

serum showed more than 70% cell death in cytopins (Figure 1C), consistent with the results obtained by FACS.⁴

We conclude that flow cytometry results with anti-HLA-DR or type II anti-CD20 antibodies are largely due to cell aggregation. Large aggregates containing 30 to 100 cells are probably mostly excluded from the analysis, falling outside the gate of scatter plots. Dead cells are in contrast enriched within the single-cell population because aggregation is an energy-dependent process. An apparent rapid loss of cells due to aggregation was confirmed in flow cytometry by counting the absolute number of events using calibrated beads. Indeed, 87% and 70% apparent decrease in total cell number was observed at 24 hours in anti-HLA-DR- and B1-treated samples, respectively (Figure 1D).

Altogether, these observations suggest that FACS analysis should be interpreted with caution in the functional study of antibodies that cause strong homotypic adhesion, unless means are found to disaggregate cells without causing cell death. They also suggest that anti-HLA-DR and anti-CD20 B1 antibody may induce much less direct cell death than previously suggested and the reason for their efficacy in vivo may reside in other properties, such as homotypic adhesion itself inducing opsonization or immune mediated mechanisms, as recently suggested for type II anti-CD20 mAbs.⁵

Josée Golay

Laboratory of Cell Therapy "G. Lanzani,"
Division of Hematology, Ospedali Riuniti,
Bergamo, Italy

Luca Bologna

Laboratory of Cell Therapy "G. Lanzani,"
Division of Hematology, Ospedali Riuniti,
Bergamo, Italy

Pierre-Alain André

Service of Nuclear Medicine,
University Hospital of Lausanne,
Lausanne, Switzerland

Franz Buchegger

Service of Nuclear Medicine,
University Hospital of Lausanne,
Lausanne, Switzerland

Jean Pierre Mach

Department of Biochemistry,
University of Lausanne,
Epalinges, Switzerland

Laurence Boumsell

Institut Cochin, Inserm U1016, Paris, France and
MAT Biopharma, Evry, France

Martino Introna

Laboratory of Cell Therapy "G. Lanzani,"
Division of Hematology, Ospedali Riuniti,
Bergamo, Italy

Acknowledgments: The authors thank MAT Biopharma for BK267 purification.

This work was supported by Associazione Italiana per la Ricerca sul Cancro and European Economic Community (Specific Targeted Research Project, Bispecific Monoclonal Antibody Technology Concept project no. 518185).

Conflict-of-interest disclosure: L. Boumsell was a consultant for MAT Biopharma under French law article 25.2. The remaining authors declare no competing financial interests.

Correspondence: Josée T. Golay, c/o Presidio Matteo Rota, Laboratory Lanzani, Ospedali Riuniti, via Garibaldi 11-13, Bergamo, Italy 24128; e-mail: jgolay@ospedaliuniti.bergamo.it.

References

- Ivanov A, Beers SA, Walshe CA, et al. Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. *J Clin Invest*. 2009;119(8):2143-2159.
- Mössner E, Brünker P, Moser S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood*. 2010;115(22):4393-4402.
- Alduaij W, Potluri S, Ivanov A, et al. New generation anti-CD20 antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell lymphoma [abstract]. *Blood (ASH Annual Meeting Abstracts)*. 2009;114(22):302a. Abstract 725.
- Golay J, Manganini M, Rambaldi A, Introna M. Effect of alemtuzumab on neoplastic B cells. *Haematologica*. 2004;89(12):1476-1483.
- Beers SA, French RR, Chan HT, et al. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. *Blood*. 2010;115(25):5191-5201.

Response

Novel lysosomal-dependent cell death following homotypic adhesion occurs within cell aggregates

Golay et al raise several important points regarding the possible pitfalls in assessing cell death by flow cytometry. In particular, they refer to experiments on anti-CD20 and HLA-DR monoclonal antibodies (mAbs) reported by our groups in the past year.^{1,2} We fully concur that more than one technique should be performed to validate any cell death phenomenon and that flow cytometry alone is insufficient. For exactly these reasons we performed real-time and snapshot light microscopic analysis, electron microscopic, and growth inhibition assays to demonstrate by 4 different techniques that type II anti-CD20 and HLA-DR mAbs elicit a novel form of lysosomal-dependent death related to homotypic adhesion (HA) and that type I anti-CD20 mAbs do not. The central argument presented by Golay et al is that cells undergoing HA become sensitive to pipetting and flow cytometric analysis, although it is unclear as to why this should be the case. Nevertheless, we reported in situ experiments with SYTOX green (Invitrogen) that excluded flow cytometry and pipetting. Importantly in these experiments, where there was no mechanical disruption, HA was accompanied

by increased SYTOX green positivity, indicative of cell death (Figure 1A and Ivanov et al¹). We also reported a clear and substantial decrease in viable cell proliferation in the presence of tositumomab or HLA-DR mAb, using XTT assays (Figure 1B and Ivanov et al¹). This assay again excludes the mechanical issues of pipetting and flow cytometry detailed by Golay et al. Both the XTT reagent used by us and the alamarBlue used by Golay et al operate through a common mechanism (reduction by metabolically active cells to give a color change). Therefore, the differences observed between our groups are hard to reconcile, but may potentially be explained by differences in cell density or dye incubation time used. Furthermore, we have previously confirmed that the loss of cell viability observed translated into a loss of clonogenic survival.³

Although HA correlates with cell death, the relationship is not direct for all mAbs. For example, the pan-HLA mAb A9-1 caused high levels of aggregation yet elicited relatively low levels of cell death compared with anti-HLA DR and type II anti-CD20 mAbs.¹ This lack of a direct relationship implies that mechanisms other

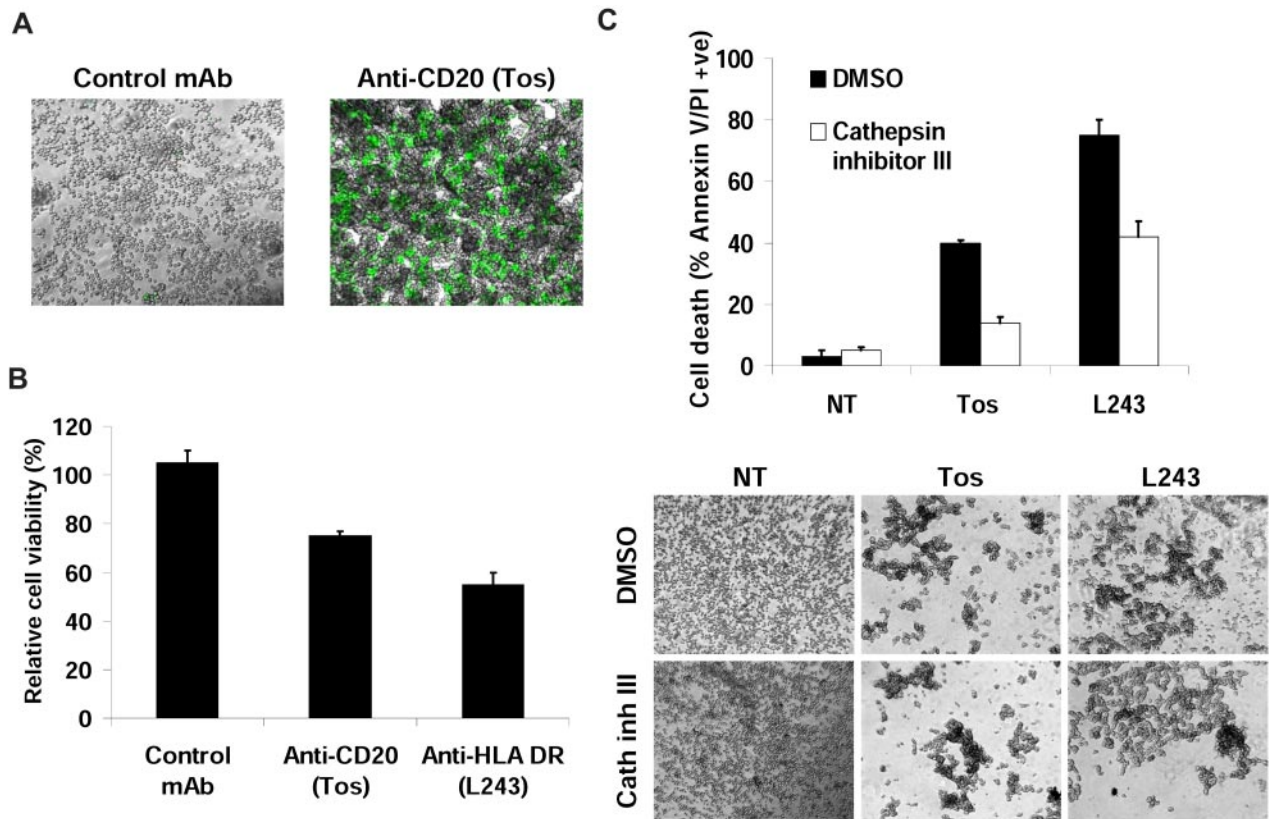


Figure 1. Monoclonal antibody (mAb)-induced cell death detected by flow cytometry is not an artefact of cell aggregation. Alternative assays used to detect mAb-induced cell death. (A) RAJI cells were treated with mAb (10 μ g/mL) for 4 hours and labeled in situ with SYTOX green. Cell death was then analyzed by fluorescence microscopy (original magnification: $\times 4$), representative of 2 independent experiments. (B) Cells were treated with mAb (10 μ g/mL) for 24 hours and cell viability determined by the colorimetric XTT assay. Cells were incubated for 4 hours at 37°C with XTT reagent, and absorbance was normalized to untreated cells. Mean \pm SEM of 3 independent experiments are shown. (C) Cells were pretreated with Cathepsin inhibitor III (100 μ M) for 30 minutes before treatment with mAb and after 4 hours cell death and cell aggregation were analyzed by flow cytometry (top panel) and microscopy (bottom panel), respectively (original magnification: $\times 4$). Mean \pm SEM of 3 independent experiments are shown. Cathepsin inhibitor III potently attenuated cell death induced by both tositumomab (anti-CD20) and L243 (anti-HLA DR) but had no effect on cell aggregation. NT indicates nontreated; Tos, Tositumomab/B1; and control mAb, anti-CD3 (OKT3).

than the simple mechanical explanation proposed link HA and cell death.

Finally, we have demonstrated that mAb-induced cell death is dependent on lysosomal permeabilization and release of cathepsins, using a specific cathepsin inhibitor that potently attenuated cell death measured by flow cytometry but had no effect on HA (Figure 1C). Therefore, the hypothesis that cell death detected by flow cytometry is an artefact of HA cannot explain the prominent inhibition of cell death by the cathepsin inhibitor.

In conclusion, the novel mechanism of antibody-induced death that we have described is definitively not an artefact of pipetting and flow cytometric analysis, but represents a distinctive form of cell death. All cell death phenomena should be validated by different techniques, and if possible, by agents that can modulate cell death without affecting HA, as we have previously demonstrated.¹

Mark S. Cragg

Tenovus Laboratory, Cancer Sciences Division,
University of Southampton School of Medicine,
Southampton, United Kingdom

Waleed Alduaij

CRUK Paterson Institute of Cancer Research,
University of Manchester,
Manchester, United Kingdom

Christian Klein

Roche Glycart AG,
Schlieren, Switzerland

Pablo Umana

Roche Glycart AG,
Schlieren, Switzerland

Martin J. Glennie

Tenovus Laboratory, Cancer Sciences Division,
University of Southampton School of Medicine,
Southampton, United Kingdom

Tim M. Illidge

CRUK Paterson Institute of Cancer Research,
University of Manchester,
Manchester, United Kingdom

References

- Ivanov A, Beers SA, Walshe CA, et al. Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. *J Clin Invest*. 2009;119(8):2143-2159.
- Mössner E, Brünker P, Moser S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood*. 2010;115(22):4393-4402.
- Chan HT, Hughes D, French RR, et al. CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. *Cancer Res*. 2003;63(17):5480-5489.