b
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90
time (minutes)
c
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90
time (minutes)

Figure 6.


a
fraction of cells lysed
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90
time (minutes)
b
1.00
0.90
0.80
0.70
0.60
0.50
0.40
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0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90
time (minutes)
c
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0 10 20 30 40 50 60 70 80 90
time (minutes)

Figure 7.