problems such as developmental delay, ovarian dysfunction and speech abnormalities persist. This suggests that the alternative pathway for the formation of UDP-galactose by UDP-glucose-4-epimerase for galactosylation is inadequate in certain cells or in the fetus. The structure of the serum disialo-transferrin and other glycoproteins in untreated and treated galactosaemia patients is under investigation.

The CDGS are a group of primary and secondary genetic and acquired disorders resulting from defects in events in the endoplasmic reticulum or Golgi apparatus. Elucidation of the mechanisms of altered glycosylation in CDGS will provide information on the regulation of glycosylation and will lead to methods for heterozygote detection, prenatal diagnosis and possibly treatment of this major new group of inborn errors of metabolism.

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Mutational analysis of the epitopes recognized by anti-(rat CD2) and anti-(rat CD48) monoclonal antibodies
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Introduction
CD2 was discovered with monoclonal antibodies (mAbs) which precipitate a 50 kDa molecule from T cells and block the formation of rosettes between these cells and sheep red blood cells (reviewed by [1]). CD2 is now one of the best characterized T-cell cell-surface molecules. On the basis of early sequence analyses it was predicted that CD2 consists of two immunoglobulin superfamily (IgSF) domains [2] and this was subsequently confirmed in structural studies of the molecule [3,4]. The rosetting phenomenon implied that CD2 functions as a recognition molecule and it is now clear that in humans and rodents CD2 interacts with CD58 [5,6] and CD48 [7,8] respectively.

The work of Meuer et al. [9] provided the first insights into the cellular events initiated by CD2 recognition. It was shown that a subset of anti-(human CD2) antibodies, now known to bind to domain 1 and referred to as T11-type antibodies, induced the appearance of a 'neo-epitope' on
domain 2, allowing the binding of a third (T113) type of antibody. Induction of the T113 epitope was observed upon T-cell activation, and combinations of T112 and T111, antibodies were shown to activate resting human T cells in an antigen-independent manner [9]. The activation of rat T cells by a pair of anti-(rat CD2) antibodies was shown later [10]. The activation of T cells with certain antibodies to surface antigens is not an uncommon finding [11], but the requirement for simultaneous binding of two antibodies to a single molecule is hereto unique to CD2.

The experiments of Meuer et al. clearly establish that CD2 is coupled to the signal-transduction apparatus of T cells. The precise nature of this coupling has been contentious, however. The induction of the 'neo-epitope' and the transmission of an activation signal were both attributed to a conformational change in human CD2 induced by the antibodies [9] and the notion that CD2 can undergo such changes still persists [12]. Given that the activation phenomenon is conserved for human and rat CD2, a prediction of the 'conformational change' hypothesis is that the locations on the structure of CD2 of the epitopes of antibodies inducing such changes will be similar for both species.

This prediction is tested in this study through a mutational analysis of the OX55 epitope of rat CD2 domain 1 which, in combination with the domain 2-binding OX54 antibody, activates rat T cells [10]. This epitope is compared with those of activating T113-type anti-(human CD2) antibodies in the context of the crystal structures of rat and human CD2 [4,13]. A similar analysis of the epitopes of ligand-blocking anti-(rat CD2) (OX34) and anti-(rat CD48) (OX45 and OX46 [14]) antibodies was also undertaken. The OX45 antibody has previously been shown to block the rat CD2–CD48 interaction [8]. The data indicate that the locations of the epitopes of ligand-blocking antibodies, but not activating antibodies, are conserved.

Materials and methods

Antibodies
The antibodies used in the study were OX34 [15], OX54 [10], OX55 [10], OX45 [14] and OX46 [14].

Protein expression and mutagenesis

For mutagenesis of rat CD2, a truncated form encoding domain 1 (consisting of residues 1–99 of the mature protein [3]) was subcloned into M13 mp18. Single-stranded template was prepared and then mutated by oligonucleotide-based in vitro mutagenesis using a commercial kit (Sculptor, Amersham, Bucks, U.K.). In addition to the mutated codon, the mutating oligonucleotides introduced silent restriction sites to facilitate selection of the mutants. After mutagenesis, the sequence of the mutant gene was confirmed by dideoxy sequencing prior to subcloning into the expression vector pGEX-2T. The construct was then transformed into Escherichia coli strain MC1061 for expression of the mutant domain as a fusion protein with glutathione S-transferase [3]. After induction the expressed protein was purified from the cultures by affinity chromatography and gel-filtration chromatography according to published methods [3].

For mutagenesis of CD48 a chimera consisting of domains 1 and 2 of CD48 fused to domains 3 and 4 of CD4 [16] was subcloned into the XbaI site of the expression vector pEFBos. Single-stranded template was prepared using helper phage and this was then mutated by oligonucleotide-based in vitro mutagenesis with a commercial kit (Muta-Gene v.2, Bio-Rad). The mutant chimera was then expressed transiently in COS-1 cells after DEAE transfection according to the protocol of Seed and Aruffo [17].

Binding experiments

Analysis of the interactions of the mutant forms of rat CD2 and rat CD48 with anti-(rat CD2) and anti-(rat CD48) mAbs respectively, was performed on a BIAcore biosensor [18] (Pharmacia Biotech AB, Uppsala, Sweden). Experiments were performed at 25°C at the indicated buffer flow rates (3–20 μl/min). The buffer used was Hepes-buffered saline (HBS) which contained (in mM): NaCl, 150; MgCl2, 1; CaCl2, 1; 0.005% surfactant P-20 (Pharmacia), and Hepes, 10 (pH 7.4).

For the analysis of the rat CD2 mutants, rabbit anti-(mouse Fc) antibody was immobilized in a CM5 sensor chip via primary amine groups (Pharmacia Biotech AB) and OX34 or OX55 antibody was then injected into the flow cell in the form of tissue culture supernatant for 5 min. After washing unbound antibody out of the flow cell with HBS, the purified mutant protein was injected for 60 s at a concentration of 50 μg/ml. Levels of binding were determined after a final wash of the flow cell with HBS to remove unbound mutant protein.

For the analysis of CD48 mutants, the mutated proteins in the form of supernatants from the transfected COS-1 cultures were immobilized via OX68, an antibody which binds to the CD4 portion of the chimeric protein (as described in [19]). The OX68 antibody was immobilized directly
to the sensor by amine coupling as previously described [19]. After injection of the mutant proteins for 12 min and a brief wash of the flow cell with HBS, OX45 and OX46 antibodies in the form of tissue-culture supernatant were injected for 4 min. Levels of binding were then determined after a final wash of the flow cell to remove unbound antibody.

Results
Mutation of the rat CD2 domain 1 antibody epitopes
Rat CD2 domain 1 can be rapidly expressed at high levels in bacteria as a fusion protein with glutathione S-transferase using the pGEX system [3]. Under these conditions the protein is stably folded and retains its ligand-binding properties [3,8]. The 1H-n.m.r. and crystal structures of rat CD2 allowed a judicious choice of out-pointing residues for mutation. Large changes to side-chain bulk and/or charge were made in order to maximally disrupt antibody binding. Also, it was anticipated that large changes would only be tolerated if they caused minimal local disruption of the structure. Thus, levels of mutant expression similar to that of the unmutated molecule were taken as evidence that the proteins were stably folded, and the fact that all of the highly expressed rat CD2 mutants used in the analysis bound to either OX34, OX55 or both antibodies is consistent with this.

Mutants were generated by in vitro mutagenesis, expressed and tested for binding to both the activating antibody OX55 and the ligand-blocking antibody OX34 on the BIAcore biosensor. This instrument uses the optical phenomenon of surface plasmon resonance to detect binding of macromolecules to ligands immobilized on a dextran matrix within a small flow cell [18]. The mutant proteins were injected into flow cells in which the OX55 or OX34 antibodies had been bound to the dextran matrix via immobilized rabbit anti-(mouse Fc) antibody. Representative binding analyses for wild-type protein and three mutants are shown in Figure 1(a); in these experiments a sustained increase in the baseline response following the injection of a reagent indicates that binding has occurred. Similar levels of OX34 binding were observed for the wild-type protein and the R87E and R70E mutants (mutants are designated by the single-letter code for the unmutated residue followed by the residue number (based on the mature polypeptide sequence) and the single-letter code for the mutant residue) but binding was disrupted by the mutation E41R. In total, 21 mutations were made and four of these were found to disrupt the binding of OX34: E41R (Figure 1a), D28K, E29R and K43E (data not shown). OX55 binding was only disrupted by the mutation R70E. The locations of all of the mutations are shown on the crystal structure of domain 1 of rat CD2 in Figure 2(a). The OX55-disrupting mutation is located in the EF loop at the base of domain 1 adjacent to the linker region between domains 1 and 2, and mutations which affect OX34 binding map to the GFCC'C' face of domain 1.

Mutation of the rat CD48 domain 1 antibody epitopes
An analogous strategy was used for the analysis of the anti-(rat CD48) ligand-blocking antibodies OX45 and OX46. Because a structure for CD48 has not been obtained this was modelled using the rat CD2 domain 1 structure as a template (P. A. van der Merwe, unpublished work). Large changes in residues predicted to be out-pointing in domain 1 of CD48 were then made by in vitro mutagenesis. The mutants were expressed transiently in the form of chimeras with domains 3 and 4 of rat CD4, immobilized in a flow cell using an antibody against the CD4 region of the chimera and then tested for binding to OX45 and OX46 antibodies injected into the flow cell. Twenty-one mutants were expressed at levels comparable with that of unmutated CD48. Representative binding analyses for wild-type protein and three mutants are shown in Figure 1(b). Similar levels of OX45 and OX46 binding were observed for the wild-type protein and the K59E mutant but binding to both antibodies was disrupted by the mutation E91K. The mutation F46D disrupted OX46 but not OX45 binding. Additional mutations that disrupted both antibodies were R31E and H90D (data not shown). The locations of all of the mutations are shown on the model of CD48 domain 1 in Figure 2(b). All of the mutations that disrupt binding to the antibodies map in the region of the putative GFCC'C' face.

Discussion
The principal aim of this study was to map the epitopes recognized by two antibodies that bind to domain 1 of rat CD2 in the context of the three-dimensional structure of rat CD2 [4]. These antibodies have very different functional effects: the first, OX34, blocks the ligand interactions of rat CD2, and the second, OX55, activates rat T cells in combination with a domain-2-binding antibody (OX54).
The epitope of OX34 is disrupted by mutations of domain 1 in the C and C' strands. This suggests that the ligand-binding site of rat CD2 is located on the GFCC'C'' face of domain 1 and this conclusion is consistent with a mutational analysis of rat CD48 binding to rat CD2 (data not shown). The current experiments also show that the binding of the OX45 and OX46 antibodies, which bind to the rat CD2 ligand (CD48) is sensitive to the mutation of residues predicted to form part of the C and C' strands and the FG loop in domain 1 of CD48. These antibodies have been shown to block the rat CD2-CD48 interaction (P. A. van der Merwe, unpublished work) [8]. Several mutational studies have shown that the ligand-binding face of human CD2 is formed by the GFCC'C'' face of domain 1 [20–22]. Thus, taken together, the results suggest that the same binding surface is used by both CD2 and its ligands in all species in which CD2 is expressed. Such a conclusion is consistent with the idea that CD2 and its ligands, CD48 and CD58, evolved by gene duplication from a distant precursor involved in homophilic interactions [23]. Duplicate homophilic interactions mediated by the GFCC'C'' face of domain 1 were seen in both the rat and human sCD2 crystal lattices [4,13].
Figure 2

Summary of the mutational analysis of anti-(rat CD2) and anti-(rat CD48) mAb epitopes

Mutations are designated by the single-letter code for the unmutated residue followed by the residue number (based on the mature polypeptide sequence) and the single-letter code for the mutant residue. The mutations are mapped on to domain 1 of the crystal structure of rat CD2 [4] (a) and on to a model of domain 1 of rat CD48 (P. A. van der Merwe, unpublished work) (b). The sites of mutations which had no effect on antibody binding are indicated by open circles. In (a) the closed circles and triangle represent the sites of mutations which disrupt OX34 and OX55 respectively. In (b) the closed circles represent mutations which disrupt the binding of both OX45 and OX46 whereas the triangle represents the site of a mutation which disrupts the binding of OX46 only. The figure was prepared with the MOLSCRIPT program [28].

Figure 3

Human CD2 epitopes recognized by mAbs T11_2, MT910 and 9.6

These antibodies are representative of the type that have been shown to activate human T cells in combination with T11_2-type antibodies [9,29]. Mutational data were taken from [20,22] and mapped on to domain 1 of the crystal structure of human CD2 [13]. The locations of mutations which disrupt the binding of the antibodies are shown as closed circles. Every tenth residue in the structure is numbered. The figure was prepared with the MOLSCRIPT program [28].
these interactions are unphysiological [24] it seems very likely that these crystal interactions mimic those of the precursor of CD2, CD48 and CD58.

The binding of the activating antibody OX55 is disrupted by a mutation in the middle of the EF loop of domain 1 of rat CD2. Mutations in the C, C', C" and D strands of domain 1 had no effect on OX55 binding. In contrast, previous studies have shown that the binding of activating anti-(human CD2) antibodies are all affected by mutations in the C, C', C" or D strands of domain 1 [20,22] (Figure 3). This result indicates that the activating effects of the anti-(rat CD2) and anti-(human CD2) antibodies are not directly related to the particular epitopes recognized by the antibodies. If conformational changes leading to cellular activation were crucial to the function of CD2 it would be expected that the structural basis of these changes would be conserved and that antibodies inducing these changes would bind to the same structural element in both species. The fact that these antibodies bind to distinct regions is inconsistent with the ‘conformational change’ hypothesis.

IgSF domains form relatively compact structures and the available data suggest that there is little scope for intra-domain flexibility that could give rise to significant conformational change. In antibodies conformational changes have been seen but these are restricted to alterations in loop configuration and to shifts in quaternary organization (reviewed in [25]). It is difficult to envisage a mechanism for the transmission of such changes between the largely independent domains of monomeric IgSF molecules such as CD2.

Other explanations for the effects of anti-CD2 antibodies can be invoked. The ability of pairs of anti-CD2 antibodies to activate T cells may be attributable to highly efficient cross-linking and aggregation of CD2 molecules on the cell surface by these antibodies. CD2 is associated with the T-cell receptor complex [26], and T-cell signalling via CD2 is entirely T-cell-receptor dependent [27]. Suitable pairs of antibodies may aggregate these complexes in a particular orientation required for signalling. The induction of the T11 'neo-epitope' has been attributed to enhanced divalent antibody binding after an increase in CD2 expression upon activation [11] and divalent binding might also be enhanced by the prior binding of T11 antibodies. Equally, these conditions could each alter the association of CD2 with other cell-surface molecules leading to exposure of the T11 epitope, which might otherwise be obscured by inter-molecular interactions. These studies of human and rat CD2 suggest that the structural effects of antibody binding should be interpreted cautiously.

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