

at the C terminus⁶. The predicted LLO sequence¹⁷ contains five such motifs (Fig. 4a). In addition, some identified foreign epitopes have valine, alanine or threonine at the C terminus. According to these criteria we synthesized 11 nonamers, representing only 18% of the LLO molecule, and tested them for their ability to sensitize P815 target cells to lysis by H-2K^d-restricted, LLO-specific CTL. Amino-acid residues 91-99 represent the H-2K^d LLO epitope recognized by B9 and this peptide can target cells at concentrations less than 10⁻¹¹ M (Fig. 4b). The precise molecular mass and partial amino-acid sequence of synthetic LLO 91-99 were confirmed by tandem ion spray mass spectroscopy. The other 10 peptides were not detected by LmT, suggesting that LLO contains only one H-2K^d-restricted peptide. The exact comigration of synthetic LLO 91-99 with the CTL targeting activity in trifluoroacetic acid extracts from infected BALB/c spleens on a very shallow acetonitrile gradient (Fig. 4c, d) indicates that we have identified the natural epitope. Other regions of the LLO molecule contain epitopes recognized by *L. monocytogenes*-specific CD4⁺ T lymphocytes¹⁸. LLO 91-99 was not, however, contained within any of the previously predicted T lymphocyte epitopes¹⁸.

Phagocytosed, virulent *L. monocytogenes* secrete LLO which disrupts the phagolysosomal membrane and allows the bacterium to enter the host cell cytoplasm¹. It has been proposed that only upon entry into the cytoplasm can *L. monocytogenes* antigens become processed and presented by class I MHC molecules¹². This would explain why *L. monocytogenes* strains or mutants lacking LLO do not induce protective immunity. Although our findings are consistent with this hypothesis, they could also be interpreted to suggest that the absence of LLO in avirulent *L. monocytogenes* is responsible for their lack of immunogenicity. Although the immunogenicity of LLO has been demonstrated^{18,19}, further studies will be required to settle this controversy.

Identification of the CTL epitopes of infectious agents is important for vaccine development²⁰⁻²². The diversity and number of proteins expressed by bacterial and protozoal pathogens has made the task of identifying CTL epitopes formidable. Determination of LLO 91-99 as a major H-2^d-restricted CTL epitope was possible because of the recent identification of the binding motif of H-2K^d for endogenous peptides⁶. Our findings demonstrate the predictive power of this motif for the determination of a pathogen-derived CTL epitope and argue the case for the systematic determination of binding motifs of other class I molecules. □

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Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement

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TRIGGERING of mast cells and basophils by immunoglobulin E (IgE) and antigen induces various biochemical signals, including tyrosine kinase activation^{1,2}, which lead to cell degranulation and the release of mediators of the allergic reaction. The high-affinity receptor for IgE (FcεRI) responsible for initiating these events is a complex structure composed of an IgE-binding α-chain, a β-chain and a homodimer of γ-chains³. It has been assumed that β and γ, which have extensive cytoplasmic domains, play an important but undefined role in coupling FcεRI to signal transduction mechanisms. Here we show that FcεRI engagement induces immediate *in vivo* phosphorylation on β (tyrosine and serine) and γ (tyrosine and threonine) by at least two different non-receptor kinases. We take advantage of unique features of this receptor system to demonstrate that the phosphorylation signal is restricted to activated receptors and is immediately reversible upon receptor

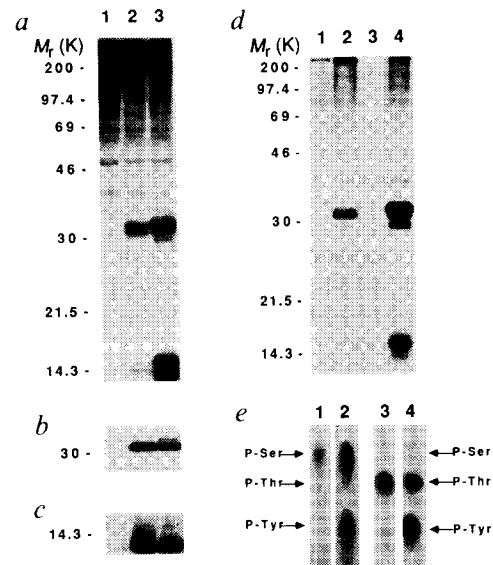
disengagement by undefined phosphatases. Rapid phosphorylation and dephosphorylation may be a general mechanism to couple and uncouple activated receptors to other effector molecules. This could be particularly relevant to other multimeric receptors containing FcεRI γ-chains or the related ζ and η chains such as the T-cell antigen receptor (TCR)⁴⁻⁶ and the low-affinity receptor for immunoglobulin G (FcγRIII, CD16) (refs 7-11).

Rat basophilic leukaemia cells (RBL-2H3) were incubated with anti-DNP IgE and labelled *in vivo* with [³²P]orthophosphate. The cells were then stimulated for one minute by a multivalent antigen dinitrophenyl[30]-human serum albumin (DNP[30]-HSA), solubilized and the IgE-bound receptors immunoprecipitated using a polyclonal anti-IgE antibody. Figure 1a shows the results of a reduced SDS-PAGE separation and autoradiography. In resting cells (lane 2), the β-chain appears as three species (relative molecular masses (M_r) around 30,000 (30K)) which are phosphorylated, although the fastest species requires a longer exposure to be clearly seen. After stimulation (lane 3), the overall level of phosphorylation of β increases substantially, particularly in the 33K species, although anti-β immunoblotting reveals equivalent amounts of β in immunoprecipitates from resting and stimulated cells (Fig. 1b). An increase in ³²P specific activity is also observed in the coprecipitating γ-chains, seen here in monomeric form of ~14K (Fig. 1a, c). Immunoprecipitations with monoclonal anti-β (ref. 12; Fig. 1d) and anti-γ-peptide antiserum⁶ (not shown) give virtually identical results. Thin-layer electrophoresis after gel extraction and hydrolysis of β- and γ-bands from similar experiments (Fig. 1e) confirms observations^{13,14} of serine phosphorylation of β and threonine phosphorylation of γ under resting conditions. But in contrast with previous studies^{13,14}, we find that receptor engagement induces tyrosine phosphorylation of both β and γ while increasing the serine phosphorylation of β and threonine phosphorylation of γ.

The direct relationship of receptor engagement and receptor phosphorylation was examined in antigen dose-response experiments (Fig. 2). The phosphorylation of β and γ increases with the dose of antigen and is maximal at 100 ng ml^{-1} of antigen. Receptor phosphorylation is not seen in ionomycin-stimulated cells (data not shown) and therefore is not simply secondary to nonspecific cell activation.

FIG. 1 Aggregation of Fc ϵ RI in RBL-2H3 cells induces tyrosine and serine phosphorylation of β -chain as well as tyrosine and threonine phosphorylation of γ -chains. *a*, Immunoprecipitates with polyclonal anti-IgE antibody from ^{32}P -labelled cells were analysed by SDS-PAGE and autoradiography. M_r calibration is shown on the left. Cells were incubated with medium alone (lane 1) or with anti-DNP mouse IgE, then challenged for 1 min with medium alone (lane 2) or DNP[30]-HSA (lane 3). *b*, The same immunoprecipitates were analysed by western blotting with monoclonal anti- β antibody and *c*, with anti- γ -peptide antiserum. Immunoblots with irrelevant monoclonal antibody or preimmune serum did not show any reactivity (not shown). *d*, Immunoprecipitates with control IgG1 (lanes 1, 3) or anti- β (lanes 2, 4) antibody from labelled cells were analysed as in *a*. Cells were incubated with anti-DNP IgE and stimulated for 5 min with medium alone (lanes 1, 2) or with DNP[30]-HSA (lanes 3, 4). *e*, Thin-layer electrophoresis of phosphoaminoacids from non-activated receptor β -chains (lane 1) and γ -chains (lane 4). **METHODS.** RBL-2H3 cells (1×10^7 cells per ml) were pre-incubated in phosphate-free medium for 4 h at 37°C before addition or not of $5 \mu\text{g ml}^{-1}$ of monomeric monoclonal anti-DNP mouse IgE (ref. 26) for 1 h at 37°C , then labelled at 3×10^7 cells per ml with $37 \text{ MBq per ml } [^{32}\text{P}]\text{-orthophosphate}$ (Amersham) for 2 h at 37°C . The cells (5×10^7 per sample) were incubated for various lengths of time with medium or with 100 ng ml^{-1} of DNP[30] conjugated to human serum albumin (Sigma). Cells were then immediately washed with 50 ml ice-cold PBS containing the following phosphatase inhibitors: 1 mM sodium vanadate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate and 5 mM EDTA. Cells were lysed in a buffer (pH 8) containing 200 mM boric acid, 160 mM NaCl, 0.3% Triton X-100, 1% BSA, the same phosphatase inhibitors and the following protease inhibitors: $10 \mu\text{g ml}^{-1}$ aprotinin, $4 \mu\text{g ml}^{-1}$ leupeptin, $10 \mu\text{g ml}^{-1}$ pepstatin, 1 mM PMSF. The post-nuclear supernatants were precleared with protein A- or protein G-Sepharose beads (Pharmacia) and immunoprecipitated with rabbit polyclonal anti-IgE bound to protein A-Sepharose or with monoclonal anti- β (JRK)¹² or control IgG1 bound to protein G-Sepharose beads. Beads were washed with lysis buffer and the immunoprecipitates analysed on 12.5% SDS-PAGE under reducing conditions before autoradiography. For western blotting, the postnuclear supernatants from 1.5×10^7 cells were immunoprecipitated and analysed by SDS-PAGE as described and the proteins transferred electrophoretically to Immobilon-P membranes (Millipore) for 6 h at 50 V. Membranes were then incubated overnight in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% BSA, before a two-hour incubation in the same buffer containing $15 \mu\text{g ml}^{-1}$ anti- β antibody or 1:200 dilution of anti- γ -peptide antiserum⁶ or the same concentration of control antibody or pre-immune serum. After

We next investigated whether β - and γ -chain phosphorylations are early events in the signalling cascade, possibly preceding other early signals such as the hydrolysis of phosphoinositides and the increase in intracellular calcium, both of which are typically seen in RBL cells after 15 s of receptor engagement^{15,16}. Figure 3*a* shows that, when the cells are saturated with IgE, phosphorylation of β and γ is almost maximal after



two washes in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, 2.5% BSA, membranes were incubated with 1:3,000 dilution of goat anti-mouse or goat anti-rabbit antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch) and developed with the chemiluminescent substrate AMPPDTM (Tropix). Thin-layer electrophoresis of phosphoaminoacids was modified from ref. 27. Gel slices corresponding to β and γ were cut and the proteins digested with $50 \mu\text{g ml}^{-1}$ of trypsin in 50 mM NH_4HCO_3 to improve the yield of elution. The eluted peptides were hydrolysed for 90 min at 110°C in 0.2 ml 6N HCl. Samples were dissolved in 5 μl thin-layer buffer (10% glacial acetic acid, 1% pyridine in water, pH 3.5) containing 5 μg each of unlabelled phosphotyrosine, threonine, -serine (Sigma) as standards. Samples were applied to cellulose plates (100- μm DC-cellulose; Merck) and electrophoresed at 1,500 V for 45 min. Standards were developed by ninhydrin and ^{32}P -labelled amino acids identified by autoradiography. We have confirmed the results of this analysis by 2-dimensional thin-layer electrophoresis. Phosphoaminoacid analysis of β and γ purified from anti-IgE, anti- β or anti- γ immunoprecipitates gave virtually identical results.

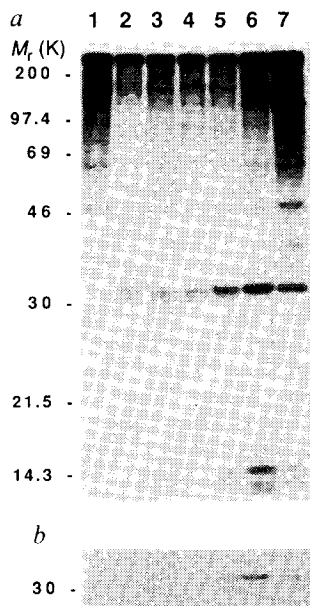


FIG. 2 Effect of increasing doses of antigen on β and γ phosphorylation. *a*, Immunoprecipitates with anti-IgE antibody from ^{32}P -labelled cells were analysed by SDS-PAGE and autoradiography as for Fig. 1. Cells were incubated with medium alone (lane 1) or with anti-DNP mouse IgE, then challenged for 5 min with medium alone (lane 2) or with 0.1 ng ml^{-1} (lane 3), 1 ng ml^{-1} (lane 4), 10 ng ml^{-1} (lane 5), 100 ng ml^{-1} (lane 6), $1 \mu\text{g ml}^{-1}$ (lane 7) of DNP-HSA. *b*, Tyrosine phosphorylation of β -chains from the same immunoprecipitates was analysed by immunoblotting with monoclonal anti-phosphotyrosine antibody 4G10 (UBI; ref. 28).

5 s stimulation with antigen (see legend). Indeed receptor phosphorylation is unaffected by the addition of EDTA at the time of stimulation, although [^3H]serotonin release is abolished under these conditions (not shown). In addition, efficient depletion of protein kinase C by prolonged incubation with phorbol esters did not modify receptor phosphorylation (not shown). Therefore it seems that the receptor-induced activation of tyrosine and serine/threonine kinases and the consequent receptor phosphorylation precede the other known biochemical signals.

One of the unique features of the IgE-Fc ϵ RI system is that it permits studies of rapid receptor disengagement. The binding of the monovalent ligand (IgE) is not sufficient for cell triggering. In fact, the β and γ from empty and liganded (but not cross-linked) receptors show similar states of phosphorylation when analysed by SDS-PAGE and subsequent thin-layer electrophoresis (not shown). To stimulate the cells, surface IgE-receptor complexes need to be engaged by a multivalent antigen. If a multihapten antigen is used as the triggering tool (here DNP[30]-HSA), then receptor disengagement can be studied by adding an excess of monovalent hapten (DNP-lysine). Figure 3a shows that within 5 s of monovalent hapten addition, β and γ are dephosphorylated, regardless of the length of the preceding triggering period (5, 15 or 60 s). The tyrosines, and to a lesser extent the serines, on β are dephosphorylated (Fig. 3b). Following disengagement by the addition of monovalent hapten, these receptors can then be rephosphorylated on β and γ (not shown)

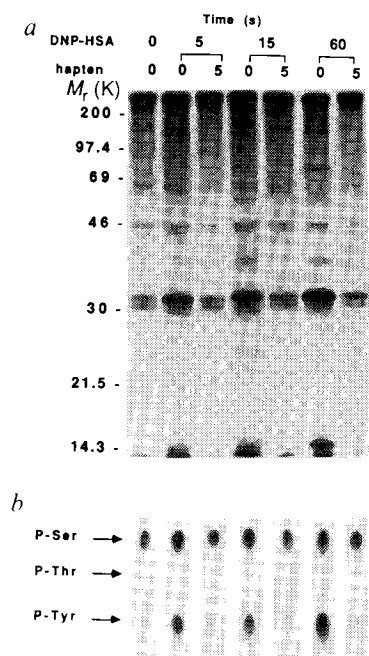


FIG. 3 Phosphorylation of β - and γ -chains is very rapid and reversible. *a*, Immunoprecipitates with monoclonal anti- β from ^{32}P -labelled cells were analysed by SDS-PAGE and autoradiography as for Fig. 1. Cells were incubated with IgE and stimulated with DNP-HSA for the time indicated. Cells were then chilled on ice or incubated for a further 5 s with 50 μM monovalent hapten (DNP-lysine). *b*, Thin-layer electrophoresis of phosphoaminoacids from receptor β chains purified from the corresponding lane of the gel shown above. Anti- β immunoblotting revealed an equivalent amount of β in the immunoprecipitates from this experiment (not shown). The levels of phosphorylation and dephosphorylation of β - and γ -chains were quantitated from similar experiments on a Betascope 603 blot analyser (Betagen). On average, the level of phosphorylation of β increases 2.5–5-fold after stimulation, whereas the level of phosphorylation of γ increases 100–400-fold. Both levels reach a maximum after 5–15 s. After disengagement with monovalent hapten, phosphorylation of β and γ decreases to resting levels. In control experiments (not shown), addition of monovalent hapten (DNP-lysine) had no effect on the phosphorylation of β and γ induced by monoclonal anti- α (BC4; ref. 17).

when re-engaged with anti-IgE. This rephosphorylation is qualitatively and quantitatively comparable to the phosphorylation of receptors freshly triggered with anti-IgE. Thus, phosphorylated receptors can be rapidly dephosphorylated by receptor disengagement and rephosphorylated following further stimulation.

Another advantage of this receptor system is the possibility to discriminate easily between activated and non-activated receptors in cells where only a fraction of receptors are bound to IgE and activated (Fig. 4). We analysed the fraction of receptors remaining empty (70%) by immunoprecipitation with monoclonal anti- α (BC4 recognizes only empty receptors)¹⁷. The level of phosphorylation in this fraction is the same whether the adjacent IgE-bound receptors (30%) are engaged (lane 7) or not (lane 6), even though an increase in phosphorylation is seen in the 30% fraction of activated receptors (lane 5). This experiment, in conjunction with anti- β (Fig. 4b) and anti- γ (Fig. 4c) immunoblots, demonstrates that the phosphorylation signal affects only β and γ associated with crosslinked receptors. This restricted phosphorylation may explain why phosphorylation is more intense when 100% (lane 3), as opposed to 30% (lane 5), of the receptors are activated.

Activation of tyrosine kinases is an early step in the signalling cascade of various receptors such as the TCR¹⁸, the B-cell antigen receptor¹⁹ and Fc γ RIII (CD16). As a consequence, receptor components such as the TCR- and CD16-associated ζ -chains^{20–22}, which are highly homologous with Fc ϵ RI

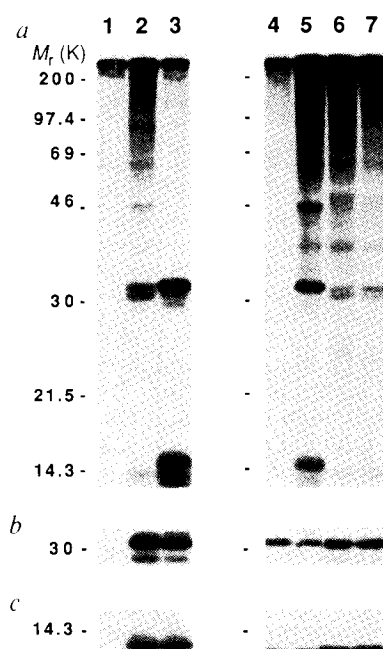


FIG. 4 Phosphorylation of β - and γ -chains is restricted to activated receptors. *a*, Immunoprecipitates from ^{32}P -labelled cells were analysed by SDS-PAGE and autoradiography as in Fig. 1. Lanes 1–5, anti-IgE immunoprecipitates: lane 1, cells unoccupied with IgE; lane 2, cells saturated with IgE and non-triggered; lane 3, cells saturated with IgE and triggered with antigen; lane 4, cells partially saturated and non-triggered; lane 5, cells partially saturated with IgE and triggered with antigen; lanes 6 and 7, immunoprecipitation with monoclonal anti- α BC4 (ref. 17) following a first immunoprecipitation with anti-IgE: lane 6, cells partially saturated and non-triggered; lane 7, cells partially saturated with IgE and triggered with antigen. *b*, The same immunoprecipitates analysed by western blotting with monoclonal anti- β antibody, and *c*, with anti- γ -peptide antiserum.

METHODS. RBL cells were incubated with medium alone (lane 1) or with saturating amount of IgE ($10 \mu\text{g ml}^{-1}/1 \times 10^7$ cells) (lanes 2, 3) or with a concentration of IgE ($0.2 \mu\text{g ml}^{-1}/1 \times 10^7$ cells) calculated to occupy about 20–30% receptors (lanes 4–7). Cells were challenged for 1 min with medium alone (lanes 1, 2, 4, 6) or with 100 ng ml^{-1} DNP-HSA (lanes 3, 5, 7).

γ -chains and clearly essential for cell activation²³, are phosphorylated on tyrosine. Indeed the genistein-induced inhibition of histamine release by RBL cells suggests a physiologically relevant role for tyrosine kinases in the Fc ϵ RI receptor activation pathway²⁴. Our results also support an important role for tyrosine kinase activity in receptor signalling. In addition, we have demonstrated that receptor phosphorylation, at least of Fc ϵ RI, is restricted to activated receptors, requires continuous receptor engagement and is immediately reversible upon receptor disengagement, presumably through the action of undefined phosphatases. We also show that Fc ϵ RI-induced activation of serine/threonine kinase(s) seems to occur as rapidly as tyrosine kinase activation. We propose that cycles of kinase and phosphatase activation are initiated and maintained by receptor engagement. The consequent phosphorylation/dephosphorylation of receptor and other substrates may promote coupling/uncoupling to other components of the signalling machinery. This model would explain the absence of an increase in phosphorylation over time, as well as the immediate receptor dephosphorylation that occurs when receptor triggering is halted.

The identification of the tyrosine and serine/threonine kinases involved in Fc ϵ RI activation will further our understanding of the system. Preliminary studies²⁵ suggest that the *lyn* and *yes* proteins of the *src* family of tyrosine kinases may be activated by Fc ϵ RI. Mutation analysis of specific residues on β and γ should enable us to assess the relative importance of tyrosine and serine/threonine receptor phosphorylations and to analyse their potentially discreet roles in coupling Fc ϵ RI to the signalling machinery in mast cells. □

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Interleukin-2 programs mouse $\alpha\beta$ T lymphocytes for apoptosis

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ANTIGEN receptor stimulation of mature $\alpha\beta$ T lymphocytes can lead either to proliferation or death¹⁻⁴. Programmed cell death, termed apoptosis, leads to the clonal deletion of both thymocytes and mature T cells that establishes tolerance⁵⁻⁹. How a mature T cell selects between proliferation and death is not understood. Here I show that interleukin-2 (IL-2) is a critical determinant of the choice between these two fates. Both CD4⁺ and CD8⁺ T cells previously exposed to IL-2 undergo apoptosis after antigen-receptor stimulation. Antibody blockade of IL-2 but not IL-4 reverses the marked reduction of lymph node V β 8⁺ T cells caused in mice by the bacterial superantigen *Staphylococcus aureus* enterotoxin B. IL-2 may thus participate in a feedback regulatory mechanism by predisposing mature T lymphocytes to apoptosis.

A.E7 is a CD4⁺, T_H1 T lymphocyte clone that constitutively expresses high-affinity IL-2 receptors and shows increased thymidine incorporation after application of 100 units per ml of exogenous IL-2 for two days^{10,11}. Antigen stimulation at 1 μ M following IL-2 treatment diminished thymidine incorporation, possibly because of an antigen-dependent block in proliferation^{12,13}. Microscopic examination unexpectedly revealed extensive death of the T cells (Fig. 1a, top panels). There were 82% fewer IL-2 pre-treated T cells following 1 μ M antigen stimulation compared with control (Table 1). Cell death was less at lower doses of IL-2 and just evident without IL-2 pre-treatment, which could be attributed to IL-2 produced by antigen stimulation (Table 1). These results suggested the possibility that IL-2 following antigen stimulation leads to proliferation, whereas IL-2 before antigen exposure causes cell death.

I therefore evaluated IL-2 and antigen stimulation in an experiment in which endogenous IL-2 was not produced. A.E7 cells require co-stimulatory signals from antigen-presenting cells in addition to T-cell receptor stimulation to produce IL-2 (refs 14, 15). Consequently, A.E7 cells were pre-treated with IL-2 and stimulated without antigen-presenting cells on dishes coated with a monoclonal antibody against CD3 ϵ (ref. 16). Anti-CD3 ϵ stimulation following IL-2 again led to T-cell death (Fig. 1a, lower panels), with 74% of the untreated cells, but only 14% of the T cells pre-treated with 10 units per ml of IL-2, surviving (Table 1). Killing caused by anti-CD3 ϵ was also dependent on the IL-2 dose, with 49% cell loss at 2 units per ml. DNA fragments of 200-base-pair multiples were evident in dying A.E7 cells after IL-2 and anti-CD3 ϵ stimulation (Fig. 1b, 2C11) and death was avoided by including cyclosporin A as described in Table 1 legend. These data strongly suggested that apoptosis was occurring^{1,2,5,7}.

I then tested whether IL-2 could predispose cells bearing particular T-cell receptors (and not bystander cells) to apoptosis in a heterogeneous lymph node T-cell (LNT) population. Because LNT cells do not constitutively express IL-2 receptors, they were first stimulated to express high-affinity IL-2 receptors with the lectin, concanavalin A. The concanavalin A was then removed and the cells exposed to IL-2. As LNT cells did not survive without any IL-2, low doses (3 units per ml) and high doses (100 units per ml) of IL-2 were compared. After two days exposure to IL-2, the LNT cells (>97% $\alpha\beta$ T cells; data not shown) were plated on dishes coated with either no antibody, the F23.1 monoclonal antibody (anti-V β 8, specific for the V β 8.1, 2, 3 receptor chains)¹⁷ or the RR4-7 monoclonal antibody (anti-V β 6)¹⁸. In low IL-2, both anti-V β 8 and anti-V β 6 antibodies caused cell number to increase, but high IL-2 caused loss of cells (Table 1). Flow cytometry revealed that anti-V β 8 antibody markedly deleted cells with cognate V β 8 receptors in LNT cells given high IL-2 but not those given low IL-2 (data not shown). The high-dose IL-2 populations were gated separately into CD4⁺ cells and CD4⁻ cells (virtually all CD8⁺ cells), which revealed that anti-V β 8 decreased V β 8⁺ cells from 38.4% to 14.1% for