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Differential expression of CD32 isoforms following alloactivation of human T cells

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SUMMARY

Receptors for the Fc region of immunoglobulin G (IgG) (FcγRs) exist in three main forms: membrane bound, soluble and cytoplasmic. The function of cytoplasmic FcγRs is poorly understood. We have previously demonstrated cytoplasmic FcγRII (cCD32) within most normal human peripheral blood lymphocytes (PBL), including T cells. In this study we have investigated the hypothesis that following lymphocyte activation, up-regulation of cCD32 occurs, resulting in increased expression at the cell surface. Normal PBL were activated *in vitro* using a two-way mixed lymphocyte reaction (MLR) and expression of CD32 monitored by flow cytometry and by immunoperoxidase staining using specific monoclonal antibodies and aggregated mouse IgG subclasses. Furthermore, we designed oligonucleotide probes specific for the three main isoforms of CD32 and looked for changes in mRNA expression throughout the MLR using an *in situ* hybridization technique. Increased surface expression of CD32 was found on both activated human T and B lymphocytes, but this was found only in the early stages of the MLR, on days 3 and 4, and was virtually absent by day 7. An inverse relationship between cell surface expression of CD32 and mRNA for the IIb isoforms was noted with strong mRNA expression for IIb isoforms occurring in the later stages of the MLR (days 6–7) when interleukin-2R (IL-2R)-positive T cells were predominant. A soluble IgG binding factor (soluble CD32?) was also detected in the MLR culture supernatant. These observations provide support for the hypothesis that synthesis of IIb isoforms of CD32 occurs following alloantigen activation of human T lymphocytes.

INTRODUCTION

Receptors for the Fc region of immunoglobulin G (IgG) provide an essential link between humoral and cellular effector mechanisms within the immune system.[1] Three main classes of human Fcγ receptor (FcγR) have now been described: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16).[2,3]

The most widely distributed of these receptors is CD32 which is found on most haematopoietic cells. CD32 expression is controlled by three distinct genes located on chromosome 1.[4] The A gene transcript is a polymorphic transmembrane protein (FcγRIIa1) and a soluble form of this receptor (s FcγRIIa2) may also be generated by alternative RNA splicing of the hydrophobic transmembrane region.[5] The A gene receptors are found on all types of white blood cell except lymphocytes. The three B gene transcripts (IIb1, IIb2 and IIb3) differ mainly at sites within the cytoplasmic tail region and are restricted to macrophages/monocytes and B lymphocytes. The IIb1 and IIb2 transcripts are identical apart from a 19 amino acid insert within the cytoplasmic tail region of IIb1 which prevents ligand-mediated receptor internalization of this isoform.[6] The IIb3 isoform lacks the information encoded on the S2 exon and may not expressed on cells *in vivo*.[7] The C gene has a cytoplasmic region homologous to A and an extracellular region homologous to B6 and has a cellular distribution similar to the A gene (i.e. it is not found on human lymphocytes).[7]

The B gene isoforms of CD32 found on human B lymphocytes are thought to play a role in the regulation of antibody synthesis.[6,7] Occupancy of FcγRIIb1 by ligand (IgG) in the form of an immune complex, results in receptor cross-linking which in turn delivers an 'off' signal to B cells.[6] In addition, it has been proposed that activated human B cells produce a soluble form of FcγRIIb1 which behaves like a immunoglobulin binding factor (IBF) with the capacity to inhibit antibody synthesis.[6] Binding of ligand to the IIb2 isoform of CD32, in contrast, results in endocytosis of the receptor/ligand moiety; a mechanism which may be important in antigen presentation by B cells.[8]

Human T cells are not thought to express any of the three major human FcγRs.[3] One study has, however, demonstrated low levels of CD32 on the T-cell surface using a very sensitive flow cytometric method and CD32 mRNA has been detected within normal human $CD8^+$ T cells.[9]

We have recently demonstrated a cytoplasmic form of CD32 (cCD32) within the majority of resting human lymphocytes (89%), including T cells.[10] The function of cCD32 is not

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known. In this study we have investigated the hypothesis that cCD32 represents an internal receptor pool which may be mobilized and expressed at the surface of human T cells following activation. Changes in CD32 expression during the time course of a two-way mixed lymphocyte reaction (MLR) were therefore monitored using flow cytometry and immunocytochemical methods. Furthermore, we designed oligonucleotide probes for the three most common isoforms of CD32 and used *in situ* hybridization to look for changes in CD32 mRNA throughout MLRs.

MATERIALS AND METHODS

Isolation of PBMC

Normal human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Lymphoprep, as previously described.[10]

Mixed lymphocyte reaction (MLR)

PBMCs isolated from two normal healthy donors were adjusted to a final concentration of 1×10^6 cells per ml in sterile Iscoves (Gibco BRL, Paisley, UK) culture medium supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin and gentamycin: 100 units/ml). Equal volumes (100 μl) of PBMCs from two donors were mixed and added to wells of a 96-well round-bottomed sterile tissue culture plate (Costar UK Ltd, Bucks, UK) and incubated at 37° in a moist CO_2 incubator (5% CO_2/95% air) up to a maximum of 7 days. Cells were harvested daily from day 3 onwards and washed in culture medium prior to analysis by flow cytometry.

Six different normal donors were selected at random and all 15 permutations of pairs used to produce the MLR response. Three pairs were found to produce a much better response (i.e. increased numbers of large blast cells as assessed by flow cytometry, see below) than the others and these donor combinations were used in all experiments described in this paper. The kinetics of each MLR was however, found to vary slightly with some progressing more rapidly than others. Although we usually performed at least six MLRs for each set of experiments it was in some instances a bit misleading to show average values because of this variation in the MLR response. For this reason we have in some cases elected to show the results of a single representative experiment rather than the average.

Flow cytometry

Cells were adjusted to 5×10^7/ml in phosphate-buffered saline (PBS) and 10 μl aliquots incubated with an equal volume of fluorochrome-conjugated primary antibody (pre-titrated to determine the optimum concentration) for 30 min at 4°. Cells were washed at 200 ***g*** for 5 min in PBS prior to resuspension in 200 μl membrane-filtered PBS. In order to assess cell viability, 5 μl of 5 M propidium iodide was added to each tube immediately prior to acquisition on the fluorescence-activated cell sorter (FACScan; Becton Dickinson). Monoclonal antibodies, secondary antibodies and control immunoglobulins used in this study were as follows: anti-CD32 (AT10) fluorescein isothiocyanate (FITC) conjugated and anti-CD64 (10·1) phycoerythrin (PE) conjugated from Serotec Ltd, Oxford, UK. Anti-CD16 (CLB gran 1) FITC conjugated from Eurogenetics, UK Ltd, Middlesex, UK. Anti-CD3 (UCHT 1), anti-CD19 (HD37) and anti-CD25 (interleukin-2 (IL-2R)-Tac), all PE conjugated and rabbit $F(ab')_2$ anti-mouse immunoglobulin–FITC conjugated from Dako Ltd, High Wycombe, UK.

Monomeric mouse IgG sub-classes (IgG1, IgG2a, IgG2b and IgG3) myeloma proteins were from Sigma Chemical Co., Poole, UK.

Ligand binding

The ox-EA *rosette assay.* Washed ox erythrocytes (E) (Tissue Culture Services, UK) were sensitized with a maximum sub-agglutinating concentration of rabbit anti-ox erythrocyte IgG (A) (Nordic, Tilburg, the Netherlands) and the EA rosette assay conducted as previously described in detail.[10]

Aggregate binding.

Mouse IgG sub-classes (Sigma) were aggregated using glutaraldehyde, as previously described.[10] Aggregates prepared in this manner form a homogeneous band between 250 and 330 kDa when run under reducing conditions on sodium dodceyl sulphate (SDS)–polyacrylamide gels.[10] Binding of aggregates was assessed by flow cytometry using FITC-conjugated rabbit anti-mouse immunoglobulin.

Immunocytochemistry

Cell suspensions at 1×10^6 cells per ml in PBS were centrifuged (Shandon Cytospin 2) onto APES (*a*mino*p*ropyltri*e*thoxy*s*ilane)-treated glass slides for 5 min at 150 ***g***. These cytospin preparations were air dried, fixed in acetone and washed in PBS prior to pre-blocking with normal horse serum (diluted 1:60 in PBS) for 5 min. Cells were incubated with primary monoclonal antibody overnight at room temperature, washed in PBS and subjected to a standard indirect Diaminobenzidine (DAB) immunoperoxidase staining method for the detection of mouse IgG (ABC Vectastain kit; Vector Labs, Peterborough, UK). Sections were counter-stained with haematoxylin and mounted in histomount.

In situ *hybridization*

Probes were based on the published sequences for human CD32 isoforms.[4] All antisense probes were 25 nucleotides in length. The FcγRIIb1-specific probe was designed to bind to sequences specific for the cytoplasmic insert encoded for by the C1 exon at positions 842–866 while probes for the mRNA of the FcγRIIb2 isoform were specific for areas across the splice site at positions 830–840/898–911. The probe for FcγRIIa was designed to bind to nucleotides 15–39 of FcγRIIa mRNA as described.[4]

FcγRIIb1 5′-CCT GCA CTC AGG GTA TCC TGG GAG A-3′
FcγRIIb2 5′-ATT AGT GGG ATT GGC TGA AAT CCG C-3′
FcγRIIa 5′-ACA TTC TGA GAC ATT TGG GTC TCC A-3′

Sequences for these probes were tested against Genebank sequences to ensure no cross-hybridization with CD16 (FcγRIIIa or b), CD32 (FcγRIIa1 or FcγRIIc) and CD64 (FcγRIa1, b1 and b2). The possibility that these probes may cross-hybridize with FcγRIIc was considered. The FcγRIIb1 probe will not bind to IIc because the cytoplasmic insert characteristic of the IIb1 isoform is spliced out of the IIc isoform. The IIb2 probe has a single nucleotide substitution

(A to G at the position shown in bold) compared with the IIc form. It is unlikely that this probe will bind to IIc because this substitution occurs in the middle of the sequence and because of the high stringency hybridization and washing conditions employed in this assay. In the absence of competitive binding assays we cannot however, entirely exclude the possibility that this probe will also hybridize to some extent with IIc. The probe for IIa was checked against Genebank sequences to ensure specificity and was based on specific probe sequence previously reported by Alevy *et al.* (1992).[11]

Probes were synthesized using an Applied Biosystems Oligonucleotide Synthesizer, labelled with digoxigenin and stored at −20° until required.

Hybridization was carried out as described by Farquharson *et al.* (1990).[12] In order to provide a positive control, initial experiments were conducted on cytospin preparations of U937 cells; a cell line known to express CD32. *In situ* hybridization was conducted on cytospin preparations obtained from a two-way MLR, from day 3 onwards, and normal PBMCs from each donor obtained on day 0 were also included to provide a negative control. Following fixation in neutral buffered formalin, cells were washed in RNAse inhibition buffer, permeabilized using proteinase K (Sigma) at 30 μg/ml at 37° for 30 min and incubated with the appropriate CD32 isoform probe at 37° overnight. Cytospin preparations were also treated in parallel with an unrelated probe or with hybridization buffer alone in place of the probe in order to provide background control levels.

Following two washes in 2× standard saline citrate (SSC) buffer at room temperature and two washes in 0·1 × SSC buffer at 42°, cells were incubated with anti-digoxigenin/alkaline phosphatase conjugate (Boehringer Mannheim), diluted 1/500 in 20% normal human serum, for 2 hr and finally incubated overnight in NBT/BCIP (*nitro*blue/*tetrazolium*/*bromochloro*-*indolyl*phosphate) detection buffer. Slides were washed three times in dH_2O and mounted in Dako glycergel.

Slides were inspected on a colour TV monitor, using a Leitz Orthoplan microscope at a magnification of ×630. Using this technique, positive staining for mRNA was indicated by a dark blue/black colour. Large cells of >30 μm in diameter, measured using an eyepiece graticule, with the characteristic morphological appearance of blast cells were counted as as 'activated cells '.

Inhibition of aggregate binding

Cells harvested from a two-way MLR on day 7 were removed by centrifugation and the cell-free supernatant obtained was concentrated 10-fold. Supernatant was stored in small aliquots at −20° until required and repeated freeze–thawing avoided.

For the detection of an IgG binding protein in these MLR supernatants we used normal human PBL as the source of FcγR bearing cells. Since CD32 is found only on about 10% of PBL we decided to use permeabilized PBL as the source of FcγR positive cells. This procedure has previously been shown[10] to expose a cytoplasmic form of CD32 which appears to be present within a high proportion of normal human PBL (70–90%). Using the cells it was therefore possible to design an aggregate binding inhibition assay.

Normal human PBMCs were permeabilized using methanol/triton-X-100, as previously described by Kurki *et al.* (1988).[13] In all experiments, permeabilization was confirmed by the uptake of PI on the FL3 channel of a Becton Dickinson FACScan flow cytometer.

Concentrated MLR supernatant was incubated with an equal volume of aggregated mouse IgG (all four subclasses) or in PBS as a control, at 37° for 1 hr. Aggregates were then added to permeabilized PBMCs at a final concentration of 1 μg/10^6 cells and incubated for 1 hr at 4°. Detection of aggregates within the cell cytoplasm was performed by flow cytometry as described above using FITC-conjugated anti-mouse immunoglobulin. Results were expressed as the percentage inhibition of aggregate binding relative to the aggregate/PBS control.

RESULTS

CD32 expression on MLR-activated lymphocytes using flow cytometry

Dot–plot analysis of MLR activated cells (forward versus side light scatter) showed that the majority of cells in these suspensions remained as normal sized lymphocytes throughout the time course of the MLR (Fig. 1a). These cells (gated as region 1 – R1) had a normal human peripheral blood lymphocyte phenotype which did not alter significantly throughout the time course of the MLR. CD32 was found on ≈10% of cells in R1 and dual immunofluorescence studies showed that CD32 expression was confined to the $CD19^+$ B-cell population.

Large cells (forward scatter >700) appeared in MLR cell suspensions from day 3 onwards and were gated as region 2 (R2). The number of cells in R2 increased steadily throughout the MLR reaching maximum levels by day seven (Fig. 1a). This increase in cell number in R2 was not observed when cells from each donor were incubated alone. Monocytes, were present in R2 on day zero but adhered to the plastic tissue culture plates and were not found in the harvested MLR cell suspensions. Their absence was confirmed by flow cytometry using monoclonal anti-CD14, by electron microscopy and by morphological inspection of cytospin preparations.

It was also evident that cells with the morphological appearance of large immunoblasts were present in MLR cytospin preparations from day 3 onwards. These cells were shown to bind monoclonal anti-CD25 (IL-2R) using the immunoperoxidase method. It was therefore concluded that MLR suspensions contain significant numbers of large 'activated' lymphocytes from day 3 onwards and that these cells are represented by those appearing in R2 of flow cytometry dot–plots of forward versus side scatter, as shown in Fig. 1a.

Expression of CD32 (FcγRII) and CD25 (IL-2R) was measured in parallel throughout the MLR by flow cytometry using fluorochrome conjugated monoclonal antibodies. As shown in Fig. 1b and c, both the proportion and total number of cells in R2 expressing CD25 increased steadily throughout the MLR but CD32 expression was highest on days 3–4. The mean fluorescence intensity (m.f.i.) of staining for CD32 on R2 cells on day 4 (m.f.i. = 140) was considerably greater than the m.f.i. for CD32 on R1 cells (m.f.i. = 18). From day 4 onwards the m.f.i. for CD32 declined rapidly although a small peak of strong staining cells was evident throughout the entire MLR (Fig. 2a). In contrast, the m.f.i. for CD25 increased steadily throughout the MLR (Fig. 2b). Further phenotyping

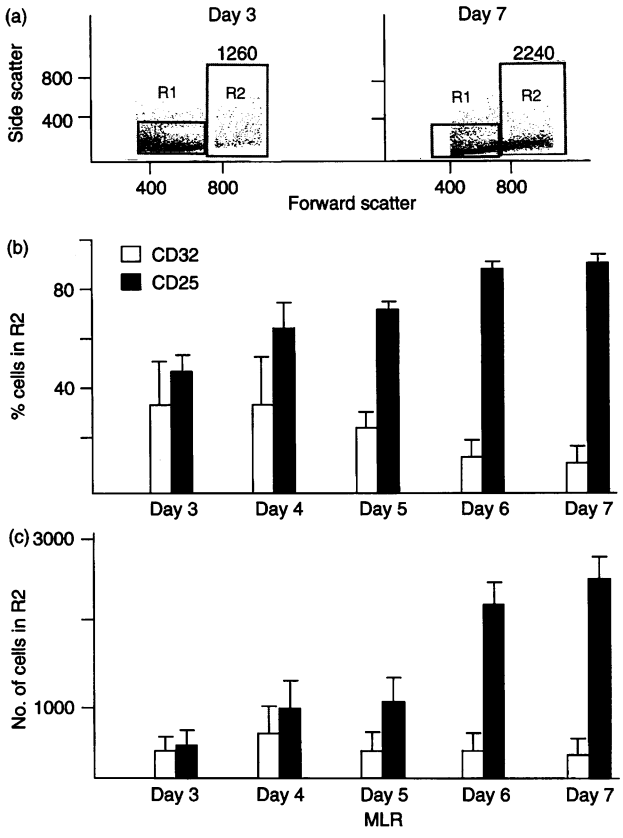

Figure 1. Flow cytometry: sequential analysis of CD32 (FcγRII) and CD25 (IL-2R) on normal human PBL following activation by alloantigens in a two-way MLR. (a) MLR-activated cells harvested cells on days 3 and 7 shown as dot–plots of forward scatter (cell size) versus side scatter (granularity). Region 1 (R1)=normal sized PBL and R2=large 'activated' cells. The number of cells in R2 per 10^4 cells acquired is also shown. Binding of fluorochrome-conjugated monoclonal antibodies (anti-CD32: AT10)* and anti-CD25 (Tac) to cells in R2 were calculated from single histogram profiles using monomeric mouse IgG1 at 20 μg/ml to set background levels of binding. The mean+SEM of four experiments is shown with results expressed (b) as a percentage and (c) as the total number of positive cells. (*Similar results were obtained with monoclonal antibodies CIKm5 and 2E1 which, like AT10, recognize all isoforms of CD32. Monoclonal antibody IV3, which binds preferentially to the FcγRIIa isoform of CD32, was found to produce weak staining on 32% of MLR blast cells (R2) on day 3 but no significant staining was detected from day 4 onwards.)

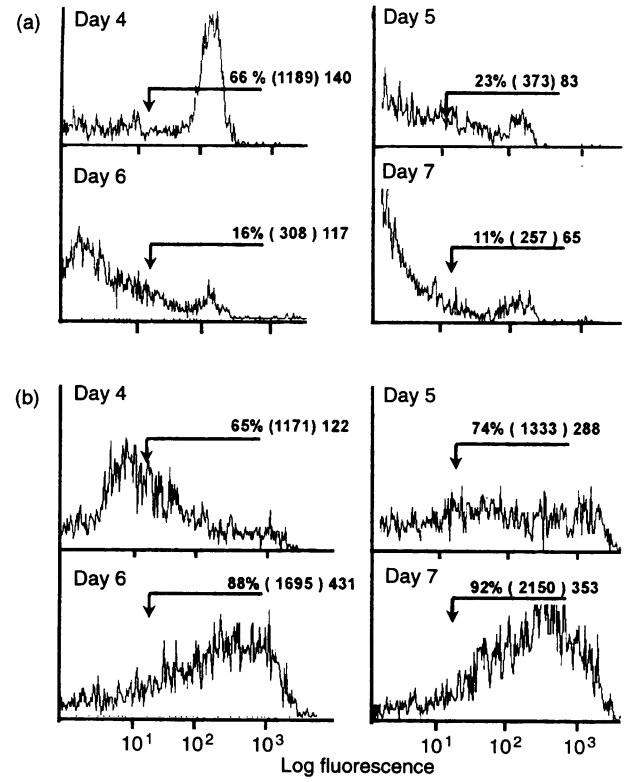

Figure 2. Flow cytometry: sequential analysis of CD32 (FcγRII) and CD25 (IL2 R) on alloactivated human lymphocytes (i.e. cells gated in region 2 (R2) as shown in Fig. 1a). Expression of CD32 on R2 cells. Single histogram profiles showing CD32 expression on R2 cells from day 4 onwards in a mixed lymphocyte reaction. The percentage, total number of positive cells per 10^4 cells acquired and the mean fluorescence intensity (m.f.i.) are shown in the following order: % (number) m.f.i., for each histogram. Background levels established using monomeric mouse IgG1 at 20 μg/ml are indicated by the arrows. (b) Expression of CD25 on R2 cells.

studies confirmed that the majority of cells in R2 were $CD3^+$ T cells (Fig. 3a). The total number of $CD3^+$ T cells in R2 increased steadily throughout the MLR as did the number of cells expressing CD25 (IL-2R). B cells ($CD19^+$) were also detected in R2 but they were present in relatively small numbers and remained constant throughout the MLR (Fig. 3a). Large cells in R2 did not show any of the markers associated with large granular lymphocytes (LGLs) including CD56 and CD16 (FcγRIII).

The total number of $CD32^+$ cells greatly exceeded the number of B cells in the early stages of the MLR (days 3–4) but in the later stages the numbers of $CD32^+$ and $CD19^+$ cells were virtually identical (Fig. 3a).

Up to 25% of large blast cells formed rosettes with IgG-sensitized ox erythrocytes (EA rosettes) in the early stages of the MLR (days 3 and 4) with levels falling to around 10% between days 5–7 (Fig. 3b). Similarly, aggregated mouse IgG1 and IgG2b bound to R2 cells, as described above for EA rosettes (Fig. 3b). Ligand binding corresponded closely to CD32 values and peaked on day 4 of the MLR (Fig. 3b). In these experiments, monomeric mouse IgG (all sub-classes) and aggregated mouse IgG2a and IgG3 did not bind to cells in R2.

Dual immunofluorescence studies, conducted throughout the MLR, showed that CD32 expression on R1 cells was confined to $CD19^+$ B cells with no double labelling observed with $CD3^+$ T cells (Fig. 4). Region 2 cells, in contrast, showed that CD32 was clearly expressed on a sub-set of $CD3^+$ T cells, particularly in the early stages of the MLR (days 3 and 4), and to a lesser extent on $CD19^+$ B cells. Some double labelling for CD32 and CD25 was also evident (Fig. 4). The proportion of double labelled cells (i.e. $CD32^+$ $CD3^+$ and $CD32^+$ $CD25^+$) fell steadily from day 4 onwards and were virtually absent by day 7 (Fig. 4). It was, however, evident in these experiments that staining for CD3 was partially inhibited by pre-incubation of cells with anti-CD32. The vast majority of large MLR blasts in R2 were found to express CD3 (90%) when this monoclonal antibody was added separately. Sequential

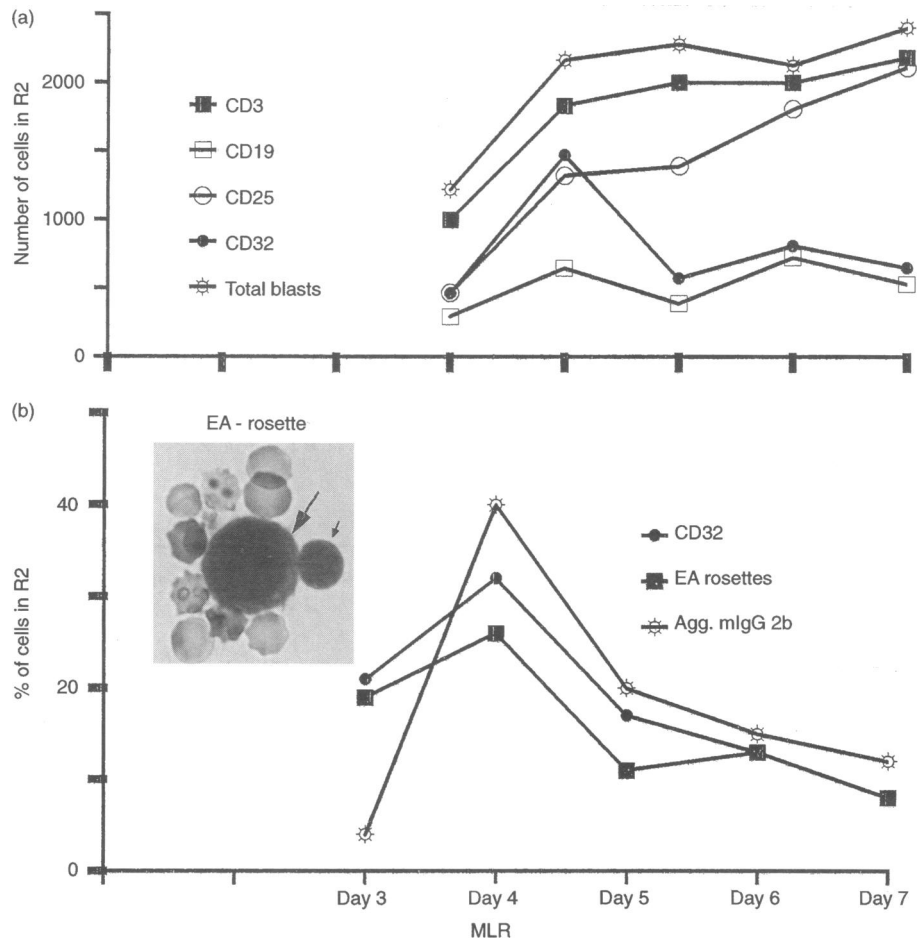

Figure 3. Flow cytometry: phenotype of alloactivated lymphocytes. (a) Cells gated as R2 cells (see Fig. 1) were phenotyped using various fluorochrome conjugated mouse monoclonal antibodies and the total number of positive cells per 10^4 cells acquired were calculated from flow cytometric analysis. For clarity, the results obtained from one typical mixed lymphocyte reaction only are shown. (b) Ligand binding was also measured by flow cytometry using aggregated mouse IgG detected indirectly using FITC-anti mouse immunoglobulin and by rosetting with IgG-sensitized ox erythrocytes (EA rosettes). Results are shown as a percentage (because rosettes are counted this way) and include values for CD32 obtained by flow cytometry in parallel for comparison. An example of rosette formation between ox EAs and a large 'activated' cell (large arrow) is also shown. A normal sized lymphocyte (non-rosetting) is also included in this photograph for comparison (small arrow).

addition and/or pre-titration of the various antibody combinations did not help to solve this problem. This blocking effect was not observed with the CD32/CD19 combination but was evident with the CD25/CD32 combination on days 6–7 of the MLR when staining for CD25 was strongest.

It therefore seems likely that the proportion of $CD32^+$ $CD3^+$ T cells may in fact be an underestimate. It is not known why these antibody combinations should inhibit each other since they are known to recognize quite distinct epitopes. Steric hindrance could account for this effect but only if the CD3 and CD32 molecules are very closely associated on the T-cell membrane.

CD32 expression on MLR-activated lymphocytes using immunocytochemistry and *in situ* hybridization

Cytospin preparations of MLR cells harvested from day 3 onwards showed strong immunoperoxidase staining for CD32 on a high proportion of cells between days 3–5 of the MLR but the number of positive cells fell considerably on days 6–7 (Fig. 5a).

mRNA for the IIa isoform of CD32 was evident on day 3 of the MLR, but declined rapidly thereafter and was absent by day 7. In contrast, mRNA for the IIb1 and IIb2 isoforms increased steadily throughout the MLR reaching maximum levels on day 7 (Fig. 5b).

Detection of an IgG binding factor in MLR culture supernatants

Aggregated mouse IgG1 bound to 90% of normal human peripheral blood lymphocytes following permeabilization of these cells using the methanol/triton-X-100 method (Fig. 6a). This has previously been shown to be due to the presence of low levels of cytoplasmic CD32 within normal lymphocytes.[10]

Pre-incubation of aggregates with day 7 MLR culture supernatant significantly impaired aggregate binding (Fig. 6b) to permeabilized lymphocytes. Identical results were obtained using aggregated mouse IgG2b. Aggregates of mouse IgG2a

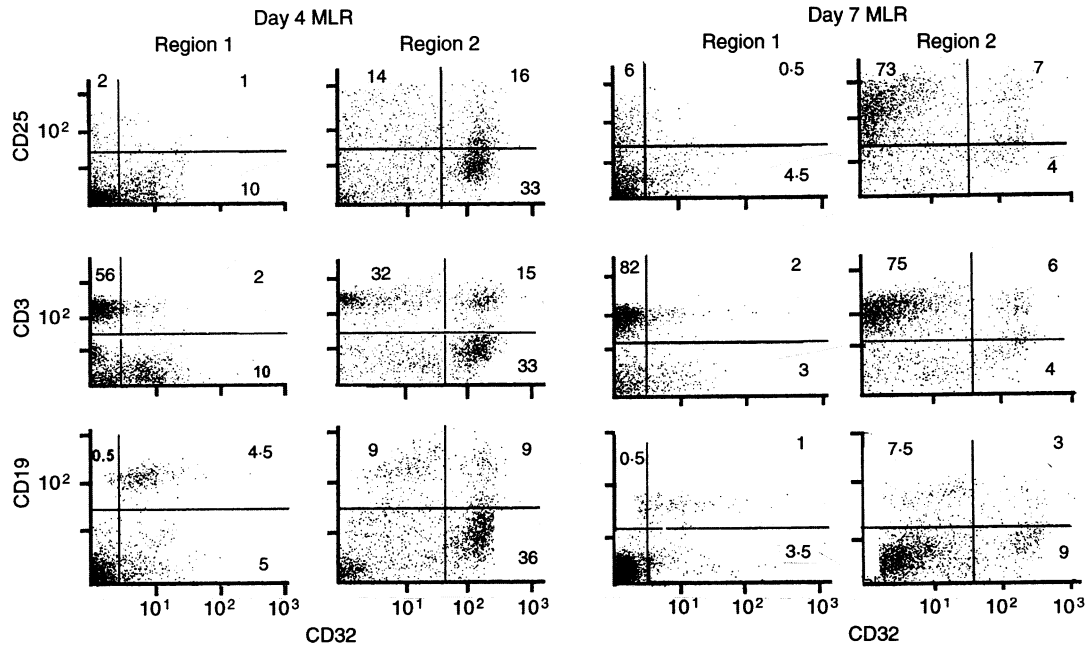

Figure 4. Flow cytometry: dual immunofluorescense. Dot–plot analysis of cells obtained from a typical mixed lymphocyte reaction (MLR) on days 4 and 7 are shown. In all experiments, dual staining using FITC-conjugated anti-CD32 is shown on the x-axis and PE-conjugated mouse monoclonal antibodies specific for CD19 (B cell), CD3 (T cell) or CD25 (IL-2R) shown on the y-axis. Dual staining for both normal sized lymphocytes (region 1) and large 'activated' lymphocytes (region 2) are included for comparison. Background levels of staining, established using monomeric mouse IgG1 at 20 μg/ml, were used to set quadrant gates. Double-labelled cells appear in the upper right quadrant. The percentage of cells in each of the three positive quadrants is shown.

and IgG3 sub-classes did not bind to permeabilized cells and were therefore unsuitable for this assay.

DISCUSSION

Human FcγRs exist in several forms: membrane-bound, soluble and cytoplasmic.[1–5,10] The function of cytoplasmic FcγRs is not clear. Human FcγR III (CD16) has been found in vesicles within the cytoplasm of polymorphonuclear leucocytes[14] and human and murine macrophage FcγRIIb2 localizes within clathrin coated pits prior to endocytosis of the ligand/receptor moiety.[5] It is however, worth noting that FcγR recycling may also occur independently of ligand with up to 20% of the total receptor pool being cytoplasmic at any one time.[15] Since internalization leads to degradation of both ligand and FcγR within lysosomes it has been suggested that an internal pool of newly synthesized FcγRs may be required to replace those lost from the plasma membrane during phagocytosis/endocytosis.[14–16] Cytoplasmic FcγRs may therefore play a role in phagocytosis and/or endocytosis of antigen.

The role of cCD32 within lymphocytes is not known.[10] Resting human B lymphocytes constitutively express both IIb1 and IIb2 isoforms of CD32 at the cell surface with the IIb2 isoform predominant.[6] Following ligand binding, the FcγRIIb2 isoform is endocytosed, thus providing B cells with a means of internalizing antigen in the form of an immune complex. This mechanism may be important in antigen presentation by B cells and in the transmission of negative signals for antibody synthesis.[8] In contrast, the IIb1 isoform, although capable of lateral movement within the plasma membrane, is not internalized and is thought to deliver a negative signal for antibody synthesis to the B cell following immune complex-mediated receptor cross-linking at the cell surface.[6,8] Furthermore, the IIb1 isoform appears to be up-regulated following B-cell activation, at the expense of IIb2, and may be released into the fluid phase as a soluble IBF with the capacity to inhibit antibody synthesis in an indirect manner.[6] Since the IIb1 isoform is not internalized it would seem likely that cCD32 represents a recycling IIb2 isoform within human B cells.

Detection of low levels of cCD32 within resting human T cells was unexpected because these cells are not thought to express cell surface FcγRs.[1–3] A few studies have however, shown that resting human T cells may indeed express low levels of CD32 or CD16 at the cell surface.[9,17] The concept of a cytoplasmic FcγR within T cells may therefore be valid. The possible function of cCD32 within human T cells is however, not known.

Since human FcγR isoforms appear to be up- or down-regulated following B-cell activation[6] and can be influenced by cytokines[1–3] we have investigated the possibility that activation of human T cells may result in up-regulation of cCD32 leading to receptor expression at the cell surface.

Flow cytometric studies showed that following *in vitro* activation of human lymphocytes, in a two-way MLR, a high proportion of activated lymphocytes were found to show very strong staining for CD32; however, this was evident only in the early stages of the MLR with levels falling from day 5 onwards. The kinetics of CD25 (IL-2R) expression was found to differ markedly from CD32 with a steady increase in IL-2R expression throughout the MLR, reaching maximum levels by day 7. Ligand binding was also found to peak on day 4 of the MLR, paralleling values observed for CD32. Increased CD32

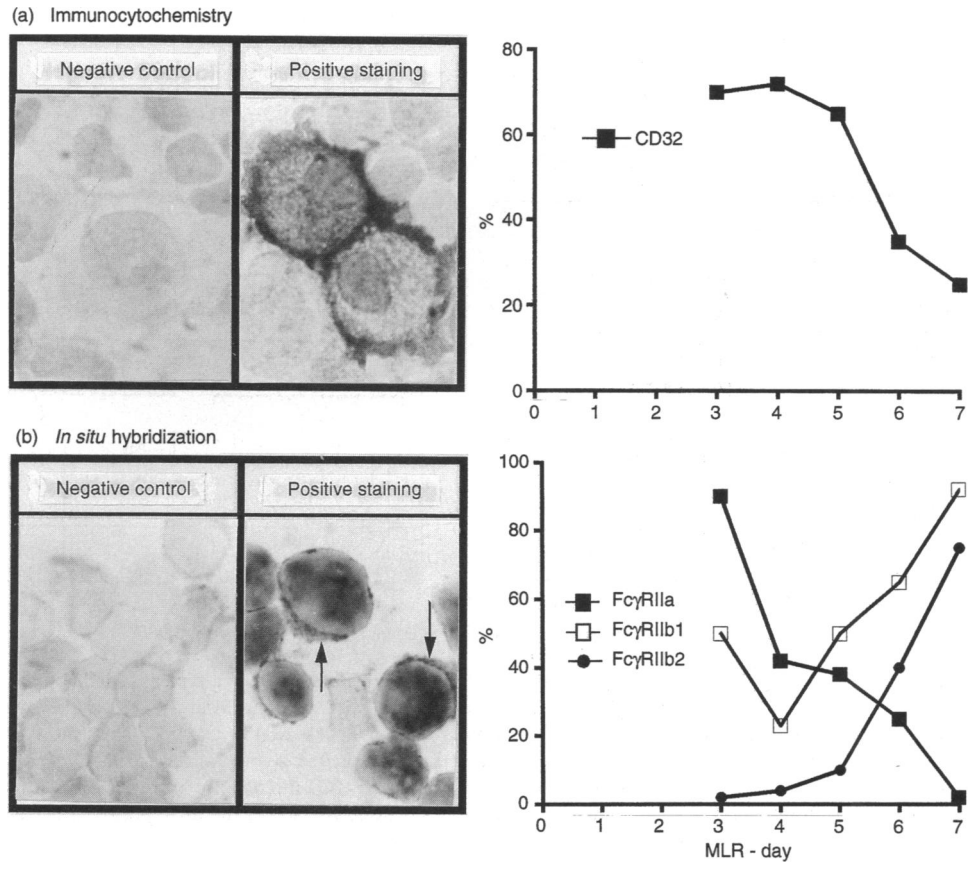

Figure 5. Demonstration of CD32 using cytospin preparations of human lymphocytes activated *in vitro* using a two-way mixed lymphocyte reaction (MLR). (a) MLR-activated cells were shown to stain for CD32 using a standard immunoperoxidase method. No staining was observed in control preparations where the primary mouse monoclonal anti-CD32 antibody (AT10) was replaced by monomeric mouse IgG1 at the same concentration. The percentage of $CD32^+$ immunoblasts, measured over the time course of a seven day mixed lymphocyte reaction (MLR) is shown. (b) *In situ* hybridization was used to demonstrate mRNA for three isoforms of CD32 (IIa, IIb1 and IIb2) in parallel with the immunocytochemistry as described in (a) above. Results were expressed as the percentage of blastoid cells showing positive (blue/black) staining within the cytoplasm, as indicated by the arrows.

expression on day 4 of the MLR could not be explained by an increase in B cell numbers since the absolute number of CD32 positive blasts exceeded the total number of $CD19^+$ blast cells. Furthermore, dual immunofluorescence studies clearly demonstrated a sub-population of $CD3^+$ $CD32^+$ and $CD32^+$ $CD19^+$ blast cells on day 4 of the MLR (i.e. activated T and B cells both express CD32 at the cell surface).

In good agreement with the flow cytometry results, strong immunohistochemical staining for CD32 was demonstrated on large MLR blasts with the proportion of positive cells falling from day 5 onwards.

In situ hybridization studies showed an inverse relationship between immunostaining for CD32 protein and expression of mRNA for the IIb1 and IIb2 isoforms of CD32 (see Fig. 5). For example, by day 7 of the MLR virtually no CD32 was detected at the cell surface but strong mRNA expression for the IIb isoforms of CD32 was evident. This observation suggests that by day 7 of the MLR the IIb isoforms of CD32 are being synthesized by activated cells and are not expressed at the cell surface. The detection of an IgG binding factor in the MLR culture supernatant (see Fig. 6) lends some support to this hypothesis.

It is not known which isoform of CD32 is expressed at the cell surface on days 3–4 of the MLR but mRNA for the IIa isoform follows most closely to the pattern observed for CD32 protein expression at the cell surface (i.e. ligand binding assays) (see Fig. 4b) and is in good agreement with flow cytometry results for CD32 expression using anti-CD32 monoclonal IV3 which is thought to bind preferentially to the FcγRIIa isoform. Detection of mRNA for the IIa isoform of CD32 within activated cells was unexpected because this isoform is not usually associated with lymphocytes.[1–3] Expression of mRNA for the IIa isoform is, however, transient and may be suppressed within activated cells as mRNA for IIb isoform expression takes precedence.

In conclusion, we have shown that CD32 can be detected on the surface of allo-activated human B and T cells, but only at certain times within the activation cycle.

We propose that following lymphocyte activation differential regulation of mRNA for CD32 occurs resulting in release of certain isoforms into the fluid phase. These findings are in good agreement with the hypothesis proposed by Gergeley *et al.* (1993)[6] that soluble FcγRIIb1 may be a human IBF derived from activated B lymphocytes. Our findings also suggest that soluble FcγRII isoforms may also be synthesized by activated T cells. This hypothesis would therefore be in

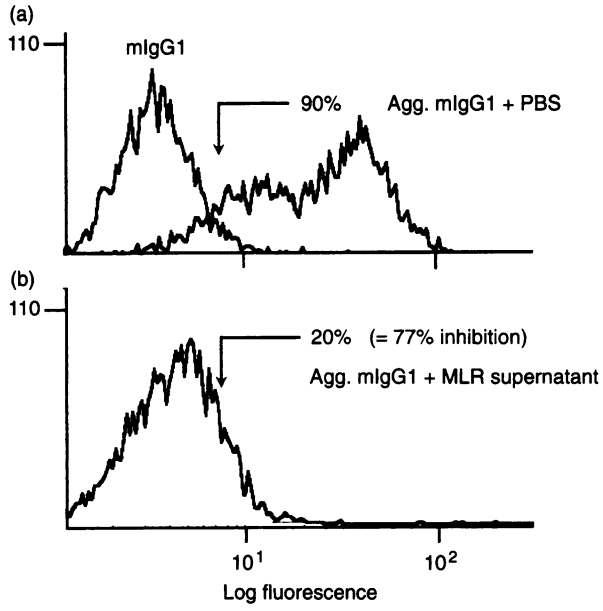

Figure 6. Demonstration of an IgG binding factor in the mixed lymphocyte reaction (MLR) culture supernatant. (a) Control values for binding of monomeric and aggregated mouse IgG1(Agg. mIgG1) to permeabilized normal human peripheral blood lymphocytes (P-PBL) were established by flow cytometry and shown as a histogram overlay. The percentage of cells binding Agg.mIgG1 relative to background (arrow) is shown. (b) As above except that Agg. mIgG1 was pre-incubated with day 7 MLR culture supernatant prior to addition to P-PBL.

good agreement with the extensive studies on the synthesis of soluble immunoglobulin binding factors by alloantigen-activated T cells in the mouse.[5] Since T cells greatly outnumber B cells we suggest that a potentially important source of human IBF is the activated T cell. This possibility is currently being investigated in our laboratory.

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REFERENCES

1. Ravetch J.V. & Kinet, J.-P. (1991) Fc Receptors. *Ann Rev Immunol* **9,** 457.
2. Van de Winkel J.G.J. & Anderson C.L. (1991) Biology of human immunoglobulin G Fc receptors. *J Leukocyte Biol* **49,** 511.
3. Van de Winkel J.G.J. & Capel P.J.A. (1993) Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunol Today* **14,** 215.
4. Brooks D.G. Qui W.Q., Luster A.D. & Ravetch J.V. (1989) Structure and expression of a human IgG FcRII (CD32): functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *J Exp Med* **170,** 1369.
5. Fridman W.H., Bonnerot C., Daeron M., Amigorena S., Teillaud J.L. & Sautes C. (1992) Structural bases of FcγR functions. *Immunol Rev* **125,** 49.
6. Gergely, J., Rozsnyay Z. & Sarmay G. (1994) New aspects of FcγRII-mediated B cell activation. Switch of FcγRIIb isoforms depends on B cell activation. *The Immunologist* **2/3,** 77.
7. Capel P.J.A., van de Winkel J.G.J., van den Herik-Oudijk I.E. & Verbeek, J.S. (1994) Heterogeneity of human IgG Fc receptors. *Immunomethods* **4,** 25.
8. Fridman W.H. (1993) Regulation of B cell activation and antigen presentation by Fc receptors. *Curr Opin Immunol* **5,** 355.
9. Mantzioris B.X., Berger M.F., Sewell W. & Zola H. (1993) Expression of the Fc receptor for IgG (FcγRII/Cdw32) by human circulating T and B lymphocytes. *J Immunol* **150,** 5175.
10. Sandilands G.P., McLaren A.P., Howie D. & MacSween R.N.M. (1995) Occult expression of CD32 (FcγRII) in normal human peripheral blood mononuclear cells. *Immunology* **86,** 525.
11. Alevy Y.G., Tucker J. & Mohanakumar T. (1992) CD32 A (FcγRIIa) mRNA expression and regulation in blood monocytes and cell lines. *Mol Immunol* **29,** 1289.
12. Farquharson M., Harvie R. & McNicol A.M. (1990) Detection of mRNA using a digoxigenin end labelled oligonucleotide probe. *J Clin Pathol* **43,** 424.
13. Kurki P., Ogata K. & Tan E.M. (1988) Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. *J Immunol Methods* **109,** 49.
14. Jost C.R., de Goede R., Fransen J.A., Daha M.R. & Ginsel, L.A. (1991) On the origin of the FcγRIII (CD16) – containing vesicle population in human neutrophil granulocytes. *Eur J Cell Biol* **54,** 313.
15. Mellman I., Plutner H. & Ukkonen P. (1984) Internalization and rapid recycling of macrophage Fc receptors tagged with monovalent antireceptor antibody: possible role of a prelysosomal compartment. *J Cell Biol* **8,** 1163.
16. Green S.A., Plutner H. & Mellman I. (1985) Biosynthesis and intracellular transport of the mouse macrophage Fc receptor. *J Biol Chem* **260,** 98.
17. Lanier LL, Kipps T.J. & Phillips J.H. (1985) Functional properties of a unique sub-set of CD3+ T lymphocytes that express Fc receptors for IgG (CD16/leu 11a). *J Exp Med* **162,** 2089.