

germinal centres in *bcl-2* mice, was moderately less than in controls (Fig. 4b). This indicates that *bcl-2* confers only incomplete protection from apoptosis caused by soluble antigen. Any difference between this result and those of Shokat *et al.*³¹ may be attributable to differences in the affinity of binding between NP-specific and HEL-specific cells.

Finally, to determine whether the observed cell death was unique to germinal centre cells, mice were injected with soluble NP₁-HSA eight days after immunization with NP-HSA and analysed as above. At this time point in this immune response, foci of extrafollicular cells forming anti-NP antibody are at maximal size³. The sections were double-stained to detect any TUNEL-positive cells in IgG1 foci (K.G.C.S., T. D. Hewitson, G.J.V.N. and D. M. Tarlinton, manuscript in preparation). A large number of cells in IgG1 foci were counted, and the number of apoptotic cells constituted only 2–3% of all cells in the foci of both the immune and tolerant spleens. Therefore, the enhanced apoptosis caused by the soluble antigen appears to be specific to germinal centres.

In conclusion, we have shown that soluble antigen causes the apoptosis of antigen-specific germinal centre B cells. This apoptosis exhibits very rapid kinetics and appears to act directly

on the B cells. It is only partially impeded by constitutive expression of *bcl-2*, and seems unique to germinal centre cells. This apoptosis is likely to be caused by antigen binding the immunoglobulin receptor directly. Whatever the mechanism, this phenomenon may reflect a special susceptibility of germinal centre cells to tolerance induction, which has evolved to cope with the generation of self-reactive B cells through somatic hypermutation. □

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Antigen-induced B-cell death and elimination during germinal-centre immune responses

Kevan M. Shokat* & Christopher C. Goodnow

Howard Hughes Medical Institute and Department of Microbiology and Immunology, Beckman Center, Stanford University School of Medicine, Stanford, California 94305, USA

DURING an immune response, hypermutation of immunoglobulin genes in B cells proliferating within germinal centres (GCs) generates variant antibodies that react with higher affinity against either foreign or self antigens^{1–9}. Several experiments suggest that self-reactive B cells may be censored at this stage of the immune

* Present address: Departments of Chemistry and Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA.

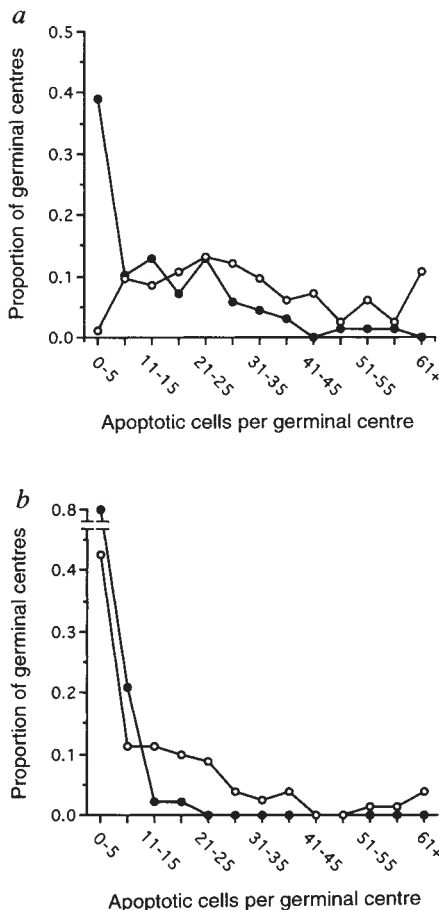


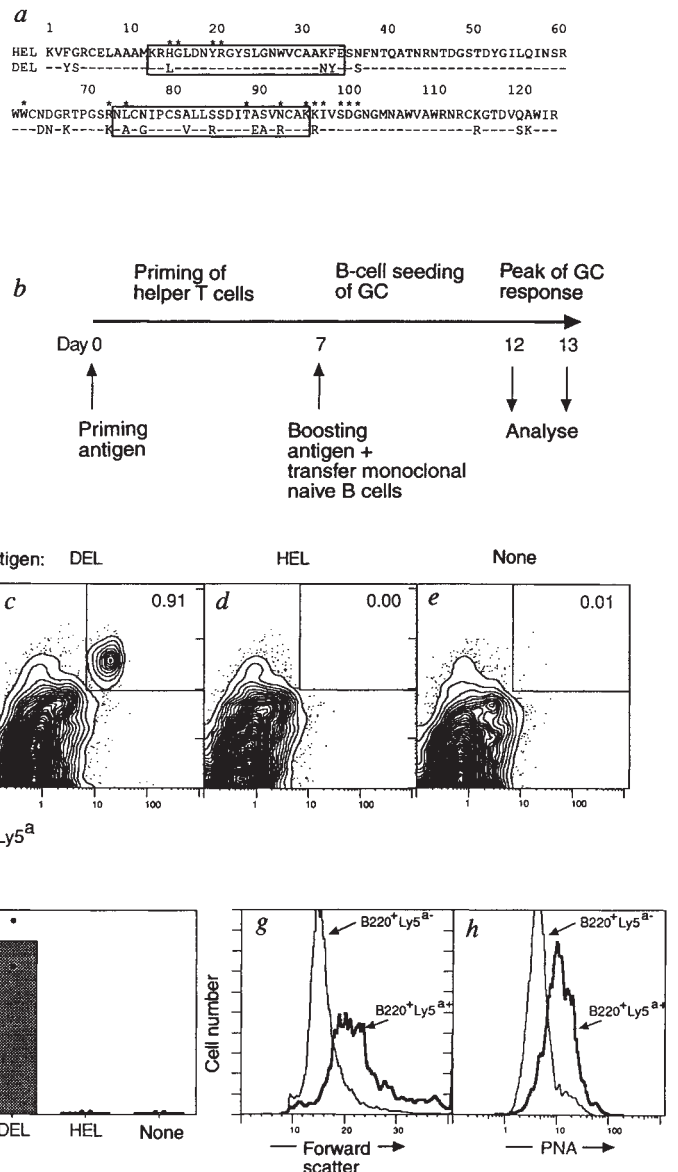
FIG. 4 The effect of *bcl-2* on apoptosis caused by soluble antigen. Mice expressing *bcl-2* constitutively in GC B cells, and their littermate controls, were immunized with NP₁₆-HSA, and injected with soluble NP₁-HSA 6 h before being killed at day 14. *a*, The littermate controls showed greatly enhanced apoptosis within a large fraction of their germinal centres. *b*, In *bcl-2* mice, there was an increase in apoptosis in a smaller, but significant fraction of the germinal centres. The residual germinal centres in the *bcl-2* mice that showed no increase in apoptosis, may represent 'background', non-antigen specific germinal centres in these mice (which were housed in conventional mouse facilities for over 24 weeks).

FIG. 1 Seeding of germinal centre (GC) reactions with uniform lysozyme-specific B cells. *a*, Sequence comparison of HEL and DEL antigens. Dashes indicate identical amino acids, and boxed segments denote regions that are dominant T-cell epitopes in the I-A^b background¹⁴. Asterisks denote amino acids in HEL that contact HyHEL10 immunoglobulin, determined by X-ray crystal structure²⁶. The differences between HEL and DEL were exploited by priming immune responses with DEL, which had three advantages: (1) in contrast to HEL, DEL elicits a strong helper-T-cell response in B6 mice, primarily due to a F→Y mutation at position 3 which affects antigen processing¹⁴; (2) DEL differs considerably from HEL within the dominant T-cell epitopes defined in HEL¹⁴, providing a pair of antigens that would be expected to crossreact poorly at the helper-T-cell level; and (3) DEL binds to the immunoglobulin expressed by the transgenic B cells with 1,000-fold lower affinity than HEL¹⁵, thus better approximating the binding affinity of B cells during primary antibody responses. *b*, Experimental protocol used to generate a synchronous burst of lysozyme-specific GC B cells. *c–e*, FACS analysis of spleen cells from DEL-primed B6 mice on day 12 of the immune response, 5 days after transfer of Ly5^{α+} immunoglobulin-gene transgenic cells and boosting with DEL (*c*), HEL (*d*), or no additional antigen (*e*). Spleen cells were analysed by three-colour flow cytometry for cell-surface lysozyme-binding receptors (HEL), and the B220 and Ly5^α surface markers. The profiles shown were first gated on B220⁺ cells. The percentage of ungated nucleated cells falling within the B220⁺ HEL⁺ Ly5^{α+} window is shown; contours are 1% probability intervals. *f*, Total number of Ly5^{α+} B220⁺ HEL⁺ cells in the spleen of pairs of mice treated and analysed as shown in (*c–e*). *g–h*, Cell size (forward scatter, *g*), and staining with peanut agglutinin (PNA, *h*) of Ly5^{α+} B220⁺ transgenic cells in DEL-primed and boosted recipients. Overlaid histograms show forward scatter or PNA staining for transferred cells (Ly5^{α+} B220⁺, thick lines) compared to endogenous non-transgenic B cells (Ly5^{α+} B220⁺, thin lines).

METHODS. Peking duck eggs were obtained from Reichardt's Duck Farm (Petaluma, California). DEL was purified as described²⁷. A mixture of DEL A and B (ref. 27) was used for immunization and boosting. Immunoglobulin-gene transgenic cells were F₁ offspring from B6 strain MD4 transgenic mice crossed with the congenic B6-Ly5^α strain. Recipients were 8- to 14-week-old B6 mice primed with 100 μg DEL in Ribi adjuvant (MPL+TDM+CWS Emulsion, Ribi ImmunoChem Research, Hamilton, MT) given intraperitoneally. After 7 days, 1 × 10⁷ sex-matched immunoglobulin-gene transgenic spleen cells in 0.3 ml RPMI-1640 medium with 2% FCS were injected intravenously in combination with 100 μg DEL or HEL, or in medium with no added antigen. Mice were killed after 5 days; half the spleen was used for immunohistochemistry, and the other half for three-colour flow cytometry as described^{23,28}. Staining with peanut agglutinin used PNA-FITC (E-Y Laboratories, San Mateo, California). Staining for B220, Ly5^α and HEL-binding B cells was performed as previously described²⁸.

response^{10–12}, but the rarity of these cells and the dynamic nature of GC reactions have prevented direct analysis. We have developed a new approach to visualize the fate of antigen-specific B cells during GC reactions by seeding an ongoing immune response with lysozyme-specific B cells from immunoglobulin-gene transgenic animals. Administration of soluble antigen at the peak of the GC response rapidly eliminates lysozyme-specific GC B cells in two waves of apoptosis, one within the GC and a second in cells that have redistributed to lymphoid zones that are rich in T cells. Elimination of these cells is inhibited by constitutive expression of the follicular lymphoma proto-oncogene *bcl-2*. These findings reveal censoring steps that may normally prevent affinity maturation of autoantibodies to systemic autoantigens, and might be used by pathogenic microorganisms or in clinical strategies to interfere with antibody responses.

To track GC cell selection, a burst of proliferating GC B cells with a uniform immunoglobulin specificity was produced



by seeding a primary antibody response with lysozyme-specific B cells from an immunoglobulin-gene transgenic mouse¹³. An immune response was initiated by priming C57BL/6 mice with the antigen duck egg lysozyme (DEL), which was chosen to exploit sequence differences from hen egg lysozyme (HEL)^{14,15} (Fig. 1*a*). After 7 days, when T-cell help would be well developed and GCs beginning to form¹⁶, 10⁷ immunoglobulin-gene transgenic spleen cells were introduced together with a boosting dose of DEL by intravenous injection (Fig. 1*b*). The transferred B cells were tracked by their lysozyme-binding receptors and a unique Ly5^α surface marker. They multiplied rapidly (Fig. 1*c,f*), and peak numbers occurred on days 12 to 13 of the response when the seeded cells were large (Fig. 1*g*), stained brightly with peanut agglutinin (Fig. 1*h*) and dominated the GC reactions. Transferred B cells did not proliferate detectably in DEL-primed mice that were boosted with HEL despite the 1,000-fold higher affinity of the B cells for HEL antigen (Fig.

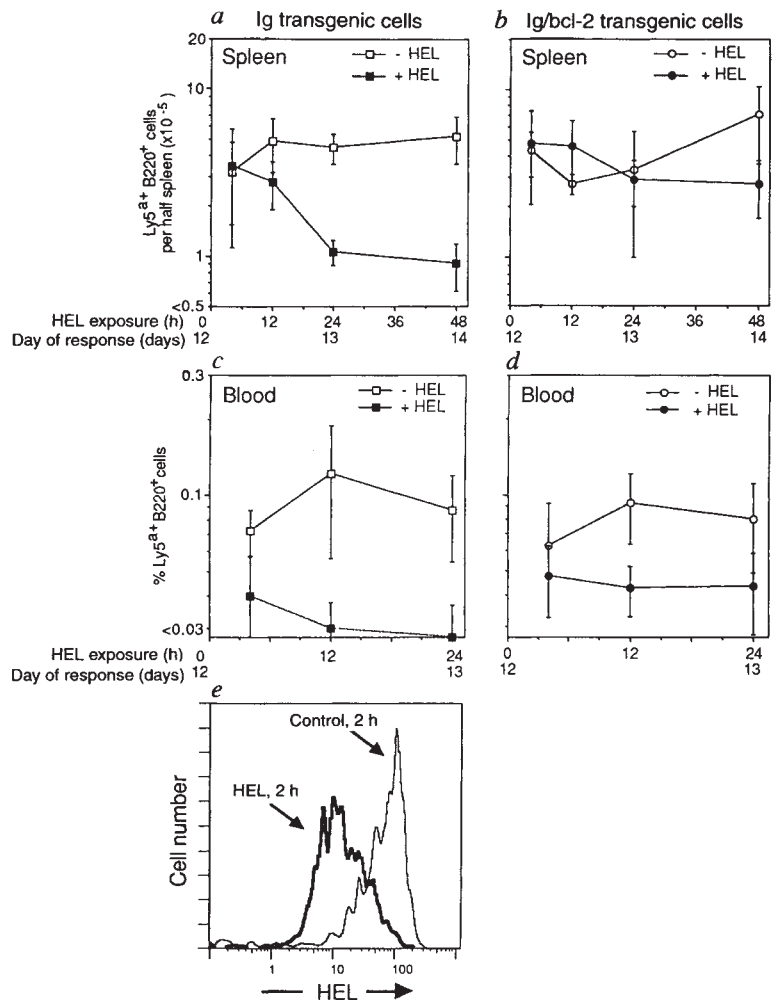
FIG. 2 Elimination of seeded lysozyme-specific GC B cells after binding HEL antigen. Groups of recipient mice were primed with DEL antigen, seeded with transgenic B cells and boosted with DEL antigen as described in Fig. 1, except that immunoglobulin-gene transgenic cells (a, c, e) or Ig/bcl-2 transgenic cells (b, d) were used. Five days after B-cell seeding (day 12 of the immune response), the mice received an intravenous injection of 5 mg HEL antigen (+HEL) or medium alone (-HEL). At the indicated times after HEL treatment, groups of 3 to 5 mice in each treatment group were killed and B220⁺ Ly5⁺ cells enumerated in spleen (a, b) and blood (c, d) by flow cytometry. Means and standard deviations for each group of mice are shown. The data shown are pooled from two separate experiments involving the same four treatment groups. Equivalent data were obtained in six other experiments with immunoglobulin-gene transgenic cells and three experiments comparing Ig/bcl-2 cells. e, Histograms showing HEL staining of Ly5⁺ B220⁺ cells 2 h after injection of HEL (thick line) or medium alone (thin line). Staining is by the sandwich technique which shows the total number of cell surface receptors regardless of the fraction occupied *in vivo*.

METHODS. B6 recipients were primed and boosted as described in Fig. 1. They were seeded with 10⁷ spleen cells from sex matched (B6 × B6-Ly5⁺)F₁ mice that were either MD-4 immunoglobulin-gene transgenic mice or also carried the Eμ-bcl-2-22 transgene¹⁷. On day 12 of the response, mice received 300 μl RPMI-1640 medium either alone or containing 5 mg HEL, via the lateral tail vein. Mice were killed with CO₂ at the indicated times, heart blood was collected in Alsever's solution and erythrocytes removed by NH₄Cl lysis, and blood leukocytes and half of the spleen was used for flow cytometry. The other half of the spleen was divided for frozen section and paraffin embedding.

1d, f), possibly because HEL-derived peptides were not recognized by DEL-specific helper T cells.

To model the fate of GC B cells that become strongly reactive to circulating autoantigens as a result of immunoglobulin gene hypermutation, soluble HEL antigen was injected on day 12 of the response to expose the GC B-cell population to soluble HEL antigen in an abrupt and synchronous manner. Cell-surface antigen receptors were rapidly modulated on splenic B220⁺ Ly5⁺ B-cell blasts following HEL antigen (Fig. 2e), but the seeded cells were not markedly reduced in number until after 24 hours (Fig. 2a). After 48 hours the number remaining was at the lower limit of detection by flow cytometry. Lysozyme-reactive B-cell blasts in the bloodstream of HEL-treated mice disappeared well before any reduction in the spleen (Fig. 2c), implying that elimination from the spleen might reflect cell death *in situ* rather than emigration via the blood. This was confirmed by seeding parallel groups of mice with immunoglobulin-gene transgenic B cells that also carried a constitutively expressed bcl-2 transgene which inhibits cell death¹⁷. The number of splenic lysozyme-reactive B-cell blasts was not reduced 24 hours after HEL injection in mice that had received Ig/bcl-2 transgenic cells, and only a twofold reduction occurred after 48 hours (Fig. 2b).

The precise sites of B-cell death were explored by locating either lysozyme-binding Ly5⁺ B cells in spleen cryosections by immunofluorescent staining, or dying cells in paraffin sections by the TUNEL method¹⁸ to detect DNA fragmentation. In the absence of HEL, lysozyme-binding B cells were located predominantly in the apical zones of GCs with scattered cells in the surrounding mantle and marginal zones (Fig. 3a, d), and occasional TUNEL⁺ nuclei were clustered in tingibile body macro-

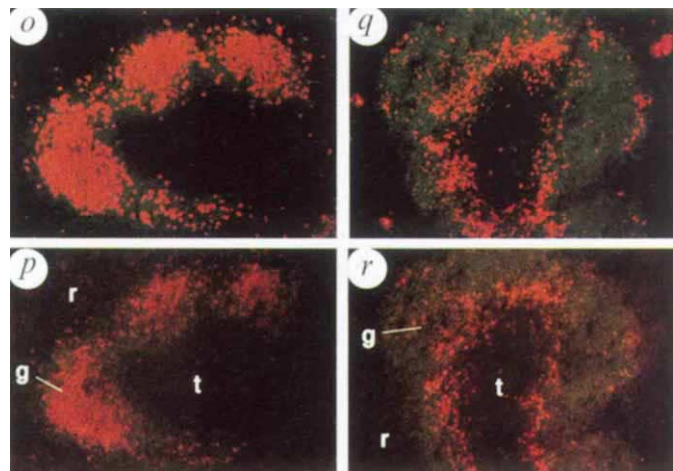
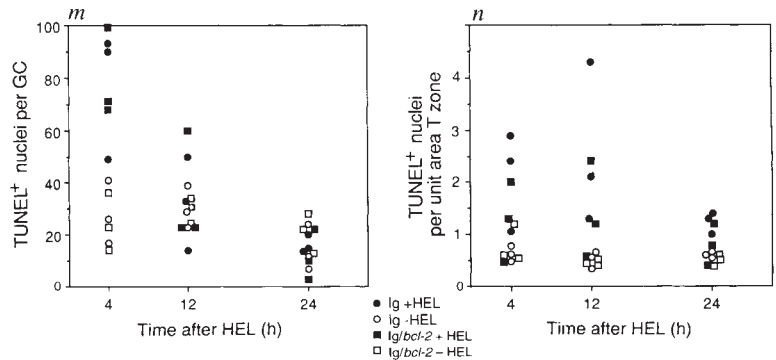
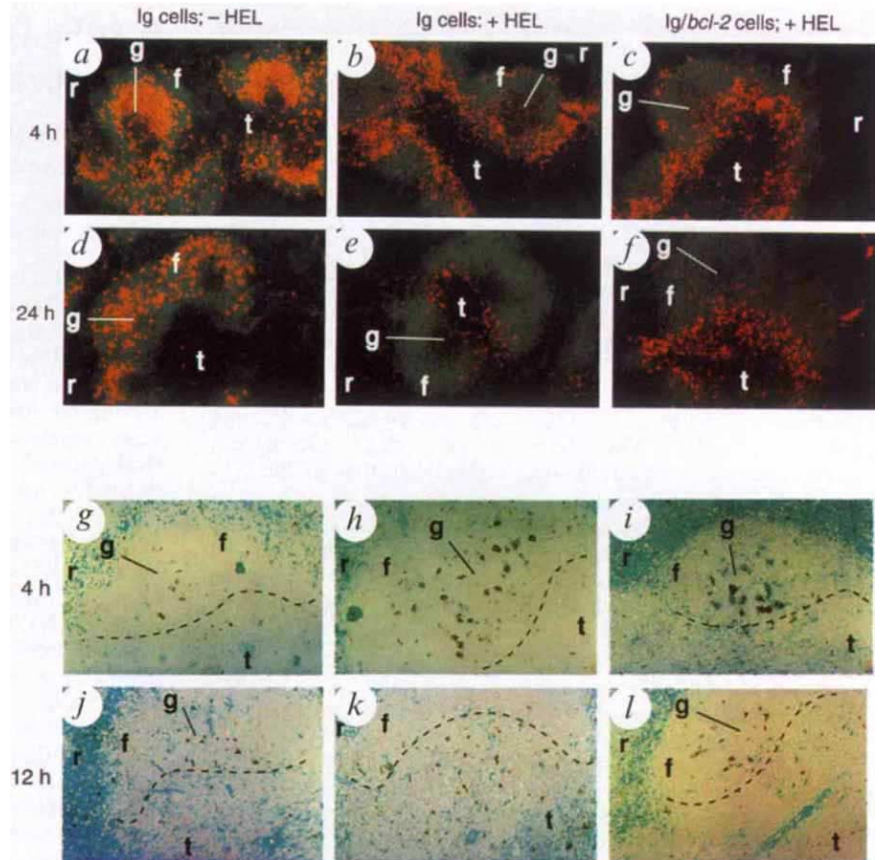


phages in GCs (Fig. 3g, j). Within 4 hours of HEL injection, however, few lysozyme-binding cells remained in the GCs (Fig. 3b) and TUNEL⁺ nuclei were markedly increased in this site, many being clustered in groups of 10 to 20 nuclei within prominent tingibile-body macrophages (Fig. 3h, m). Large numbers of lysozyme-binding B cells were also redistributed to the outer part of the T-cell zones at this time (Fig. 3b) and after 12 hours (data not shown), but few remained by 24 hours after HEL treatment (Fig. 3e). Staining of serial sections for Ly5⁺ showed comparable cell distributions (Fig. 3o-r), confirming that the transferred B cells were rapidly depleted from the GCs and concentrated in the T-cell zones following soluble HEL administration. TUNEL⁺ nuclei were more frequent in the T-cell zone 4 to 12 h after HEL treatment (Fig. 3k, n), but had mostly disappeared by 24 hours consistent with the elimination of HEL-

FIG. 3 Location of B-cell death. Spleen sections from the mice analysed in Fig. 2 were analysed by immunofluorescence to detect seeded B cells (a-f, o-r) and by the TUNEL method to detect apoptotic cells with fragmented DNA (g-n). a-f, Two-colour fluorescence micrographs of 8-μm spleen cryosections stained for HEL-binding cells (orange) and a pan-B cell marker, CD79β (B29/Igβ; green). T-cell zones (T), germinal centres (G), follicular mantle zones (F) and red pulp (R) are indicated. In recipients of immunoglobulin-gene transgenic cells that did not receive HEL antigen (-HEL), HEL-binding cells are found predominantly in the apical zones of GCs with scattered cells in mantle and marginal zones (a, d). Cells in the basal zone of GCs carry few immunoglobulin receptors of any type^{16,20}, as they also lack staining for CD79β (green). Note that in a the T-cell zone is sectioned tangentially and the scattered

HEL-binding cells near the T-cell zone are in follicular mantle zones. In mice treated with HEL (+HEL), few HEL-binding cells remain in the GC at 4 h (b, c), and none are detected after 24 h (e, f), and most HEL-binding cells are concentrated in the outer part of the T-cell zone. g–l, Transmission micrographs of TUNEL staining in 3- μ m paraffin sections showing apoptotic nuclei containing nicked DNA (brown), counterstained with methyl green. The edge of the T-cell zone is outlined with broken lines, and was determined by viewing with phase contrast to detect the characteristic interdigitating dendritic cells of the T-cell zone and from adjacent serial sections stained with haematoxylin and eosin. In the absence of HEL (g, j), few apoptotic nuclei are present in the GCs and very few are in the T-cell zones. By contrast, large numbers of clustered apoptotic nuclei are present in GCs 4 h after HEL treatment (h, i). Apoptotic nuclei are also scattered through the T-cell zones 12 h after HEL treatment (k, l). Equivalent results to those shown in a–l were obtained from each of the mice shown in Fig. 2, and in four separate experiments. m, n, Mean number of apoptotic nuclei in GCs (m) and T-cell zones (n) of individual mice at each time point. o–r, Two-colour immunofluorescent staining as in a–g, except that orange staining of serial sections represents either HEL-binding (o, q) or staining for Ly5^a (p, r). Note that staining for Ly5^a is not as bright as HEL staining and hence is less sensitive in tissue sections.

METHODS. One-quarter of each spleen from the mice shown in Fig. 2 was cut into 5-mm sections and snap-frozen in OCT (Biomedica, Foster City, California) for cryosections. Another quarter was cut into 5-mm sections, fixed in 10% neutral buffered formalin, and embedded in paraffin. Immunofluorescence staining was as described²⁹. HEL staining used a three-layer sandwich of sequential staining with HEL, HyHEL9-biotin and streptavidin-PE. Ly5^a staining was with biotinylated AS-20 followed by streptavidin-PE. CD79 β was detected with affinity-purified rabbit antiserum raised against a glutathione S-transferase (GST)-B29 cytoplasmic tail fusion protein (generous gift of J. G. Cyster) followed by donkey anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC; Jackson Immunoresearch). Two-colour fluorescence was visualized and photographed through a dual filter system: excitation 480–500 nm/emission 517–532 nm (FITC) and excitation 545–578 nm/emission 585–620 nm (phycoerythrin, PE; Chroma, Brattleborough, VT). The method of Gavrieli *et al.* was used for TUNEL staining with minor modifications¹⁸. Paraffin was removed using Hemo-De (Fisher Scientific) under the same conditions as described for use of xylene. Endogenous peroxidase activity was blocked with methanol: 3% aqueous H₂O₂ for 10 min. Following the TUNEL reaction, the sections were counterstained with an aqueous solution of 0.5% lissamine green for 45 s, followed by 3 rinses in water, 3 rinses in *n*-butanol, and dehydration using Hemo-De. For quantitation, slides were coded, randomized, white pulp cords sketched under phase contrast and by viewing haematoxylin and eosin-stained serial sections, and apoptotic nuclei counted in 10–20 GCs and 4–6 T-cell zones from each mouse. The area of each counted T-cell zone was measured using a grid in one eyepiece containing 56 μ m \times 56 μ m squares, and the number of apoptotic nuclei in each T-cell zone was divided by the number of 56 μ m \times 56 μ m squares to give a value per unit area.



binding Ly5⁺ cells by this time. Equivalent changes occurred after HEL treatment in recipients of Ig/*bcl-2* transgenic cells (Fig. 3c, f, i, l, m), except that many lysozyme-binding Ly5⁺ cells remained in the T-cell zone after 24 hours (Fig. 3f) and fewer TUNEL⁺ nuclei were present in this site (Fig. 3l, n).

These results show that circulating antigen causes apoptosis and elimination of specific GC B cells in two waves. The data do not address how apoptosis is triggered in each case, but it is tempting to speculate that the rapid cell death, within 4 hours in GCs, may reflect early centrocytes emerging from the GC basal dark zones that were prevented from binding DEL antigen displayed on follicular dendritic cells (FDC), because recognition of FDC-bound antigen may deliver short-term survival signals to centrocytes^{19, 21}. The *bcl-2* transgene was unable to block this phase of HEL-induced B-cell death (Fig. 3i, m), but may inhibit comparable death of GC B cells in response to the hapten nitrophenyl (NP)²². The affinity for HEL in the GC B cells studied here is 100 to 1,000-fold higher than that of the NP-reactive cells^{3, 5}, and may thus exceed the capacity of *bcl-2* to interfere with apoptosis at this selection step. The slower phase of B-cell disappearance in the T-cell zones, which was inhibited by *bcl-2*, may represent older centrocytes that had recognized FDC-bound DEL antigen and received survival signals before HEL exposure. Their apparent migration to the T-cell zones resembles the redistribution of marginal-zone memory B cells following restimulation with antigen¹⁶, and implies that antigen-receptor engagement induces a tropism of B cells towards T-cell zones. Premature death of primed HEL-binding cells in the T-cell zone resembles competitive elimination of self-reactive B cells in the preimmune repertoire²³, and may reflect exclusion from follicular niches and absence of T-cell help²³. The two censoring steps identified here may normally eliminate B cells that, through hypermutation, bind systemic autoantigens such as DNA too strongly^{6, 8, 9, 24}. The ability to abort B-cell affinity maturation with soluble antigen also suggests clinical strategies to curtail undesirable antibody responses, and might be exploited by pathogenic organisms, such as malaria, which produce appreciable amounts of circulating antigen²⁵. □

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A role for Rac in Tiam1-induced membrane ruffling and invasion

Frts Michiels, Gaston G. M. Habets, Jord C. Stam, Rob A. van der Kammen & John G. Collard*

The Netherlands Cancer Institute, Division of Cell Biology, 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands

RHO-LIKE GTPases have been implicated in the regulation of the actin cytoskeleton which controls the morphology, adhesion and motility of cells^{1–3}. Like Ras proteins, they become activated when bound GDP is exchanged for GTP, a process catalysed by GDP-dissociation stimulator (GDS) proteins⁴. Several GDS proteins specific for Rho-like GTPases have been identified^{5–8}. Most of these contain a conserved catalytic domain, the DBL-homology (DH) domain⁹, and activate Cdc42 or Rho but not Rac^{5–8}. We have isolated the invasion-inducing *Tiam1* gene, which also encodes a protein with a DH domain¹⁰. Here we show that *Tiam1* is a GDS protein for Rho-like GTPases *in vitro*. In fibroblasts, *Tiam1* induces a similar phenotype as constitutively activated (V12)Rac1, including membrane ruffling, and this is inhibited by dominant negative (N17)Rac1. Moreover, T-lymphoma cells expressing V12Rac1 become invasive, indicating that the *Tiam1*–Rac signalling pathway could be operating in the invasion and metastasis of tumour cells.

To investigate whether *Tiam1* is a GDS for Rho-like GTPases, *Tiam1*–glutathione *S*-transferase (GST) and Myc-tagged RhoA and Rac1 fusion proteins were purified from transfected COS-7 cells. Isolated Rac1 and RhoA proteins were preloaded with ³H-GDP and incubated with *Tiam1*–GST or GST alone to determine the release of bound ³H-GDP as described^{6, 11}. Incubation of Rac1 with *Tiam1*–GST resulted in a pronounced release of ³H-GDP when compared to the incubation of Rac1 with GST (Fig. 1a). A similar, though slower, release was observed for RhoA (Fig. 1a). The *Tiam1*-induced release of ³H-GDP was evident within the first 5 minutes of incubation. To substantiate these findings further, we analysed the effect of *Tiam1* on the exchange of preloaded GDP for GTP-γ³⁵S on Rac1 and RhoA. Indeed, *Tiam1* acted as a more potent GDS for Rac1 than for RhoA as both the rate of exchange and the total amount of bound GTP-γ³⁵S were higher for Rac1 than for RhoA (Fig. 1b). Similar results were obtained with GST–RhoA and GST–Rac1 fusion proteins isolated from *Escherichia coli* (Fig. 1c). In the latter experiment we included GST–hCdc42, a Rac-related GTPase¹², which was also efficiently activated by *Tiam1*. These experiments indicate that *Tiam1* is a GDS protein for Rho-like GTPases and that *in vitro* *Tiam1* activates Rac1 and hCdc42 and, to a lesser extent, RhoA.

We next determined whether *Tiam1* could also activate Rac and Rho proteins in fibroblasts. Constitutively activated RhoA (V14RhoA) induces focal contacts and stress fibre formation in 3T3 cells¹³, whereas V12Rac1 induces the formation of actin filaments at the plasma membrane leading to membrane ruffling¹⁴. *Tiam1*-encoding constructs were transfected into NIH3T3 cells and the resulting transfectants were compared with V12Rac1- and V14RhoA-transfected cells. Cells that transiently expressed the full-length or N-terminally truncated *Tiam1* (ΔN-*Tiam1*) showed extensive membrane ruffling (Fig. 2a, b), as was found for V12Rac1-transfected (Fig. 2d) or -microinjected cells¹⁴. Membrane ruffling was observed in more than 80% of the *Tiam1*-transfected cells and hardly at all (less than 5%) in untransfected cells. V14RhoA induced the formation of large bundles of stress fibres (Fig. 2c), which was not seen in *Tiam1*-transfected cells. The membrane ruffling in established ΔN-*Tiam1*-transformed NIH3T3 cell lines, was inhibited in most (>70%) of the cells that co-expressed dominant negative Rac1

* To whom correspondence should be addressed.