Intracellular Targeting of Antigens Internalized by Membrane Immunoglobulin in B Lymphocytes

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Summary

An important function of membrane immunoglobulin (mIg), the B cell antigen receptor, is to endocytose limiting quantities of antigen for efficient presentation to class II-restricted T cells. We have used a panel of mIg mutants to analyze the mechanisms of mIg-mediated antigen presentation, and specifically to explore the ability of mIg to target internalized antigen to intracellular processing compartments. Transfected mIgs carrying substitutions for the transmembrane Tyr_{587} residue fail to efficiently present specifically bound antigen. However, these mutants internalize antigen normally, and their defect cannot be attributed to a lack of mIg-associated Igα/Igβ molecules. A novel functional assay for detecting antigenic peptides in subcellular fractions shows that wild-type mIg transfectants generate class II-peptide complexes intracellularly, whereas only free antigenic peptides are detectable in the mutant mIg transfectants. Furthermore, an antigen competition assay reveals that antigen internalized by the mutant mIgs fails to enter the intracellular processing compartment accessed by wild-type mIg. Therefore, mIg specifically targets bound and endocytosed antigen to the intracellular compartment where processed peptides associate with class II molecules, and the transmembrane Tyr_{587} residue plays an obligatory role in this process. Targeting of internalized antigen may be mediated by receptor-associated chaperones, and may be a general mechanism for optimizing the presentation of specifically bound and endocytosed antigens in B lymphocytes and other antigen-presenting cells.

APCs such as macrophages, dendritic cells, and B cells actively take up extracellular proteins for processing by a number of nonspecific and specific receptor-mediated pathways. As opposed to macrophages, B cells have relatively slow rates of fluid-phase pinocytosis. Instead, membrane Ig (mIg)[1] on the surface of mature B lymphocytes binds specific antigen and efficiently internalizes it for subsequent processing into peptides and association with class II MHC molecules (1–4). The intracellular compartment where processing and class II association occur appears similar to, but distinct from, early endosomes or lysosomes (5–7). Depending on the cell line and assay, this compartment may (6) or may not (7) contain transferrin receptors, which is interesting in light of work showing that targeting antigens to early endosomes by conjugation to transferrin does not always promote efficient processing and presentation (8, 9). After internalization of antigen-mIg complexes, mIg recycles back to the cell surface. where it can bind additional antigen (10, 11).

The remarkable efficiency with which mIg targets antigens for processing was originally attributed to the role of mIg as a high affinity receptor. It is, however, possible that mIg may also direct the intracellular traffic of internalized antigen. The structural basis of such regulated trafficking is not known. Cytoplasmic tail motifs that mediate internalization of a variety of plasma membrane receptors have been identified. These motifs typically contain tyrosines or phenylalanines, as well as a variable number of other polar residues (12–14), which are critical for efficient internalization, perhaps by mediating attachment to clathrin, adaptins, or other cytoskeletal elements. However, these motifs do not appear to be involved in targeting at least some internalized receptors to specific intracellular sites (15). Furthermore, the cytoplasmic tails of mIgM and mIgD lack any identifiable internalization motifs. An alternative possibility is that mIg-associated proteins, such as Igα and Igβ, function to control the internalization and intracellular trafficking of mIg and mIg-bound ligands (16).

Elucidating the structural basis of mIg-mediated antigen

[1] *Abbreviations used in this paper:* CIIV, class II-containing vesicles; GAHμ, goat anti-human μ; GAMγ2a, goat anti-mouse γ2a; GGG, goat gamma globulin; mIg, membrane Ig; OVA, ovalbumin; PC, phosphorylcholine; RAMG, rabbit anti-mouse Ig; RGG, rabbit gamma globulin; TM: Y/F, transmembrane mutant, try to phe; TM: YS/VV, transmembrane mutant, tyr-ser to val-val; t-OVA, tryptic fragments of OVA.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/05/1705/10 $2.00
Volume 181 May 1995 1705–1714

internalization and intracellular trafficking is clearly important for understanding how antigen is targeted to class II–rich vesicles in B lymphocytes (6, 7, 17). The same mechanisms may also be operative in other APCs that internalize antigens via specific receptors for class II–associated presentation. We have used a mutational approach of mIg to address this question, using a panel of murine B lymphoma cell lines transfected with wild-type or mutant forms of a phosphorylcholine (PC)-specific human IgM (18). Three of the mutants we analyzed showed markedly reduced abilities to present PC-conjugated proteins to protein antigen–specific T cells. One mutant lacks the three–amino acid cytoplasmic tail (Lys-Val-Lys) of the μ heavy chain, and it is expressed as a phosphatidylinositol-linked protein that is internalized very slowly (19). More interesting are two constructs in which a transmembrane tyrosine residue, Tyr_{587}, is mutated, either with the adjacent serine to valines (denoted TM: YS/VV), or individually to phenylalanine (TM: Y/F). Both these mutants bind and internalize antigen, but they fail to efficiently present it to class II–restricted T cells (18, 19). Moreover, the TM: YS/VV mutant does not associate with Igα and Igβ, and it fails to transduce intracellular signals upon antigen binding; conversely, the TM: Y/F mutant associates with Igα and Igβ, and it is competent at signaling (20, 21). Other workers have generated similar mutations of murine μ heavy chain and transmembrane regions (22, 23), but these have not been analyzed for their effect on antigen presentation. Chimeric mIgs with the human TM: YS/VV mutations and either Igα or Igβ cytoplasmic tails have also been constructed; these show normal intracellular signaling upon antigen ligation (20), but have not been previously examined for their ability to mediate antigen presentation. Thus, we have available a panel of human mIg mutants that provides an opportunity for analyzing the structural constraints on antigen internalization and processing, as well as the role of the mIg-associated proteins, Igα and Igβ, in these processes.

Materials and Methods

Cell Lines. A20 murine B lymphoma cells ($\gamma 2a^+$, κ^+, H-2^d) were transfected with PC-specific human μ heavy chain constructs; high expressing cells were separated by cell sorting and cloned by limiting dilution, as described previously (18). Cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS (GIBCO Laboratories, Grand Island, NY), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 50 μM 2-ME, and 0.6 mg/ml G418 (GIBCO) in a 37°C, humidified, 5% CO_2 environment. Chimeric proteins containing Igα or Igβ linked to the TM: YS/VV mutant were expressed in A20 cells as described (20). The I-A^d–expressing M12.4.1 cell line and its class II^- variant, M12.C.3 (24), were generous gifts of Dr. Laurie Glimcher (Harvard School of Public Health, Boston, MA). DO.11, a T cell hybridoma specific for ovalbumin (OVA) peptide fragment $OVA_{323-339}$ in the context of I-A^d (25), and D1.1, a T cell clone specific for processed rabbit Ig (RGG) in the context of I-A^d (26), were maintained and used as described previously (18).

Antibodies. $F(ab')_2$-fragments of rabbit anti–mouse Ig (RAMG) and RGG were obtained from Cappel (Organon Teknika, Durham, NC), and intact RGG and goat gamma globulin (GGG) were obtained from Sigma Chemical Co. (St. Louis, MO). M5/114, a rat mAb specific for I-$A^{b,d,q}$/I-$E^{d,k}$ used as the supernatant of the hybridoma, was obtained from American Type Culture Collection (Rockville, MD). Goat anti–human μ (GAHμ), goat anti–mouse IgG2a (GAMγ2a), and horseradish peroxidase–conjugated goat anti–rat Ig were obtained from Southern Biotechnology Associates (Birmingham, AL).

Preparation of PC-conjugated and Radioiodinated Proteins. OVA (Sigma Chemical Co.) and $F(ab')_2$-RGG were conjugated with PC using the protocol described previously (18). ^{125}I-PC-$F(ab')_2$-RGG was prepared using Iodo-Beads (Pierce Chemical Co., Rockford, IL), with 2 ml of PC-$F(ab')_2$-RGG at 2 mg/ml in PBS and 500 μCi of ^{125}I-KI (Amersham Corp., Arlington Heights, IL) for 15 min at 25°C. After removing the beads, free KI was removed by passing the material over a Sephadex G50-80 column (Sigma Chemical Co.; molecular weight exclusion = 30,000) preequilibrated with 5 mg/ml RGG in PBS. Radioactive fractions were collected and extensively dialyzed against 5 mg/ml BSA in PBS. The recovery of protein averaged 50%, and specific activities averaged 0.25 μCi/μg PC-$F(ab')_2$-RGG, of which >90% was precipitable in 10% TCA.

Antigen Presentation Assays. An antigen pulse protocol was used to study antigen presentation because this assay permits a direct comparison among clones for the rate of antigen uptake, intracellular processing, and subsequent surface expression of peptide–class II complexes. Transfected A20 cell clones were incubated on ice for 15 min at 10^6/ml with 100–300 μg/ml PC-OVA. The cells were washed and warmed to 37°C for 0–120 min; internalization and processing of antigen were stopped by adding an excess of ice-cold medium. The cells were fixed at 2 × 10^6/ml in 1% paraformaldehyde in PBS for 30 min at room temperature, washed thoroughly, and then 10^5 cells were incubated with 10^5 DO.11 cells in a total volume of 0.2 ml. After a 24-h incubation, the IL-2 concentration in the supernatants was measured as described (18).

Antigen Internalization and Catabolism. Washed A20 clones at 2.5 × 10^7/ml in 5 μg/ml RGG/PBS were incubated at 4°C for 30 min with 10 μg/ml ^{125}I-PC-$F(ab')_2$-RGG. After washing twice in ice-cold PBS, cells were suspended to 10^7/ml in culture medium and incubated in duplicate at 37°C for varying lengths of time. Incubations were terminated by adding an equal volume of ice-cold PBS containing 0.2% sodium azide. After centrifugation, supernatants were precipitated at 4°C with TCA at a final concentration of 10%, and the radioactivity in the TCA pellet and supernatant material was counted. Pelleted cells were incubated in 2 ml 150 mM NaCl, pH 1.2, at 4°C for 30 min to remove any surface-bound material (10), and released and residual cell-associated radioactivity was counted. Results are expressed as a percentage of total radioactivity recovered at each time point from each of the four fractions (TCA-precipitable and soluble cpm in supernatants, acid-releasable and intracellular cpm from cells); total recovered radioactivity varied only 4–7% at all time points.

Identification of Processed Antigen in Subcellular Fractions. The method is described in detail in Barnes and Mitchell (27). In brief, 1–2 × 10^8 transfected A20 cells were incubated with 100 μg/ml PC-OVA at 37°C for 30 min, washed, and ruptured by nitrogen cavitation after equilibration at 450 psi for 5 min at 4°C. After removing nuclei (850 *g* for 10 min), the postnuclear supernatants were sterilely separated over 25.4% self-forming Percoll gradients for 1 h 45 min at 20,000 rpm (J2-21 M/E centrifuge, JA-20 rotor; Beckman Instruments, Palo Alto, CA). 22 0.8-ml fractions were sterilely harvested by gravity siphon. To assay for peptide–class II complexes, or for free peptides generated intracellularly, Percoll gradient fractions were sonicated, and 0.25 ml of each fraction was incubated with fixed and washed APC (either M12.4.1 or M12.C.3 cells) at 10^6/ml for 4 h at 37°C. After washing, the fixed cells

were incubated at 10^5 per well with 10^5 DO.11 cells in a volume of 0.2 ml, and IL-2 production was assessed after 24 h.

Characteristic enzyme distributions for plasma membrane (alkaline phosphodiesterase I [28]) and lysosomes (β-hexosaminidase [29]) were defined in each experiment. In addition, the distribution of class II in subcellular fractions was determined by solid-phase ELISA. In brief, 25–50 μl of each fraction was dried onto flexible microtiter plates (Falcon MicroTest III; Becton Dickinson & Co., Mountain View, CA). The wells were then blocked with 2% BSA in borate-buffered saline and extensively washed with PBS/0.01% Triton-X. M5/114 hybridoma supernatant was then added at 1:100 dilution for 1 h at 37°C, followed by extensive washing and incubation with 1:1,000 dilution of horseradish peroxidase–conjugated goat anti–rat Ig antibody for 1 h at 37°C. After further washing, peroxidase activity was assessed using 2,2′-azino-d-[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt (Sigma Chemical Co.) as a substrate.

Competition for Antigen Presentation. Washed A20 cell clones (10^5 per well) were incubated with 10 μg/ml of primary antigen (PC-OVA, PC-RGG, or $F(ab)'_2$-RAMG) and various concentrations of competing antigen (e.g., GAMγ2a, $F(ab)'_2$-RAMG, GAHμ, or PC-OVA), or with 1 mg/ml nonspecific antigen (GGG, RGG, or OVA; internalized by fluid-phase pinocytosis). For PC-OVA as the primary antigen, 10^5 DO.11 cells were added per well; for PC-RGG or $F(ab)'_2$-RAMG as the primary antigen, 2×10^4 D1.1 cells were added per well, all in a total volume of 0.2 ml. There is no cross-reactivity of the D1.1 cells with OVA or goat Ig, or DO.11 for rabbit or goat Ig (data not shown). Response of the T cells was assessed by IL-2 secretion.

In experiments analyzing the ability of cells to present preprocessed OVA, A20 cells were fixed at 2×10^6 cells/ml in 1% paraformaldehyde/PBS for 30 min at 25°C, followed by extensive washing with culture medium. These fixed cells were then incubated at 10^5 per well with 10–100 μg/ml tryptic fragments of OVA (t-OVA; gift from Dr. Ken Rock, Dana-Farber Cancer Institute, Boston, MA) and DO.11 cells at 10^5 cells per ml; IL-2 secretion was assessed as described above.

Results

Antigen Presentation via Transfected mIg. We have previously shown that the cytoplasmic tailless mutant, Cyto:Δ, and mutants with substitutions for Tyr_{587}, namely TM: YS/VV and TM: Y/F, all fail to efficiently present specifically internalized antigen ([18]; summarized in Fig. 1). The TM: Y/F mutant is particularly interesting because it associates with Igα and Igβ, and it produces normal calcium and phosphorylation signals upon cross-linking ([21]; and Nussenzweig, M. C., unpublished data). The inability of this mutant to present antigen suggests that the Igα and Igβ mIg-associated proteins are not sufficient for presentation of antigens internalized by mIg. To further examine this, we assayed the antigen presenting ability of the TM: YS/VV mutant to which either Igα or Igβ cytoplasmic domains were fused to generate chimeric molecules. Under conditions in which the bulk of the antigen is taken up by receptor-mediated endocytosis, A20 cells expressing the chimeric Ig molecules also failed to efficiently present PC-OVA to OVA-specific T cells (Fig. 2 *A*). All the transfected cells showed comparable levels of IL-2 induction after a 24-h incubation with PC-OVA (Fig. 2 *B*) or nonhaptenated OVA (not shown) because during this interval, significant amounts of antigen are internalized by fluid-phase pinocytosis. Therefore, even the transfectants that fail to present antigen internalized by mIg have the capacity to pinocytose, process, and present the same protein antigen to specific T cells. By flow cytometry, all these clones exhibited comparable surface expression of transfected mIg ([18, 20]; data not shown), indicating that the inability to efficiently present antigen is not attributable to diminished antigen receptor expression. Since fusing the cytoplasmic tails of either Igα or Igβ to the TM: YS/VV mutant restores its ability to transduce signals (20), we conclude that Igα and/or Igβ

	Transmembrane domain / Cytoplasmic Domain	Intracellular Signalling	Igα/Igβ Association	Antigen Presentation
wild-type	N L W A T A S T F I V L F L L S L F Y S T T V T L F K V K–COOH	+	+	+
Cyto: Δ	- COOH	−	−	−
TM: YS/VV	- - - - - - - - - - - - - - - - - - **V V** - - - - - - - - - - COOH	−	−	−
TM: Y/F	- - - - - - - - - - - - - - - - - - **F** - - - - - - - - - - - COOH	+	+	−
YS/VV-Igβ	- - - - - - - - - - - - - - - - - - **V V** - - - - - - - Igβ 181–228	+	−	−
YS/VV-Igα	- - - - - - - - - - - - - - - - - - **V V** - - - - - - - Igα 160–220	+	−	−
	569 … 587 … 597			

Figure 1. Sequences and functions of wild-type and mutant human mIgM constructs. Amino acids are represented by their single-letter code. *Cyto*, cytoplasmic tail; *TM*, transmembrane region. YS/VV-Igβ and YS/VV-Igα are chimeric constructs using the extracellular and transmembrane domains from the TM: YS/VV mutant, with the indicated domains from the Igβ and Igα sheath molecules, respectively. Italicized residues in the wild-type sequence represent amino acids conserved among all isotypes; the underlined residue is a threonine in the transmembrane region of murine mIg. Dashes indicate identity with the wild-type sequence; residues in bold face represent specifically mutated amino acids. The numbers indicate the positions of the μ heavy chain amino acids. The results of functional analyses and Igα/Igβ associations are summarized from references 18, 20, 21, and this article.

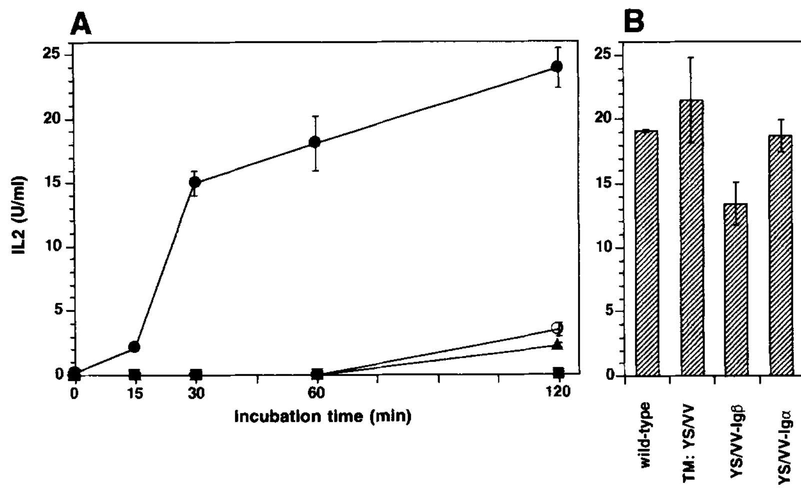

Figure 2. Antigen presentation by the transfected A20 clones. (*A*) A20 transfectants were pulsed with 300 μg/ml PC-OVA for the indicated times. After washing, the cells were fixed and used as APCs for 10^5 DO.11 cells in triplicate. After 24-h culture, the supernatants were assayed for IL-2. (*B*) A20 transfectants were continuously cultured for 24 h with DO.11 and 300 μg/ml of PC-OVA, and supernatants were assayed for IL-2. Comparable results were obtained by continuous incubation of all A20-transfectant clones with DO.11 and 1,000 μg/ml nonhaptenated OVA (not shown). –●–, Wild type; –○–, TM: YS/VV; –■–, YS/VV-Igβ; –▲–, YS/VV-Igα.

are required for the signaling function of mIg, but are not sufficient to restore efficient antigen presenting function to a defective mutant mIg.

Antigen Internalization and Degradation in mIg Transfectants. Inefficient presentation of antigen that binds to mutant mIg molecules could result from a failure to internalize antigen, process it, or form complexes of processed peptides with class II molecules. In the next series of experiments, each step in this sequence was examined. The internalization and catabolism of antigen were analyzed by following the fate of ^{125}I-labeled PC-conjugated protein in A20 clones expressing the various transfected PC-specific mIgs. The antigen we chose for these experiments was PC-conjugated $F(ab')_2$ RGG because it can be readily radioiodinated and it

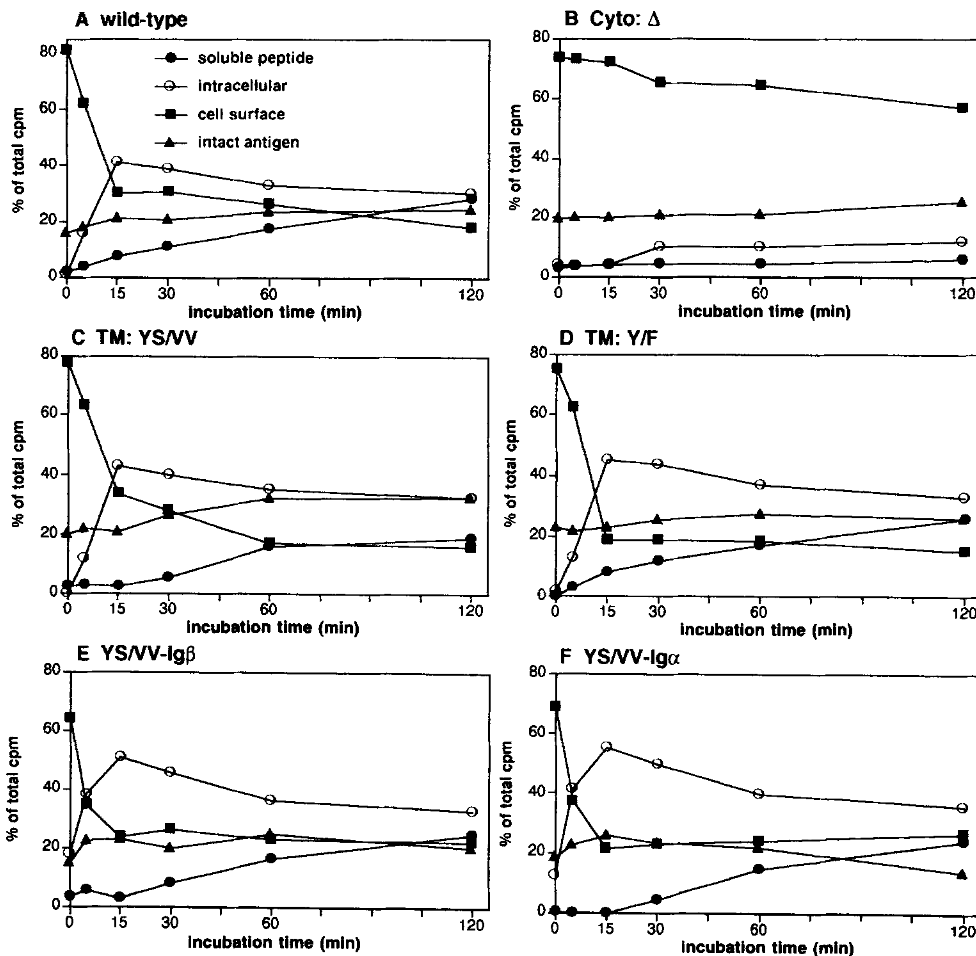

Figure 3. Internalization and degradation of PC-conjugated ^{125}I-F(ab)'$_2$-RGG by transfected A20 clones. After binding antigen and washing, cells were warmed for varying intervals at 37°C before terminating internalization with an equal volume of ice-cold PBS containing 0.2% azide. Cell-associated radioactivity released by acid treatment is identified as cell surface (■); acid-resistant cell-associated radioactivity is denoted as intracellular (○); radioactivity in the supernatant precipitable with 10% TCA is identified as intact antigen (▲); TCA-soluble supernatant radioactivity is denoted as soluble peptide (Δ). Results are expressed as percentage of total recovered radioactivity at each time point (total recoveries being 70–90% of initially bound radioactivity).

behaves identically to other PC-haptenated proteins (such as PC-OVA) in antigen presentation assays (not shown). After binding PC-F(ab')$_2$-RGG at 4°C and washing extensively, cells were warmed to 37°C for varying times. Radioactivity in the supernatant was separated into TCA-precipitable ("intact antigen") and TCA-soluble ("soluble peptide") fractions; cell-associated radioactivity was separated into "cell surface" (released by treatment with acid) and "intracellular" (resistant to acid). As shown in Fig. 3 *A*, wild-type mIg rapidly internalized specific antigen with maximal uptake by 15 min, and degraded (TCA-soluble) antigen continued to accumulate in the supernatant at a steady rate over the 2-h time course. The rates of uptake and catabolism are very similar to those described previously for specific antigen uptake by human B cell clones (10). By comparison, the Cyto:Δ mutant (Fig. 3 *B*), which is anchored to the plasma membrane by a phosphatidylinositol linkage (19), showed slow internalization of bound antigen with a correspondingly slow generation of TCA-soluble degraded fragments. This behavior is consistent with a phosphatidylinositol-anchored molecule lacking the appropriate transmembrane or cytoplasmic domains to signal efficient internalization, and it provides an explanation for the inefficiency of this construct in mediating antigen presentation. Interestingly, both the tyrosine transmembrane mutants (TM: YS/VV and TM: Y/F, Fig. 3, *C* and *D*, respectively), which also fail to efficiently mediate antigen presentation, showed rates of antigen internalization and catabolism that were virtually identical to the wild-type construct. Similar results were seen with chimeric mIgs containing the YS/VV transmembrane mutation fused to Igβ (Fig. 3 *E*) or Igα (Fig. 3 *F*) cytoplasmic tails. These mutants must therefore retain the signal for rapid ligand-induced endocytosis. Moreover, at the level of sensitivity of this assay, all the tyrosine transmembrane mutants directed internalized antigen into compartment(s) where comparable levels of antigen catabolism occurred. The slight apparent lag in the generation of TCA-soluble fragments in the TM: YS/VV mutants (relative to wild-type or TM: Y/F mutants) was not a consistent finding. In fact, in some experiments, wild-type–transfected constructs lagged behind the TM: YS/VV mutants in release of degradation products. These results indicate that the tyrosine-transfected mIgs, including one (TM: YS/VV) that fails to associate with Igα and Igβ, all efficiently internalize and mediate the degradation of bound ligand.

Generation of Processed Peptides in A20 Transfectants. The bulk degradation of protein antigens, as measured by the release of TCA-soluble radioactivity (Fig. 3), is clearly not a reliable indicator of antigen processing and generation of functionally relevant peptides. Qiu et al. (17) and our group (27) have recently developed an assay in which B lymphoma cells are allowed to process an antigen, and subcellular fractions are subsequently assayed for the presence of peptide–MHC complexes capable of stimulating T cells. In our assay, the majority of the processed peptides detected from wild-type mIg-transfected cells are already associated with class II, since these peptides can be presented to T cells by fixed, class II–negative APCs or when bound to culture wells (27). By incubating subcellular fractions with APCs that do or do not express class II in the assay, this method enables one to distinguish free peptides from class II–associated peptides generated intracellularly.

To determine if wild-type and mutant (TM: YS/VV) mIg transfectants differed in the generation of class II–associated peptides, the A20 clones were pulsed with PC-OVA for 30 min at 37°C, ruptured, and fractionated on self-forming continuous Percoll gradients, as described (27). Fractions were incubated with fixed class II$^+$ M12.4.1 or class II$^-$ M12.C.3. cells. The pulsed APCs were washed, incubated with DO.11 cells, and IL-2 production was measured after 24 h. When PC-OVA was internalized by wild-type mIg, the subcellular fractions contained peptides that were presented identically by fixed class II$^+$ and class II$^-$ APCs (Fig. 4 *A*; ref. 27). Therefore, antigen is processed and peptide–class II complexes are efficiently generated intracellularly in the wild-type transfectant. In contrast, in the TM: YS/VV transfectants, the antigen was processed into peptides that were presented only by the fixed class II$^+$ APCs (Fig. 4 *B*). Therefore, the TM: YS/VV mutant not only internalizes antigen, but it delivers the antigen into a proteolytic compartment. The defect is that the peptides fail to associate with class II molecules. Note also that in both wild type and mutant transfectants, most of the processed peptide was detected in fractions consistent with the low to intermediate density class II–containing vesicles that have been described recently (6, 7). Furthermore, in both cell lines, most of the class II was in low-density fractions, particularly the plasma membrane, and not in denser organelles. This pattern of class II localization is characteristic of A20 and other B lymphoma cells (6, 7, 17).

Competition for Antigen Presentation by Endogenous Versus Transfected mIg. In the final set of experiments, we used a functional assay to ask if wild-type and mutant mIg molecules internalize and concentrate antigen in the same compartment in intact cells. By virtue of having two distinct mIgs on their surface, transfected A20 cells may specifically internalize ligands bound to either the endogenous murine IgG2a (antigen specificity unknown) or to the transfected PC-specific human IgM construct. We found that antigens internalized by one wild-type mIg reduced the concurrent presentation of antigens internalized by the other.

Thus, in wild-type cells, presentation of antigen specifically internalized by the transfected wild-type mIg (e.g., PC-OVA or PC-RGG) was inhibited ~65% by coincubation with F(ab')$_2$-RAMG or GAMγ2a (Table 1). Similarly, presentation of ligands internalized via the endogenous mIg was inhibited by antigens internalized via the transfected wild-type mIg (see below). Competition was seen only for antigen internalized specifically in association with mIg; even a 10-fold excess of antigen internalized by nonspecific fluid-phase pinocytosis (e.g., GGG or RGG) did not effectively compete for antigen presentation (Table 1). The implication is that there is some limiting step in the sequence of antigen internalization/targeting/processing/class II association/surface reexpression. When two antigens are specifically targeted to the appropriate intracellular processing compartment by inter-

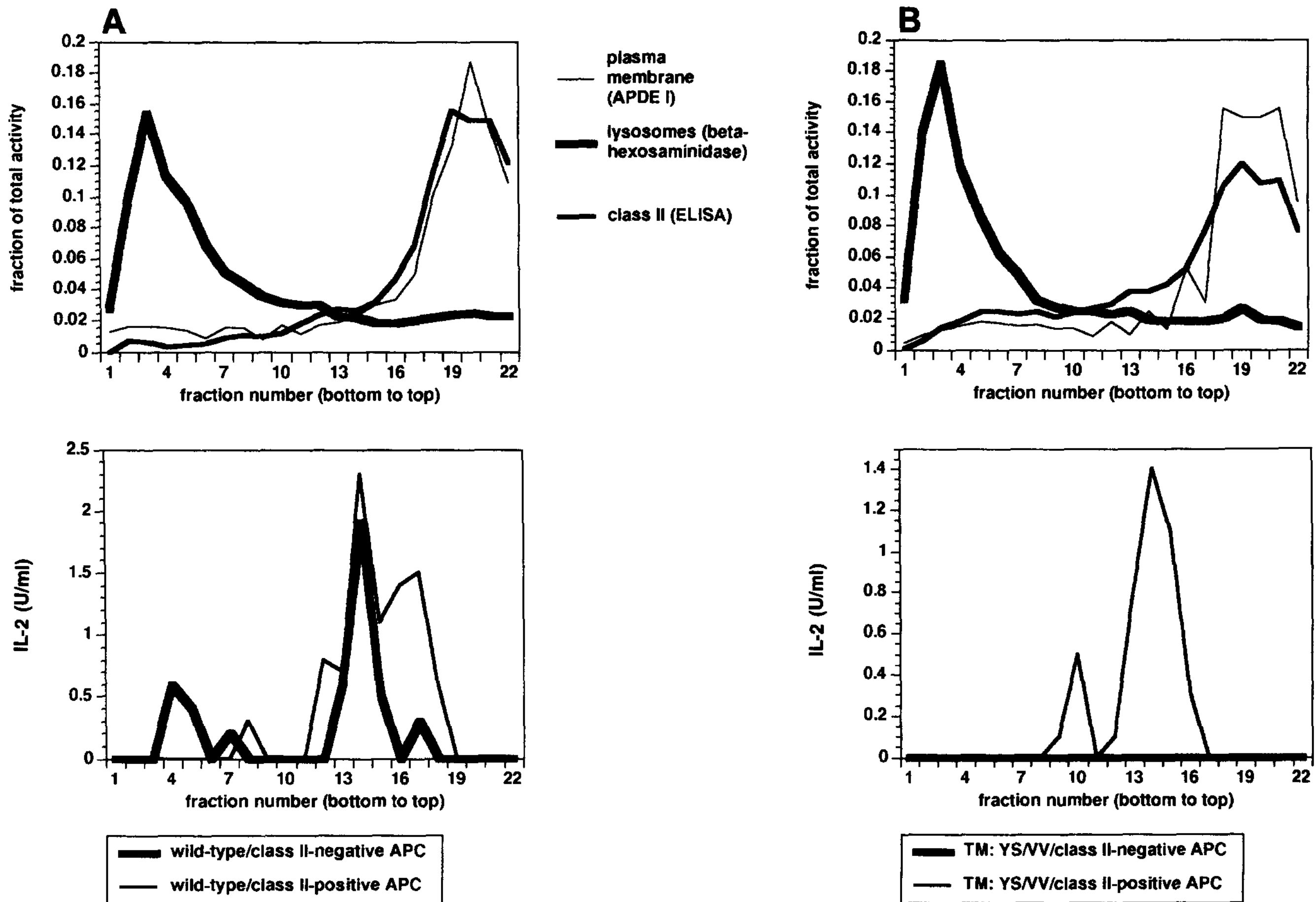

Figure 4. Identification of processed peptides and peptide-class II complexes in A20 transfectants. Wild-type (*A*) or TM: YS/VV (*B*) transfectants were incubated with 100 μg/ml of PC-OVA for 30 min at 37°C, ruptured by nitrogen cavitation, and fractionated on Percoll gradients. Individual fractions were incubated with paraformaldehyde-fixed M12.4.1 (class II$^+$) or M12.C.3 (class II$^-$) B cell lines and washed. The cells were incubated with the DO.11 T cell hybridoma, and IL-2 levels were assayed in 24-h supernatants (*bottom panels*). Fractions were also assayed for plasma membrane and lysosomal markers, and for class II, as described in Materials and Methods (*top panels*). Controls showed that fixed M12.4.1 cells presented t-OVA but not intact OVA to DO.11 (IL-2 levels of 3.5 and 0 U/ml, respectively), whereas M12.C.3 cells presented neither (IL-2 = 0 U/ml).

nalization via two mIgs, there is competition for one or more steps, and a net reduction in the surface expression of the processed peptide–class II complexes detected by the responding T cell. Control experiments using fluorimetry to evaluate receptor-mediated endocytosis showed that the rate of internalization of one mIg was not affected by simultaneously incubating the cells with antibody to the other mIg (not shown). We conclude that the transfected μ and endogenous γ2a mIgs internalize bound ligands independently of each other, and that competition for antigen processing and presentation does not occur at the level of internalization. Additional controls showed that whereas $F(ab')_2$-RAMG or GAMγ2a inhibited presentation of PC-OVA (Fig. 5 *A*), neither inhibited the presentation of preprocessed t-OVA to DO.11 T cells (Fig. 5 *B*). Therefore, antigen competition in this system is not due to competition for binding to a limiting number of surface class II molecules, since class II molecules are clearly available to bind and present exogenous peptides.

Antigenic competition is therefore another way of assessing whether the transfected mIgs deliver antigen to the same intracellular compartment as the endogenous (normal) mIg. The results of antigen competition studies were strikingly different with the Tyr_{587} transmembrane mutants. In wild-type transfected cells, presentation of $F(ab')_2$-RAMG (internalized via the endogenous mIg) to D1.1 cells was inhibited ~65% by coincubation of the cells with GAHμ or PC-haptenated proteins, both of which are internalized via the transfected mIg (Fig. 6 *A* and Table 2). However, in both the TM: YS/VV (Fig. 6 *B*) and TM: Y/F (Fig. 6 *C*) clones, presentation of $F(ab')_2$-RAMG was not inhibited by coincubation with GAHμ or PC-OVA (see also Table 2). Therefore, antigens internalized by the tyrosine mutant mIgs do not effectively interfere with the processing and presentation of antigen internalized via the endogenous murine mIg, consistent with the conclusion that mutant mIg delivers antigen to a different intracellular compartment.

Discussion

In this study, we have used a panel of cloned A20 murine B cell lines, transfected with various human mIg constructs,

Table 1. *Antigen Internalized by Endogenous mIg Competes for Processing of Antigen Internalized with Wild-type mIg*

Primary antigen	Competitor	Percentage of response to primary antigen alone
PC-RGG	GAMγ2A	**33.4 ± 16.2 (4)**
	GGG	124.5 ± 32.5 (3)
PC-OVA	GAMγ2A	**36.4 ± 21.6 (9)**
	GGG	110.1 ± 13.9 (6)
	$F(ab')_2$-RAMG	**36.7 ± 12.5 (7)**
	RGG	93.5 ± 9.7 (5)

For experiments with PC-RGG as the primary antigen, 10^5 wild-type transfected A20 cells were incubated in triplicate for 24 h with 2×10^4 D1.1 cells in the presence of 5 μg/ml PC-RGG and the indicated competitors (10 μg/ml GAMγ2A or 1 mg/ml GGG).

For experiments with PC-OVA as the primary antigen, 10^5 wild-type transfected A20 cells were incubated in triplicate for 24 h with 10^5 DO.11 cells in the presence of 30–100 μg/ml PC-OVA and the indicated competitors (10 μg/ml GAMγ2A, 1 mg/ml GGG, 10 μg/ml $F(ab')_2$-RAMG, or 1 mg/ml RGG).

After 24 h, supernatants were harvested and assayed for IL-2. Results are expressed as the percentage of IL-2 generated in the absence of competitor ± 1 SD; the numbers in parentheses represent the number of experiments. Data in boldface are statistically significant ($p \leq 0.01$).

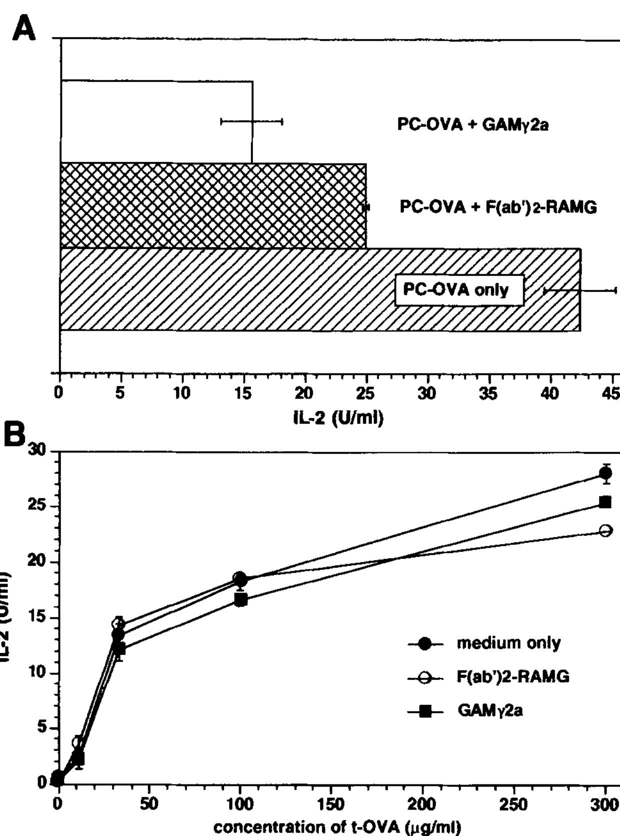

Figure 5. Competition for antigen presentation. (*A*) 10^5 wild-type A20 transfectants were incubated with 50 μg/ml PC-OVA alone or with PC-OVA plus 10 μg/ml $F(ab)'_2$-RAMG or 10 μg/ml GAMγ2a, and 10^5 DO.11 cells; IL-2 levels were assayed from 24-h supernatants. (*B*) Wild-type A20 transfectants were incubated at 10^6 cells/ml for 24 h at 37°C in medium alone or with 10 μg/ml $F(ab)'_2$-RAMG or 10 μg/ml GAMγ2a. The cells were then fixed, and 10^5 were incubated in triplicate with 10^5 DO.11 cells and the indicated concentrations of t-OVA; IL-2 levels were assayed from 24-h supernatants.

to examine the effects of mIg transmembrane structure on the ability of the B cell antigen receptor to efficiently mediate presentation of bound antigen. The results indicate that wild-type mIg internalizes bound antigen into a compartment(s) where peptides are generated and can associate with class II molecules. It is likely that this represents the same low density endocytic compartment (CIIV) recently described by Amigorena et al. in A20 cells (6), and by West et al. (7) in a human B-lymphoblastoid cell line. In contrast, the Tyr_{587} mutants of mIg, including those that interact with the Igα/β mIg-associated molecules, internalize antigen to a site where proteolytic degradation occurs, but peptide-class II complexes are not generated. This result suggests either that antigen processing and class II association occur in distinct intracellular compartments, or that the compartment entered by wild-type mIg is capable of both activities, and that it is not efficiently accessed by the Tyr_{587} mutants. Moreover, our experiments establish that the transmembrane Tyr_{587} is essential to target mIg to an antigen processing compartment, and that neither Igα, Igβ, nor the combination of both is sufficient to direct appropriate receptor trafficking.

Our results differ significantly from those of Patel and Neuberger (16), who found that association of mIg with Igβ was critical for efficient receptor-mediated endocytosis, and that portions of the cytoplasmic domain of Igβ were sufficient to direct mIg and bound antigen for efficient antigen processing and presentation. In our experiments, changes in the transmembrane domain were limited to YS/VV and Y/F substitutions, whereas these investigators altered large blocks of contiguous amino acids. The more extensive substitutions resulted in Igs that did not associate with Igα/Igβ, and were also no longer rapidly internalized upon cross-linking. In the constructs we have analyzed, even in the absence of Igα and Igβ association, internalization of the mIg was maintained. This is in agreement with others who found maintenance of antigen internalization even after replacing the entire transmembrane region with a class II transmembrane cassette (30, 31). The mutants described in those papers likewise failed to efficiently present antigen, but this was attributed to inappropriate intracellular trafficking associated with increased rates of receptor–ligand recycling to the cell surface (31). Furthermore, Patel and Neuberger (16) also found that linking the cytoplasmic domain of Igβ to the mutants restored rapid internalization, as well as efficient antigen presentation. In contrast, our TM: Y/F mutant mIg associated with Igα and Igβ, and it displayed normal signal transduction (18, 21), as well as a normal rate of internalization, yet did not mediate efficient antigen presentation (18). Similarly, our chimeric constructs, composed of mIg with the TM: YS/VV mutation

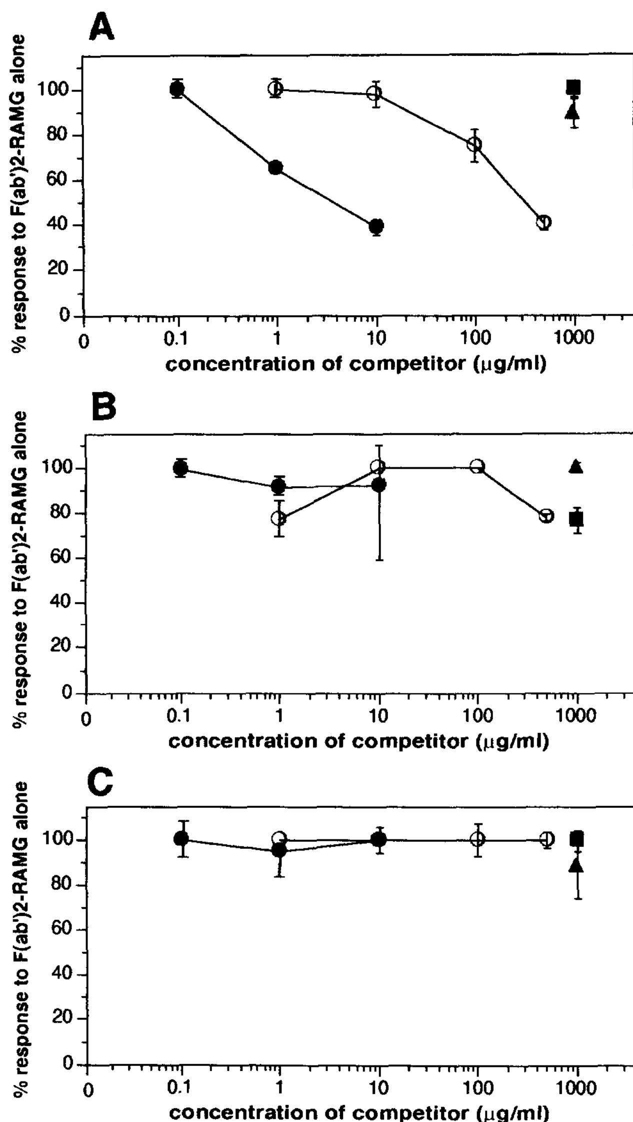

Figure 6. Tyrosine transmembrane mutants do not compete for antigen presentation. 10^5 transfectants (A, wild type; B, TM: YS/VV; C, TM: Y/F) were incubated with 2 × 10^4 D1.1 cells and 10 μg/ml F(ab)'$_2$-RAMG alone or with the same antigen plus increasing concentrations of competitors that bind to the transfected mIg (GAHμ [●] or PC-OVA [O]), or with 1 mg/ml of competitors that are internalized by fluid-phase pinocytosis (GGG [■] or OVA [▲]). IL-2 levels were measured in 24-h supernatants and are expressed as the percentage of response to F(ab')$_2$-RAMG alone. Maximal response (IL-2 secretion in response to F(ab')$_2$-RAMG in the absence of competitor) was as follows: wild type, 216 U/ml; TM: YS/VV, 220 U/ml; TM: Y/F, 25 U/ml. Competitors: –●–, GAHμ; –O–, PC-OVA; –■–, GGG; –▲–, OVA.

and Igα or Igβ cytoplasmic domains, showed the same signaling-competent but presentation-defective phenotype.

One way to reconcile the differences between our results and those of Patel and Neuberger is to postulate that an additional receptor component is necessary for the correct intracellular targeting of internalized mIg. In this model, either Tyr_{587} or other transmembrane amino acids could interact with the as yet unidentified receptor component (see below). Another possibility is that extensively mutated mIg constructs may be expressed on the cell surface as phosphatidylinositol-linked molecules; such molecules will be poorly internalized, fail to interact with Igα/Igβ, and will not mediate efficient antigen presentation (19, 20, 21). In support of this possibility, Wienands and Reth (32) have demonstrated a spontaneous variant of the J558L myeloma cell line (which normally does not exhibit surface mIg because of an absence of Igα) that expresses IgD on its surface as a phosphatidylinositol-linked molecule. The fact that efficient antigen presentation can be restored by appending portions of the cytoplasmic tail of the Igβ molecule to mutant mIg (16) may then be attributable to expression of that chimeric construct as a transmembrane molecule, as opposed to a phosphatidylinositol-linked Ig.

From an immunologic point of view, it makes sense for B cells to ensure that the bulk of their class II is occupied by peptides derived from proteins to which they are capable of making antibodies. Therefore, protein antigens bound to the mIg should be efficiently and preferentially shuttled from the plasma membrane to the appropriate intracellular site (such as the recently described CIIV compartment; [6]) where processing and class II association will occur. This intracellular site should also be sequestered from the normal endocytic pathway, although high concentrations of extracellular antigens may induce a "spillover" into the compartment. This, in fact, may explain why antigens targeted to various B cell surface molecules, including the transferrin receptor, may be presented more efficiently than pinocytosed proteins; binding to cell surface molecules may lead to high local concentrations of antigen (3, 8). In addition, there may be a low efficiency delivery of molecules from, for example, the early endosome to the processing compartment (6).

The ability of mIg to preferentially target antigen to the intracellular-processing/class II–binding organelle(s) raises the issue of what molecule(s) mediate this targeting. One possibility is that the transmembrane domain of mIg, and specifically the transmembrane Tyr_{587}, provides a binding site for an as yet unidentified chaperone-like molecule that trafficks to the class II–rich processing vesicle. If this model is correct, then our results indicate that Igα and Igβ are not sufficient for correct intracellular trafficking. Alternatively, the transmembrane domain may have a specific sequence directing the retention of internalized mIg within appropriate intracellular organelles. It is tempting to speculate that similar mechanisms, involving other receptor molecules in other APCs, may play a role in efficiently delivering antigens to specific processing compartments. We are currently using morphologic and cell fractionation techniques (27) to directly examine the intracellular traffic of internalized wild-type and mutant mIg molecules, as well as the mIg-associated proteins that may regulate this traffic. Such mIg mutants provide unique tools for analyzing the mechanisms of antigen processing, and more general issues of the structural constraints on patterns of receptor-mediated endocytosis and intracellular trafficking.

Table 2. *Antigen Internalized by Tyrosine Transmembrane Mutants Does Not Compete for Processing of Antigen Internalized with Endogenous mIg*

A20 transfectant	Percentage of response to F(ab')$_2$-RAMG alone Competitor			
	GAHμ	GGG	PC-OVA	OVA
Wild type	**33.2 ± 26.1 (10)**	123.1 ± 20.6 (10)	**68.0 ± 19.1 (8)**	94.8 ± 11.6 (4)
TM: YS/VV	94.9 ± 14.8 (5)	93.6 ± 21.8 (5)	92.9 ± 22.4 (10)	106.0 ± 0.8 (2)
TM: Y/F	82.9 ± 9.1 (4)	98.2 (1)	102.2 ± 19.5 (4)	94.3 ± 8.1 (2)

A20 transfectants were incubated in triplicate for 24 h with 2 × 10^4 D1.1 cells in the presence of 10 μg/ml F(ab')$_2$-RAMG and the indicated competitors (10 μg/ml of GAHμ, 30–100 μg/ml of PC-OVA, or 1 mg/ml of GGG or OVA). Supernatants were then harvested and assayed for IL-2. Results are expressed as percentage of IL-2 generated in the absence of competitor ± 1 SD; the numbers in parentheses represent the number of experiments. Data in boldface are statistically significant ($p \leq 0.05$).

This work was supported by National Institutes of Health grants GM-47726 (R. N. Mitchell), AI-22802 (A. K. Abbas), and AI-33890 (M. C. Nussenzweig). M. C. Nussenzweig is an Assistant Investigator of the Howard Hughes Medical Institute.

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Received for publication 15 August 1994 and in revised form 18 January 1995.

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